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Mitochondrial Function as a Tool for Assessing Function, Quality and Adulteration in Medicinal Herbal Teas

Woodley, S.

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Mitochondrial Function as a Tool for Assessing Function, Quality and Adulteration in Medicinal Herbal Teas

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Steven Woodley

May 2024

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

Abstract:

Introduction: A review of current and historical herbal quality assessment methods revealed adulteration with low potency herbal material to be challenging. Mitochondrial functional analysis presents a novel opportunity to address this deficiency. Liquorice (*Glycyrrhiza glabra* L., *Fabaceae*) was selected as an initial herb to profile due to its ubiquity in herbal medicine and commercial teas.

Methods: MCF-7 and MCF-10A cells were seeded and treated for 24 hours with an original liquorice tea extract (OLTE; Three Licorice Tea; Pukka, UK) and subjected to mitochondrial functional assays. Use of pre-extracted material was selected as a method of functional adulteration and the effects of a pre-extracted liquorice tea extract (XLTE) was then compared to OLTE assays with significant results. HPTLC was used for comparison to existing industry methods.

Results: 2000 µg / mL OLTE induced a 14.88% ($p < 0.05$) rise in reactive oxygen species (ROS) in MCF7 cells after 3 hours followed by a 14.73% ($p < 0.05$) drop at 72 hours. Oxygen consumption (OCR) rate dropped 72.6% ($p < 0.01$) and 85.16% ($p < 0.01$) in MCF10A cells treated with 500 and 2000 µg / mL OLTE respectively. The effects of mitochondrial modulating drugs were reduced in MCF7 cells pre-treated with 2000 µg / mL OLTE (oligomycin: 29.94%, $p < 0.01$; FCCP: 43.2%, $p < 0.01$; antimycin A & rotenone: 32.09%, $p < 0.01$). Further investigation revealed improved survival in 2000 µg / mL OLTE pre-treated MCF10A cells given cisplatin (50 µM: 11.79%, $p < 0.05$; 100 µM: 8.186%, $p < 0.001$), but had no effect on MCF7 cells. XLTE also demonstrated a 18.7% ($p < 0.0001$) drop in ROS in MCF7 cells treated with 2000 µg / mL at 72 hours but no effect at 3 hours. XLTE also decreased basal OCR in MCF10A cells at all doses (10 µg / mL: 21.12%, $p < 0.05$; 500 µg / mL: 53.52%, $p < 0.0001$; 2000 µg / mL: 56.41%, $p < 0.0001$), but no effect on drug resistance. HPTLC comparison revealed that differentiating OLTE from XLTE at identical strength was challenging.

Discussion: Possible mechanisms are discussed, proposing electron capture for the reduction in ROS and OCR without other modulations being observed, and efflux transporter expression explaining drug resistance. Drug resistance was the most consistent quality marker and is related to clinical use. Implications, further tests and generation of a mitochondrial functional testing pipeline are presented.

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The majority of the work in this thesis was performed by the author. Any assistance and collaborations are detailed below:

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The initial samples were prepared and MTT assays were performed by Dr Meliz Sahuri-Arisoylu due to laboratory restrictions during Covid-19. Subsequent samples, MTT assays and the analysis of all acquired data was performed by the author.

Plate Seeding of Adulteration Assays:

The plates for assays relating to the adulterated teas were performed by Grace Penelli. The assays and analysis of the data was performed by the author.

HPTLC Assay:

Dr Anthony Booker assisted in the preparation of solvents, acquisition of some of the control samples, and derivatization of the plates for the HPTLC assay. The preparation of all samples and analysis of the data was performed by the author.

Photographs:

The photographs of liquorice, cloves and forsythia in the introduction were taken by Hazeena Azeez using samples acquired by the author.

List of Abbreviations:

ΔΨm	Mitochondrial membrane potential
ABC	ATP-binding cassette
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bak	Bcl-2 antagonist killer
Bax	Bcl-2 associated X
BCA	Bicinchoninic
Bcl-2	B cell lymphoma 2
BCRP	Breast Cancer Resistance Protein
BOLD	Barcode of Life Data
BSA	Bovine Serum Albumin
C	Carbon atom
Ca^{2+}	Calcium ion
CAT	Catalase
CHM	Chinese herbal medicine
Cisplatin	Cis-diamminedichloroplatinum(II)
CO_2	Carbon dioxide
CYP450	Cytochrome P450
DCF	2',7'–dichlorofluorescein
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate

eATP	Extracellular ATP
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ETC	Electron transport chain
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide plus hydrogen
FBS	Foetal Bovine Serum
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
Fe^{2+}	Ferrous ion
FRAP	Ferric Reducing Antioxidant Power
GC	Gas chromatography
GPx	Glutathione peroxidase
GSH	Glutathione
GR	Glutathione reductase
H	Hydrogen atom
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HIF-1α	Hypoxia inducible factor 1α
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IL	Interleukin
IMM	Inner mitochondrial membrane
IRI	Ischaemia-reperfusion injury
IRS	Infrared spectrometry

Keap1	Kelch-like ECH-associated protein 1
LC	Liquid chromatography
MAPK	Mitogen-activated protein kinase
MCF10A	Michigan Cancer Foundation 10A
MCF7	Michigan Cancer Foundation 7
MCU	Mitochondrial Calcium Uniporter
MDA	Malondialdehyde
MEM	Modified Eagle's medium
MRP	Multidrug Resistance-associated Protein
mPTP	Mitochondrial permeability transition pore
MS	Mass spectroscopy
mtDNA	Mitochondrial DNA
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
Na^+	Sodium ion
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide plus hydrogen
NCBI	National Center for Biotechnology Information
NCCIH	National Center for Complementary and Integrative Health
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP	Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOX	NADPH oxidases
Nrf2	Nuclear factor erythroid 2-related factor 2
O	Oxygen atom
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide radical
OGD/R	Oxygen-glucose deprivation / reperfusion

•OH	Hydroxyl radical
•OOH	Peroxyl radical
OES	Optical emission spectroscopy
OLTE	Original Liquorice Tea Extraction
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
P	Phosphate
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PC12	Rat adrenal medulla pheochromocytoma
P-gP	P-glycoprotein
PGC-1α	PPARγ coactivator-1α
PPARγ	Peroxisome proliferator activated receptor-gamma
PRX	Peroxiredoxins
Rhod-2	Rhod-2 acetoxymethyl ester
RIPA	Radioimmunoprecipitation assay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SHMT2	Serine hydroxymethyltransferase 2
SIRT	Silent information regulator
SOD	Superoxide dismutase
TAC	Total anthocyanin content
TCM	Traditional Chinese Medicine
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor-α
TPC	Total phenolic content
TRX	Thioredoxin
UV	Ultraviolet
UVS	Ultraviolet-visible spectrophotometry

UPLC Ultra-high performance liquid chromatography

WHM Western herbal medicine

WST-1 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

XLTE Pre-Extracted Liquorice Tea Extraction

Introduction

Chapter I

1. The Issues Associated with Quality in Medicinal Herbal Teas

1.1. The Modern Herbal Tea Industry

The global trade in medicinal herbs was estimated to be worth US$60 billion in 2017 involving 29,000 herbal substances and growing by 15% each year (Srirama et al., 2017). The Covid-19 pandemic saw an unprecedented rise in demand across all regions resulting in an evaluation of US$151.91 billion in 2021 and estimated to grow to US$165.66 billion in 2022 and US$347.50 billion by 2029 (Fortune Business Insights, 2022). This is thought to be due to people taking a greater interest in their health and immunity during the pandemic. Pharmaceutical and nutraceutical products account for the majority of this share, at 56.49% with food and beverages occupying only 12.17%. Herbal tea accounts for US$3.29 billion in 2021 (Data Bridge Market Research, 2022) but does not include the US$207.1 billion global market in conventional tea (*Camellia sinensis* (L.) Kuntze, *Theaceae*) over 2020 (Statista, 2023), or a US$30.2 billion market in Traditional Chinese Medicine (TCM) in 2023 (Persistence Market Research, 2023), which comprised 18.5% of the global herbal medicines market and are predominantly also extracted in boiling water.

Decoctions and infusions are some of the oldest methods of extracting the medicinal properties from herbs and still one of the most popular today (Wang, Sasse & Sheridan, 2019). Both are aqueous extraction techniques and only differ in that decoctions simmer the water while the herbs are being soaked, while infusions are made by pouring freshly boiled water over the herbs (Bjorn, 2021). Infusions are most popular in the Western world due to their convenience and

familiarity, essentially being the same as making a cup of tea, but are best suited for soft materials such as leaves and flowers, with root and stem material needing to be finely cut to ensure a reasonable extraction. Decoctions have a long history in Chinese medicine for their ability to extract constituents from tough plant materials such as roots, barks and fungi (Wilde, 2020) but take time to prepare. Although the increasing pace of life in Asia has seen a decline in people actually decocting herbal medicines themselves, many Chinese Herbal Medicine (CHM) companies have developed convenient instant powders and granules which are still mostly derived from dehydrated decoctions (Sun Ten, 2015).

Aqueous extractions have maintained their popularity throughout history for their ability to extract polar compounds using a solvent that is safe, even in large doses. One of the reasons herbal medicines have grown in popularity in recent years is their perception as being “more natural” (Welz, Emberger-Klein & Menrad, 2018), often erroneously equated with being more safe (Sax, 2015). They often seem familiar from family traditions where water extractions would have been easily available to any household. In contrast, other solvents such as chloroform, acetone, ether or n-Hexane which can extract non-polar compounds, are usually toxic and cannot be entirely eliminated requiring maximum safe limits to be set and determined by analysis (ICH, 2019) making them the domain of professional laboratories preparing supplements. Alcohol is the main exception and retains popularity in Western herbal medicine (WHM) to make tinctures (Abubakar & Haque, 2020), but is also sometimes used in Chinese medicine where herbs may be soaked over weeks or months in a jar of high strength rice wine or other spirit (Flaws, 1994). Alcohol can also be exploited for use in decoctions and is used in CHM by pre-treating certain herbs with a wine-washing or wine-frying technique to free non-polar constituents before being added to the decoction (Sionneau, 1995; Dong et al., 2006).

Despite the popular perception of herbal medicines being natural and safe, a great deal of adulteration has been reported. While much of it is accidental, at the level of foraging or purchasing at markets, there is evidence to suggest that it is also deliberate and economically motivated, including the addition of drugs to enhance the effects of supposedly “natural” supplements (Booker, Jalil et al.,

2016), dyes that make the colour of vibrant herbs look more potent (Müller-Maatsch, Schweiggert & Carle, 2016; Booker et al., 2018), sometimes using toxic colour pigments such as lead chromate in turmeric (*Curcuma longa* L., *Zingiberaceae*) to cover blemishes and increase weight on sale at the risk of poisoning millions of end consumers (Cowell et al., 2017; Newby 2023), and substitution with cheaper herbal material which may have similar chemical profiles and so evade detection by all but the most sophisticated tests (Booker et al., 2018; Frommenwiler et al., 2019). In some supplements, as many as 94% of commercial products may be adulterated, such as Booker, Frommenwiler et al. (2016) found when testing 35 *Ginkgo biloba* L. *Ginkgoaceae* supplements. Other estimates have found as much as 68% of herbal products having some form of adulteration (Newmaster at al., 2013). Raw herbs and teas are less vulnerable to adulteration with estimates ranging from 12% (Kumar et al., 2018) to 35% (Stoekle et al., 2011), and in some cases as high as 80% (Srirama et al., 2017). Although not technically a herb but frequently included among traditional herbs as a natural remedy, a recent survey of honey from the UK found 100% of tested samples were adulterated (European Commission, 2023). This is not a new problem and adulteration of raw herbal medicines for economic gain has been a persistent problem ever since medicinal herbs have been traded.

In this introduction, the history of herbal adulteration and its methods of detection will first be summarised, followed by a literature review of contemporary methods of detection and their limitations. The concept of functional adulteration will be introduced and the role of mitochondria in health, disease and traditional medicine will then be outlined to demonstrate the potential relevance of these organelles when evaluating functional potency of herbal medicines. Finally, the initial herb to be evaluated, liquorice (*Glycyrrhiza glabra* L., *G. uralensis* Fisch. ex DC. and *G. inflata* Batalin, *Fabaceae*) will be introduced, with its constituents, uses, risks, current quality evaluation, adulteration and known effects on mitochondria.

1.2. A Brief History of Herbal Adulteration and Quality Control

1.2.1. Adulteration and Quality Testing in the Ancient World

Quality control of medicinal herbal teas has been an issue for as long as humans have been using plants to heal (Foster, 2011; Woodley, 2021). C. 50 CE, in Dioscorides' *De Materia Medica*, one of the earliest written examples of a systematic pharmacopoeia, the issue of adulteration through both accidental and fraudulent practices was acknowledged, including 40 examples of specific tests on how to detect them (Riddle, 1985, pp.74-76). The majority were organoleptic, detecting adulteration through the senses, but several employed the technologies of the time in chemico-physical tests such as dropping balsam (probably *Commiphora opobalsamum* (L.) Engl., *Burseraceae*) into a woollen cloth and then washing it out which will come clean if the balsam is pure, while its adulterants stain. His contemporary, Pliny the Elder, also wrote of widespread adulteration of food, wine and medicinal remedies in the markets with methods of detecting them (Bush, 2002). In the 2nd century CE the Roman physician Galen also complained about the quality of supplies purchased in the marketplace and suggested using animal testing to evaluate the quality of the antivenom formula Theriac (Thorndike, 1922). This issue was not unique to the WHM tradition with Tao Hongjing in 5th century China complaining that as medicine became more specialised, physicians were no longer able to tell what really mattered to determine a herb's therapeutic efficacy and were often tricked by market sellers into buying medicines that looked impressive but were grown, harvested or stored in poor conditions making them less effective (Liu, 2021). Tao attempted to remedy the situation by expounding upon the currently used pharmacopoeia with detailed commentaries to educate practitioners regarding the attributes to look for in potent medicinal herbal materials. These guides continued to be published throughout China's history with later volumes being compiled by government sponsored teams, including sections on processing, illustrations that may be hand painted for superior accuracy, or

mass published versions using block-printing (Liang & Zhao, 2017) (figure 1). *Materia medica* texts like these are still in use today using colour pictures to assist herbal pharmacists with the identification of their raw materials (Leon & Lin, 2017) but they are of little use once the herbs are processed into powders or extracts.

Figure 1: Illustrations from Chinese Pharmacopoeias
Block printed illustrations (Tang & Cao, 1249, left; work in public domain) and full colour pictures (right, illustration from the *Ben Cao Pin Hui Jing Yao*, 本草品彙精要, 1505; photographed by Eric Brand, reproduced with permission).

The link between adulteration and economic gain became an important issue in the saffron (*Crocus sativus* L., *Iridaceae*) trade where saffron could easily be worth its weight in gold. During the 5th to 15th centuries, an armed guard was employed to inspect supplies entering Venice and in Nuremberg in 1358 this became encoded in the first known food law demanding the death penalty for adulteration of saffron, often by being burned alive with your own adulterated produce (Farhan et al, 2020). In 9th century Baghdad, the medicinal properties of aromatic herbs and spices were recognised and a emerging class of professional

pharmacists developed, writing new pharmacopoeias such as those of Yuhanna Ibn Masawayh (يوحنا بن ماسويه, Romanised: Johannes Mesue), which focused on aromatics and detecting their adulteration (Tschanz, 2003). These pharmacists were held to higher standards than regular spice traders and inspected by government appointed officials, al-Muhtasib (محتسب), to ensure their quality and to curb excessive profiteering.

1.2.2. The Scientific Revolution

The scientific revolution brought in new technologies to enable the identification of correct herbal species, most notably microscopy and taxonomy.

Magnification had been documented by Euclid in his treatise on optics ca. 300 B.C., but it took a further 1900 years before the invention of the compound microscope (Bardell, 2004). The exact inventor remains a mystery but its ability to reveal the details of cellular structure opened up a new world to biologists and botanists. Nehemiah Grew (1682) gave the first vivid descriptions and drawings of magnified plant anatomy in his *Anatomy of Plants* which demonstrated the complexity of plant life and led to the possibility of species being differentiated by their minutiae (figure 2). Improved design and the development of methods such as phase-contrast, differential interference contrast, confocal and super-resolution microscopy, along with advances in staining agents, including fluorescence staining have led to the ability to detect ever increasing detail (Somssich, 2022). It remains one of the most common methods of detecting adulteration in plant materials, in part due to its simplicity that enables people from non-scientific backgrounds involved in the growing, harvesting and trade of herbal materials to understand the images presented in books on herbal materials. As a result magnified images of plant materials are a staple in books on herbal quality (Leon & Lin, 2017).

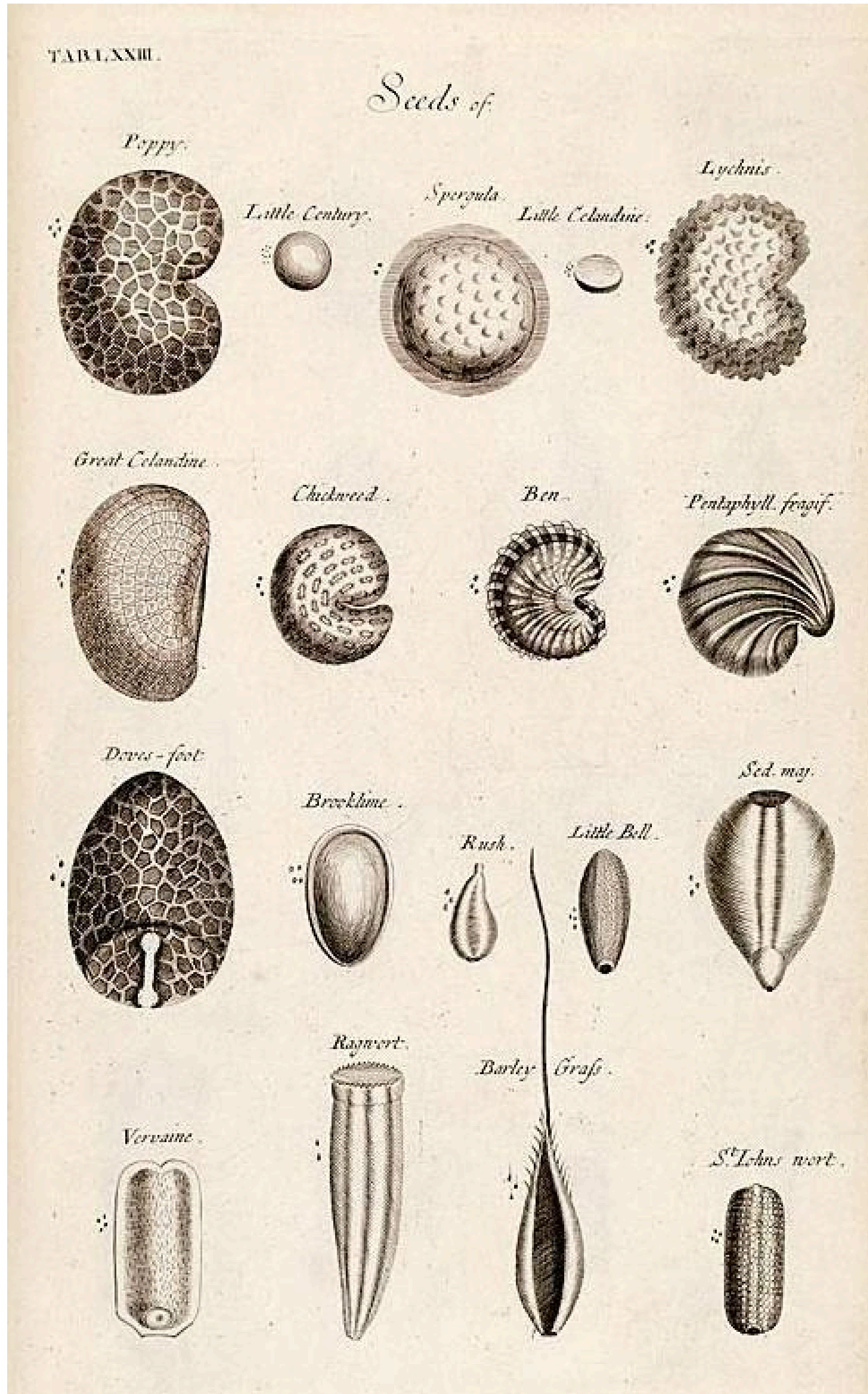


Figure 2: Illustration comparing seeds under magnification from Nehemiah Grew's *Anatomy of Plants* (1682, p.532; work in the public domain)

Linnaeus' taxonomy, first presented in his *Systema Naturae* (1735) and extended to plants in his *Species Plantarum* (1753), also represented a major milestone in herbal quality control. Systematic classification of plants had been attempted before, first by Aristotle's student Theophrastus (370-285 B.C.) who developed a natural system of dividing plants according to the habitat or form (Woodland, 2003). This system was followed, with some improvements and additional classifications, such as the recognition of monocots and dicots by Albertus Magnus (1200-1280), but all following a natural system of classifying according morphological characteristics. By contrast, the Linnaean system was an artificial system constructed by selecting a few characteristics, primarily sexual ones in plants, and categorising them according to these characteristics (Bremer, 2008). All organisms were sorted via a hierarchical system of kingdom, class, order, genus, and species using a binomial nomenclature. Since then, the original five level system has been expanded to include domain, phylum or division, and family to make eight levels, and the sexual system has been largely replaced with phylogenetic characteristics used by the Angiosperm Phylogeny Group (APG IV, 2016), but the Linnaean system intensified research into plants and the binomial nomenclature enables botanists to identify and categorise plants referred to under many different colloquial names according to precise features and place them in relation to others. For those interested in herbal quality assessment, this means not only identifying correct species, but also common adulterants and the unique features that make them similar enough to get confused with the correct species, yet different enough to detect when this has happened.

1.2.3. The Industrial Age

The industrial age saw a similar leap in the technologies that could be used to identify a plant species through the use of chemometrics.

Chromatography originated with Mikhail Tsvet in 1903 who percolated an alcohol and ether extract through a column of calcium carbonate and discovered that the different pigments separated into different bands (Chromatography Today, 2014). Previously it was thought that there were only two kinds of pigments in

plants, chlorophyll and xanthophyll, but his work showed two different kinds of chlorophyll and an additional eight other pigments. Although his work was largely overshadowed by the political events of the early 20th century, it was revived by Kuhn and Lederer (1931) and then further developed by Martin and Synge (1941) who experimented with different solvents, mobile and stationary phases to devise partition chromatography. This earned them a Nobel Prize in chemistry and led to many different specialised forms of this technology.

Another area of technology which enjoyed a renaissance during the 20th century was spectroscopy. Observations on the interaction of light and matter had been recorded since Roman times and developed into a science with Newton's observations in his work on *Opticks* (1730). Further observations by Fraunhofer during the 19th century using a prism spectroscope revealed dark bands in the spectrum of light (figure 3) leading to an understanding that the spectrum is discrete and not continuous (Sack, 2018). A subsequent superior design of spectroscope by Kirchhoff and Bunsen then enabled precise measurements of wavelengths, and using this new precision, they were able to demonstrate that the black bands created by the sun were the same as the line produced by salts introduced to a flame (Sella, 2013). In a series of painstaking experiments, they introduced as many substances as possible to a flame and filtered the resulting light, discovering that this method could identify microgram quantities of elements.

The turn of the 20th century saw the beginning of quantum theory when, in 1900, Max Planck proposed that atoms and molecules can emit or absorb energy in discrete quantities (Planck, 1967). Bohr's (1913) discovery of atomic structure and Einstein's (1917) proposition, that electrons can absorb and emit radiation of a specific frequency that corresponds to the energy required to make their electrons shift from their ground state to an excited state, laid the theoretical foundations for modern quantum theory and new applications for spectroscopy. This enabled the identification of elements within unknown compounds to be identified and, combined with the separating of different compounds with chromatographic analysis, allowed for complete chemometric fingerprinting to be developed.

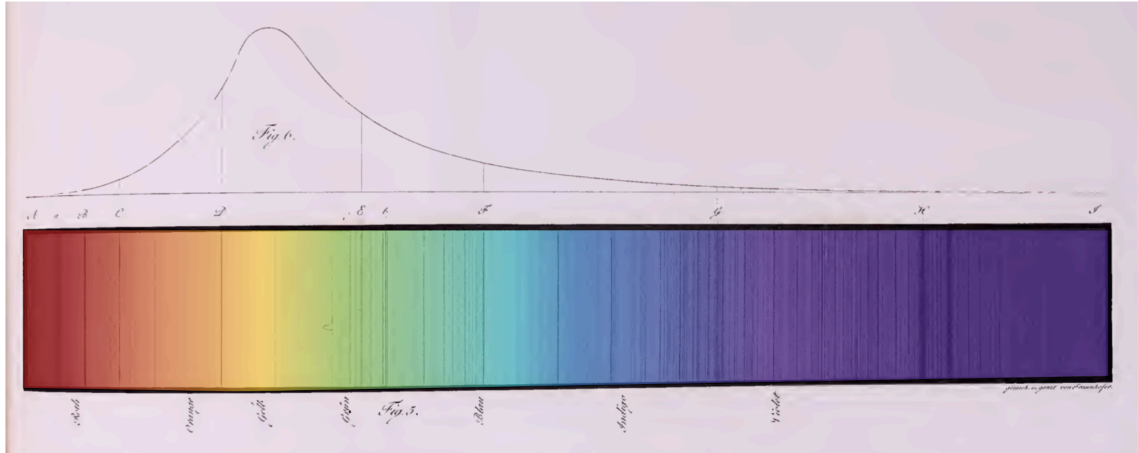


Figure 3: Fraunhofer’s observation of dark lines within the sun’s spectrum (created in Biorender, adapted from Fraunhofer, 1815, p.230; work in the public domain).

The latter part of the 20th century saw another revolution in fingerprinting medicinal plants using genetics. Just a few years after Jeffreys, Wilson & Thein (1985) published the first paper on human genetic fingerprinting, the method was being applied to plant, animal and microorganism identification (Ryskov et al, 1988). These early efforts relied predominantly on restriction fragment length polymorphism technique which was time consuming and so mainly applied to economically important crops (Nybom, Weising & Rotter, 2014). The advent of polymerase chain reaction based technologies by Saiki et al. (1988) soon gave rise to a host of techniques that were simpler, faster and required smaller samples of DNA, expanding and diversifying the potential applications of genetic markers. High-throughput sequencing machines have enhanced the speed of genetic profiling even further, allowing biologists to develop huge databases of genetic information used to identify and authenticate plants of commercial, medicinal and ecological interest.

Although organoleptic testing and microscopic examination is still performed by herbalists, the majority of scientific literature on the detection of adulteration and quality evaluation is based on modern technologies. To assess the efficacy and drawbacks of these methods, a literature review of the most frequently employed techniques in the scientific literature will be undertaken.

Chapter II

2. Literature Review of Existing Methods of Quality Control in Medicinal Herbal Teas

2.1. Search Methods

PubMed and Google Scholar databases were searched for literature on the existing methods of examining herbal teas for quality control. The search terms used were:

PubMed:

((chromatography) OR (spectrometry) OR (spectroscopy) OR (NMR) OR (ultraviolet) OR (infrared) OR ((DNA) OR (genetic) barcoding) OR (bioassay)) AND ((herbal tea) OR (tisane) OR (decoction))
AND ((adulteration) OR (quality) OR (contamination))

With “Full Text” and “English” filters applied, obtaining 616 results on 26th April 2024.

Google Scholar was searched with the term:

allintitle: "herbal tea" (chromatography OR HPLC OR HPTLC OR GC OR spectrometry OR spectroscopy OR MS OR NMR OR ultraviolet OR infrared OR "DNA barcoding" OR "genetic barcoding" OR bioassay)

Obtaining 76 results on 28th April 2024 for a total of 692 papers.

PRISMA literature review process was then conducted (figure 4) and 15 duplicates removed.

Exclusion criteria for screening was that the papers:

- Contained no authors, title, date of publication or abstract (n=1)
- Full text not available in English (n=34)

Inclusion criteria for eligibility was that the publications were:

- Research papers (n=23 excluded)
- Investigating teas, decoctions or water extractions (n=26 excluded)
- Investigating quality control (n=196 excluded)

A total of 397 papers were included in the analysis.

An earlier discussion of 245 papers published before 2020 has been published in Woodley et al. (2021). The new information provided here, reviewing an additional 152 papers with further discussion, extended tables and additional figures, combined with the open access nature of the earlier work, meant that an editor for the journal decided there would be no copyright issues in this update, provided the earlier work was referenced.

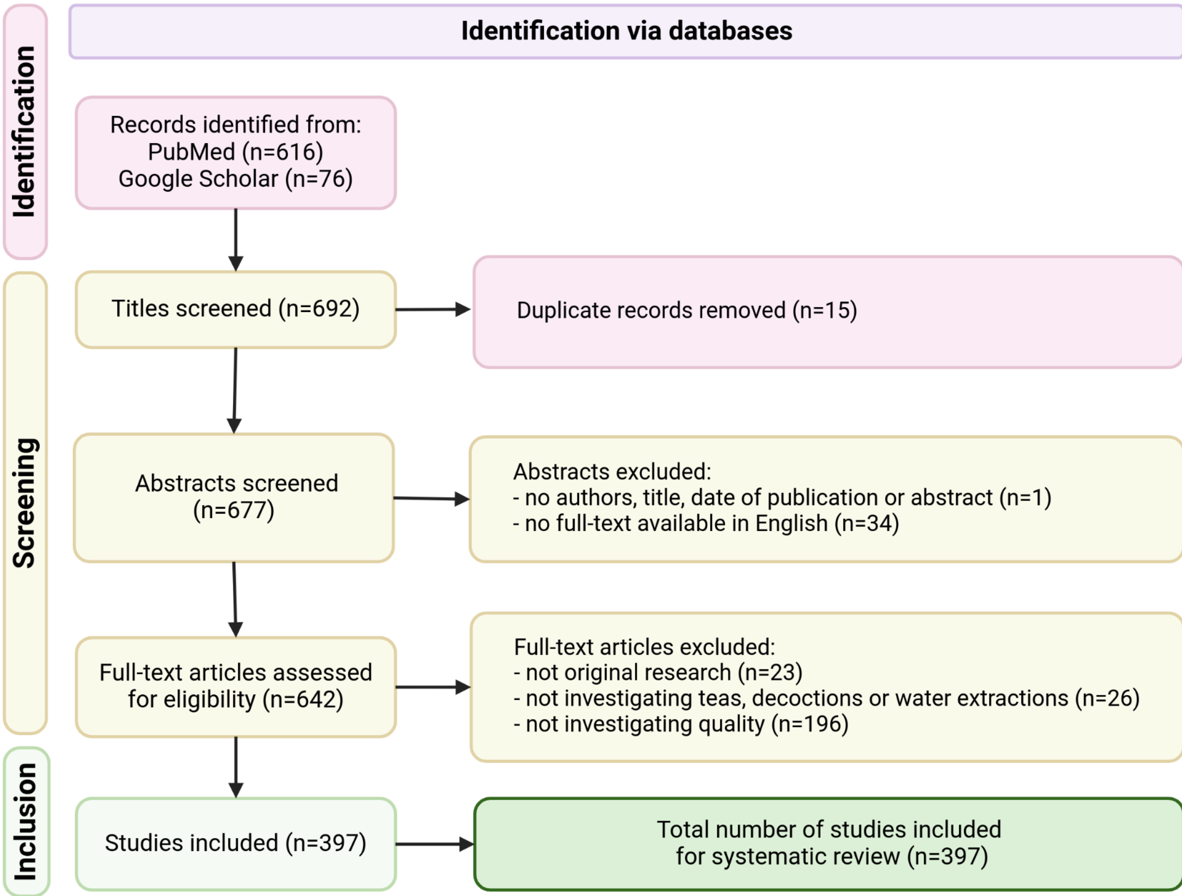


Figure 4: PRISMA Flow Diagram of the Literature Review Process.
Created with Biorender.com, adapted from Moher et al. (2009).

Table 1 reveals a breakdown of the methods and their popularity:

Method	Number of Studies	Percentage
Liquid Chromatography (LC)	303	76%
Gas Chromatography (GC)	34	9%
Thin Layer Chromatography (TLC)	16	4%
Mass Spectrometry (MS)	240	60%
Nuclear Magnetic Resonance (NMR)	17	4%
Ultraviolet-Visible Spectrophotometry (UVS)	88	22%
Infrared Spectroscopy (IRS)	16	4%
Optical Emission Spectrometry (OES)	8	2%
Atomic Absorption Spectrometry (AAS)	7	2%
Deoxyribonucleic acid (DNA) Barcoding	26	7%
Biological Assays	65	16%

Table 1: Popularity of Methods Used in Quality Control of Herbal Teas

From this we can easily see that chromatography is the most popular technique with liquid chromatography being the most favoured method overall, followed by mass spectrometry. These two techniques are far ahead of all others. This is mainly due to the fact that both of these techniques can be used to separate as well as analyse which is a very useful quality when analysing complex mixtures as is generally found in herbal mixtures such as herbal tea blends. Each individual method shall now be described with its advantages and disadvantages and a summary of what the literature revealed.

2.2. Chromatography

Chromatographic methods are some of the most popular for identifying adulteration of herbs due to the fact that they can separate complex compounds into their constituent components, creating a chemical fingerprint. All chromatographic methods achieve this through the same principle of separating a mixture by distributing its components between two phases: a mobile phase, which carries the components through a medium, and a stationary phase that remains fixed in place causing the various constituents to separate as they migrate at different speeds (Liu, 2011, pp.129-130). The fingerprint that this creates can then be compared against an authenticated sample or a suspected adulterant.

This reveals one of the main limitations of chromatography: without a reference standard to compare the peaks to, it is limited in its ability to identify or analyse the structure of the eluted compounds, for which spectroscopic methods are normally required. This is clearly illustrated by Xiao et al. (2022) who used LC to find specific constituents of Three Whites Decoction (*San Bai Tang*, consisting of *Atractylodes macrocephala* Koidz, *Asteraceae*; *Paeonia lactiflora* Pall, *Paeoniaceae*; *Poria cocos* Fr. (1822), *Polyporaceae* and *Glycyrrhiza uralensis* Fisch. ex DC., *Fabaceae*) and correlate these peaks with their antioxidant capacity, determined by a 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, but were only able to list the relative antioxidant capacities of peaks and not identify what those peaks were. This is also the strength of chromatography, for many forms of spectroscopy require some prior separation before analysis, making it a common preparatory step to further analysis. The combination of chromatographic and spectroscopic techniques is referred to as a hyphenated technique and is very popular in natural product research for its ability to simultaneously separate and analyse.

There are many different types of chromatography, usually named after one of their phases. Liquid and gas chromatography are named after the mobile phase, while paper and thin layer chromatography refers to the type of stationary phase used. Of particular interest in the analysis of herbal medicine are “High

Performance" varieties of liquid and thin-layer chromatography which use automated techniques to get more accurate, reproducible readings. Each technique has its own advantages and disadvantages.

2.2.1. Liquid Chromatography

This is one the most popular techniques found in the literature with a total of 303 papers included in the review. While LC simply refers the state of the mobile phase, the majority of papers analysing herbs used "High Performance Liquid Chromatography" (HPLC) or "Ultra-High Performance Liquid Chromatography" (UPLC) where the liquid mobile phase is pumped through a solid adsorbent stationary phase in a column under high pressure enabling sufficient resolution to be used quantitatively (figure 5), while LC is primarily used as a preparative technique for other forms of analysis. The only difference between the HPLC and UPLC is the amount of pressure used and the resolution achieved, UPLC being a later development of HPLC with superior pressure, resolution and speed (Dyad Labs, 2018).

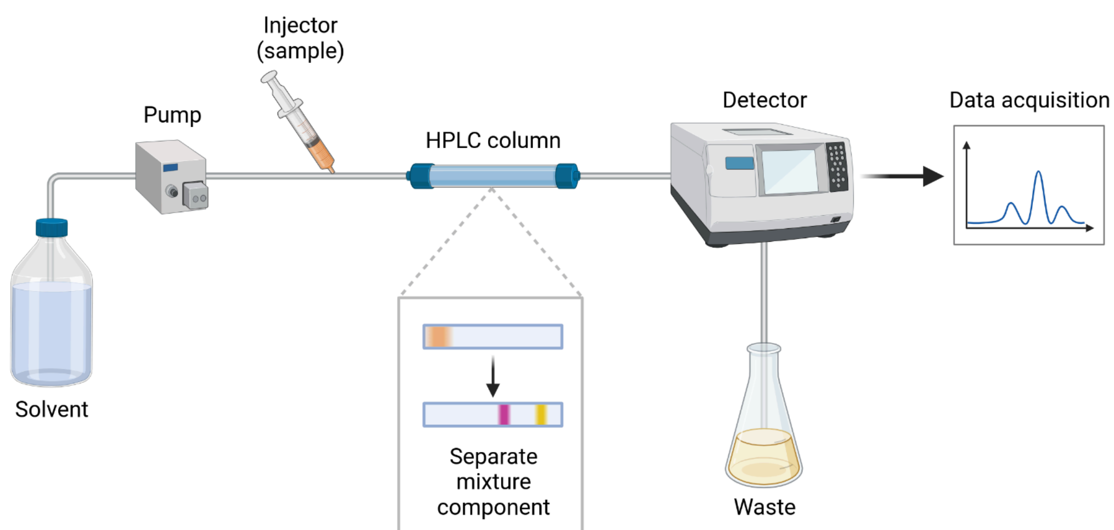


Figure 5: Overview of HPLC (created with BioRender.com)

HPLC and UPLC account for 256 of the 303 papers (84%) with only 56 (18%) using the standard form (9 using both). It is especially useful for quality

control because of the process being largely automated which makes the results highly reproducible. They are usually presented as a line graph where the peaks can be compared to show the presence of various compounds and their relative quantity making it ideal for generating a chemical “fingerprint” against which other samples can be compared (figure 6).

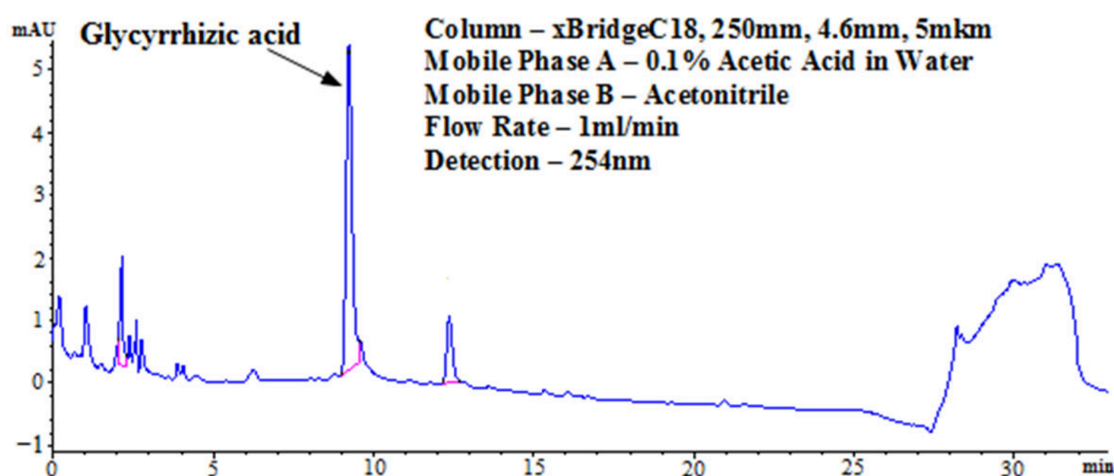


Figure 6: Example of an HPLC Chromatogram of *Glycyrrhiza glabra* (Zhurinov et al., 2023: reproduced with permission).
Glycyrrhizic acid is shown as a peak of interest.
G. glabra will be used for all chromatograms and spectrograph examples due to the relevance of this herb to the remaining thesis, and to enable comparison.

This ability to detect specific compounds by their elution peaks is reflected in the overwhelming majority of the papers using LC to identify quality markers in order to standardise market products.

Purpose	**Number of Studies**
To identify quality markers	231
To detect pyrrolizidine alkaloids	23
To detect adulterant species	15

To detect mycotoxins	15
To detect pesticide residues	11
To detect tropane alkaloids	9
To detect adulterant drugs	5
To detect heavy metals	3
To polycyclic aromatic hydrocarbons (PAH)	3
To detect adulterant toxins	3
To detect adulterant parts of correct plant species	1

Table 2: Uses of Liquid Chromatography in the Analysis of Herbal Teas

The primary use of LC is to separate out compounds for further analysis with spectroscopic techniques for which HPLC features ultraviolet (UV) detection as an integral part of the system to detect when different compounds are eluting from the column. However, 254 (84%) of the studies using LC also hyphenated it with another spectroscopic technique such as MS, NMR or IRS. MS was the most popular with 204 papers (67%) combining it with LC while only 11 used NMR and 9 of those used it in conjunction with MS to provide additional data. 6 papers used IRS spectroscopy with LC, and in all cases this was used alone, without any other spectroscopic technique.

LC seems the obvious choice in analysing herbal teas since they are naturally delivered in liquid form. It therefore does not require the solution to be subjected to additional processing, preserving less stable compounds while GC is superior at analysing more volatile compounds that are unchanged by heat (Ng, 2017). It is especially useful for analysing samples containing salts or carrying a charge which cannot be analysed with GC (Painter, 2018). It also cannot analyse inert substances that do not dissolve in the usual solvents used making it unable

to detect the addition of substances like sand, silica and maltodextrin (Gafner et al., 2023).

Illustrating these limitations, Karioti et al. (2014) used procyanidin content to develop detailed reports of the chemical composition of *Tiliae flos* (*Tilia cordata* Miller, *T. platyphyllos* Scop. & *T. x vulgaris* Heyne, *Tiliaceae*) but could not record the volatile content, despite these being important markers of quality. Yi et al. (2007) also had difficulty detecting volatile carvacrol and Z-butylidenephthalide in a quality control test for *Dang Gui Bu Xue Tang* (a combination of *Astragalus membranaceus* (Fisch.) Bge., *Fabaceae* and *Angelica sinensis* (oliv.) Diels, *Apiaceae*) despite these being important components of the effects of *A. sinensis*.

2.2.2. Gas Chromatography

GC works along the same principles as LC, except that the mobile phase is vaporised and carried by an inert gas such as helium, hydrogen, nitrogen or argon (Drawell, 2023) into the stationary phase which is located inside an oven where the temperature can be controlled (Turner, 2020) (figure 7). The stationary phase is usually a liquid coated on a solid support contained within a glass column which elutes the components of the mobile phase at different rates depending on their different interactions with the stationary phase. The resulting extraction is then passed to a detector which displays the results as a chromatogram. Various types of detector can be used, such as flame ionisation detectors, electron capture detectors, and mass spectrometers, enabling the simple chromatogram, similar to that obtained by LC (figure 6) to be combined with three-dimensional information on the analytes to be obtained.

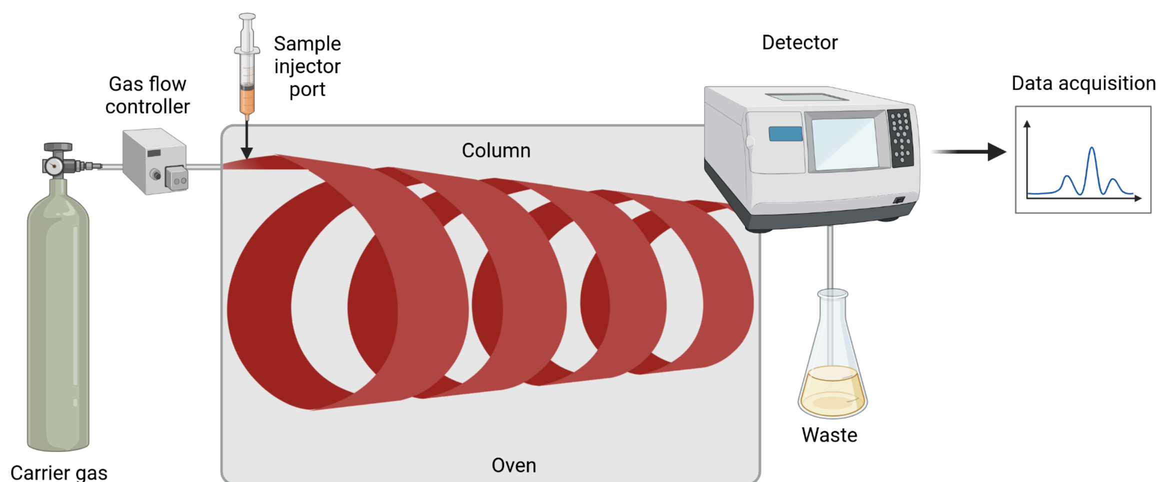


Figure 7: Overview of Gas Chromatography (created with BioRender.com)

Of the 34 studies included in the review that used GC, 27 (79%) of them connected it with MS. Of the remaining 7 studies, two used a flame ionisation detector (Farajzadeh et al. 2020; Ugoeze et al., 2023), two used an ion mobility spectrometer (Wang et al., 2021; Li et al., 2024), one used an e-nose (Qin et al., 2024) and one used both an electron capture detector and a flame photometric detector together (Bicci et al., 2003). Only one study used results based on a chromatograph without any additional detector (Naithani & Kakkar, 2004).

Purpose	Number of Studies
To identify quality markers	20
To detect pesticide residues	9
To detect heavy metals	5
To detect PAH	3
To detect adulterant drugs	1

Table 3: Uses of Gas Chromatography in the Analysis of Herbal Teas

One of the most notable trends in the literature of gas chromatography is that there are more papers dedicated to detecting pesticides than with any other technique. Out of 17 papers that searched for pesticides, 9 used GC (53%), with a similar proportion of studies detecting PAH also using GC (3 out of 6, or 50%). This comprises 18% of the total GC papers, compared to the other 3 which used LC, comprising only 1% of the total LC papers. This is because it can be both selective and sensitive with simultaneous detection of many residues at lower concentrations than other techniques (van der Hoff & van Zoonen, 1999) giving it an important specialist role in the detection of volatile compounds that includes many environmental pollutants.

GC is also useful in the analysis of storage methods because the volatile components are the most affected by storage. One example of this is Han et al. (2023), who used GC-MS to compare the effects of different drying methods on the volatile oil content of flower scented tea (*Bletilla striata*, (Thunb.) Rchb.f. (1878), *Orchidaceae*) in order to preserve the scent. They found that freeze drying without steam fixation retained the most volatile oils, compared to air drying and stove drying. This method retained the best scent and flavour profile, while also retaining the most flavonoids, polysaccharides and phenolic components, as well as having the best antioxidant and antibacterial profiles.

2.2.3. Thin Layer Chromatography

TLC uses a thin layer of material made of small particles for the stationary phase. This causes the mobile phase to rise along the surface via capillary action at a constant rate and the compounds adhere to the vacant spaces (termed adsorption) at differing rates depending on their relative solubility in the mobile phase and their affinity for the stationary phase (figure 8). These then remain in place after the mobile phase has evaporated and can be derivatized by various treatments to make all the analytes detectible (CAMAG, n.d).

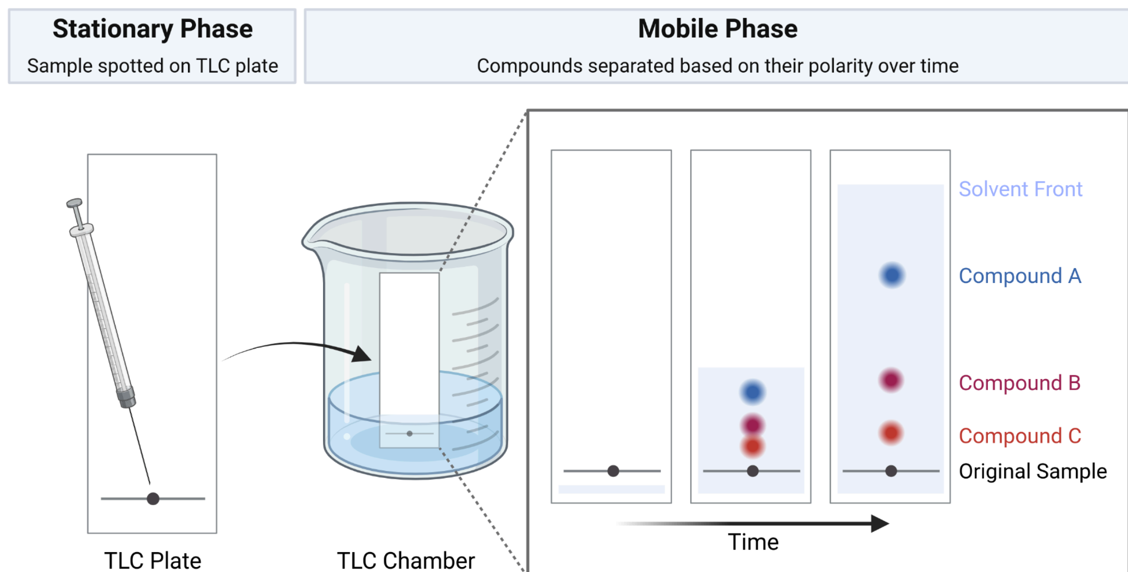


Figure 8: Overview of TLC (created with BioRender.com).

TLC results can then be displayed visually in a series of compact bands as the mobile phase travels up the plate which can easily be compared to reference standards or other samples on the same plate to obtain a relative picture of quality or adulteration (figure 9). This also facilitates a high throughput with roughly half the development time of HPLC and the option of developing multiple samples at the same time. The small size of the mobile phase means that it is also relatively inexpensive on materials. This has facilitated the growth of a High Performance Thin Layer Chromatography (HPTLC) atlas of plants (HPTLC Association, 2020) which can be used for reference standards for all herbal products.

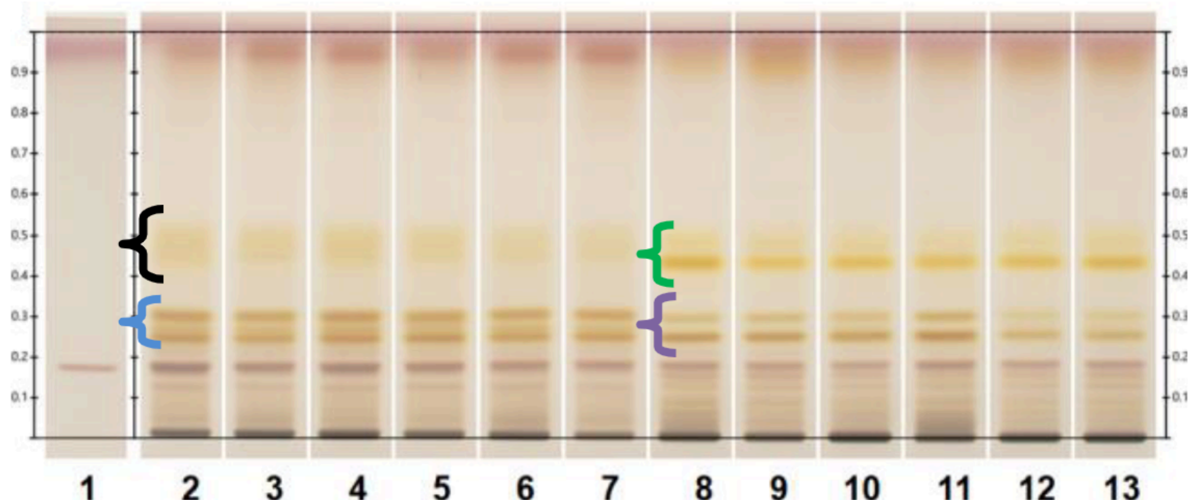


Figure 9: Example of an HPTLC chromatogram (HPTLC Association, 2019: reproduced with permission).
Track 1 shows glycyrrhizin used as a reference standard, tracks 2-7 show *G. glabra* and tracks 8-13 show *G. uralensis* with additional quenching zone at 0.43 Rf that can enable species identification.

TLC is a less popular method of analysing herbal teas than LC with only 16 papers included in the review utilising this method but deserves a thorough examination because its use for analysing herbal materials is expanding.

The most common reason for using TLC was to find identifying quality markers to confirm a correct species, in 10 of the papers (63%); of the others, the uses were as varied as detecting adulterant species, adulterant drugs or moulds and mycotoxins. Of those detecting adulterant species Guzelmeric et al. (2017) compared HPLC with HPTLC to detect adulteration of chamomile (*Matricaria chamomilla* L., *Asteraceae*) with other asteraceae (*Bellis sp.*, *Anthemis sp.*, *Tanacetum sp.* & *Chrysanthemum sp.*) and found HPTLC to be the superior method; Shen et al., (2012) analysed Chinese star anise (*Illicium verum* Hook.f., *Schisandraceae*) for adulteration with neurotoxic Japanese star anise (*Illicium anisatum* L. *Schisandraceae*); and Lam et al. (2016) used HPTLC to differentiate snow chrysanthemum (*Coreopsis tinctoria* Nutt., *Asteraceae*) from regular chrysanthemum (*Chrysanthemum × morifolium* (Ramat.) Hemsl., *Asteraceae*). Two studies used HPTLC to screen for adulterant drugs, including Miller & Stripp

(2007) who screened New York outlets selling Chinese herbal medicines for prescription drugs and found some in patent pills but none in teas, while Wang, Y. et al. (2023) used HPTLC combined with a bioluminescent bacterial solution for derivatization to detect hyperlipidemic drugs in a selection of herbal teas. Two more studies used HPTLC to detect mycotoxins, with Halt (1998) using TLC to find 1 of the 11 teas contaminated with *Aspergillus flavus* Link, *Trichocomaceae*, and trace amounts of ochratoxin, while Haq et al. (2024) used TLC to detect aflatoxin and ochratoxin in herbal teas, but then used HPLC to quantify these findings. These show the potential for TLC to be almost as varied as for liquid chromatography but also reveal some of its limitations.

The main limitation of TLC and HPTLC is that they are mainly qualitative and only semi-quantitative. This means that while it can determine the presence of a compound, it is difficult to determine the amount. This is demonstrated in the study by Haq et al. (2024) who used TLC to determine the presence of mycotoxins and HPLC to quantify them. Quantification with TLC can be attempted through the appearance of faded or absent bars on a chromatogram which might alert the analyst but intelligent adulterers can attempt to replace these with a purified chemical marker from a cheaper source that elutes onto the plate at the same point as the desired compound (Gafner et al., 2023). You et al. (2022) found this issue in 5 of 14 turmeric (*C. longa*) supplements which were adulterated with curcumin from synthetic sources and detected only by the use of ^{14}C radiocarbon analysis via spectroscopic methods. Some of these dyes are not only ways to increase the value of poor products but can be a significant safety issue. A number of turmeric products from Bangladesh were found to be deliberately contaminated with lead chromate which mimics the brilliant orange-yellow colour or fresh turmeric but results in increased lead and chromium in the blood of people who consume it (Forsyth et al., 2019; Newby 2023) and a similar outbreak was reported in the U.S.A. among cinnamon containing drinks for children (U.S. Food and Drug Administration, 2024). While this could be easily detected through TLC, it shows that adulterers often do not consider the consequences of their actions on the end users of their products and if toxic dyes can best mimic the compounds they wish to emulate, then they will likely be used.

As can be seen from these papers, a High Performance variety of TLC has been developed similar to the High Performance version of LC. It is a relatively new technique evidenced by the fact that far fewer papers have been written using it, with only 7 included in the present search, with all but one (Ramachandran et al., 2012) being written in the last 8 years. Its high throughput speed, low cost and options for analysing the results visually or by computer readout means that it is rapidly becoming the technique of choice for verifying species and detecting adulteration in herbal medicines (Frommenwiler et al., 2019; Omicron UK, 2018). The number of studies found in the present search belies the popularity and potential of HPTLC in herbal medicines research due the fact that it was restricted to teas while most studies investigate supplements using other forms of extraction and raw herb materials. If the Pubmed search is expanded to include herbal medicine that is not in tea form using the phrase "(HPTLC) AND (herbal medicine) AND ((adulteration) OR (quality) OR (contamination))" then 196 results were returned on 28th July 2024. Some of these may include raw materials used in teas without specifically mentioning the term and there is no reason why HPTLC cannot be used with water extractions, so the literature using this method is almost certainly larger than this search implies and likely to expand in coming years.

2.3. Spectroscopy / Spectrometry

Spectroscopy is the study of how radiated energy and matter interact; spectrometry is the measurement of this interaction (Verichek Tech Services, 2016). The common principle to all methods involves projecting some form of energy at the matter being analysed which absorbs it at a specific wavelength creating an excited state and emits it when it returns to its ground state. The specific wavelengths absorbed or emitted provide detailed qualitative and quantitative information about the substance.

2.3.1. Mass Spectrometry

MS measures the mass-to-charge ratio (m/Q) of ions in order to plot a mass spectrum which reveals the elemental or isotopic signature of a molecule or

compound and enables its quantification. First the substance under investigation is ionised in order to make it susceptible to influence by a magnetic field, then the charged ions are accelerated to a known speed and deflected using a magnetic field (Clark, 2019) (figure 10).

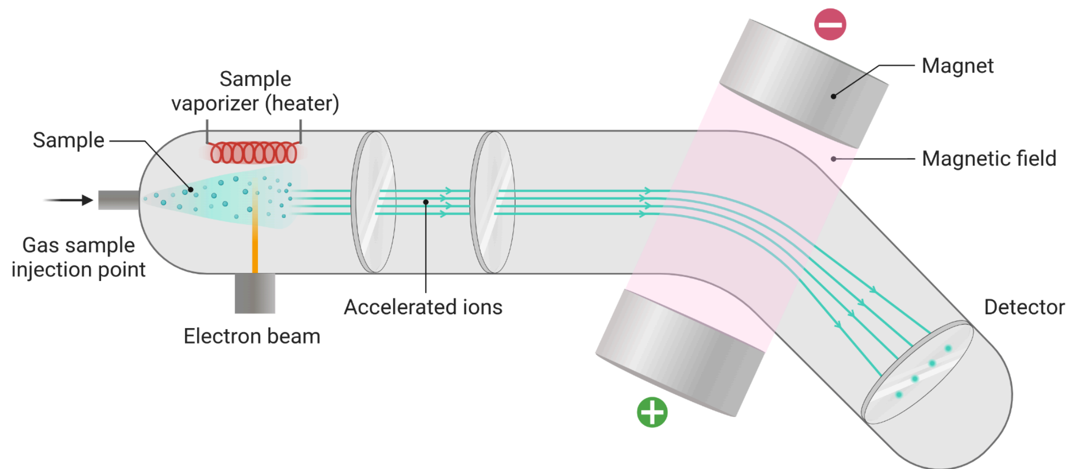


Figure 10: Functional Diagram of a Mass Spectrometer (created with BioRender.com).

The degree to which the magnetic field deflects the ionised molecules from their course is detected and used to determine the molecular weight (figure 9).

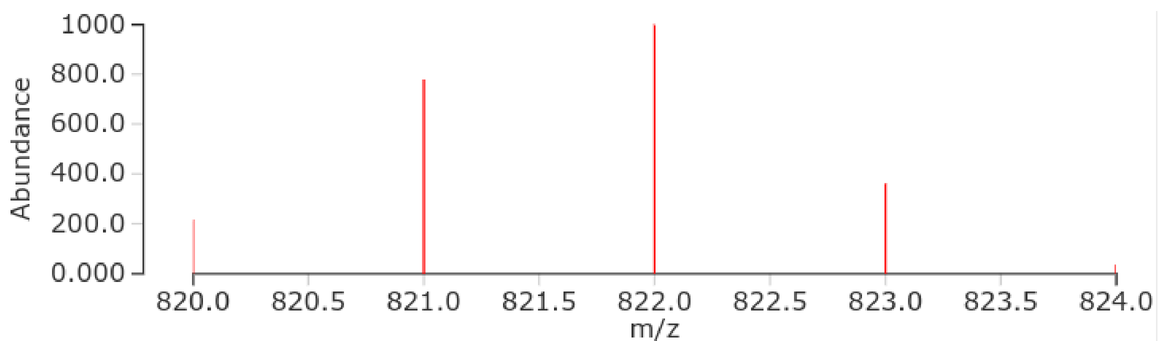


Figure 11: Mass Spectra of Glycyrrhizin, the main constituent of *Glycyrrhiza spp.* (Sawada, Matsuda & Hirai, 2008: reproduced under licence).
The base peak corresponds to glycycrrhizin’s molecular weight of 822.9 g/mol.

This process can be further enhanced by adding an additional mass analyser to filter the ions and take further measurements as they travel, known as tandem mass spectrometry, MS/MS or MS^2 (Mittal, 2015). The most common forms of tandem mass spectrometry involve measuring the time it takes to pass through the detector, their Time of Flight, based on the principle that heavier ions travel more slowly than lighter ions (Gilhaus, 2005), and by selecting specific ions based on the stability of their trajectories as they pass through oscillating electrical fields generated by four cylindrical rods (a quadrupole). Quadrupoles are often set up in a triplicate formation, called a Triple Quadrupole, where the first quadrupole is used to select certain ions, the central quadrupole acts as a collision chamber to fragment them and the last selects the fragments to be analysed (Schreiber, 2017). These setups are quite common in biomolecular research of complex molecules.

MS is the most popular spectroscopic method in the reviewed papers for analysing herbal teas with a total of 240 papers using some form of this method. This is mainly due to its powerful ability to identify and characterise unknown compounds which gives it an enormous edge compared to chromatographic techniques when analysing a previously undescribed plant compound, or forensically analysing an adulterant substance whose identity is uncertain.

Purpose	**Number of Studies**
To identify compounds as quality markers	165
To detect pyrrolizidine alkaloids	23
To detect adulterant species	15
To detect heavy metals	13
To detect mycotoxins	10
To detect pesticide residues	10
To detect adulterant toxins	7

To detect adulterant drugs	4
To detect PAH	3

Table 4: Uses of Mass Spectrometry in the Analysis of Herbal Teas

We can see this reflected in the main usage being similar to LC, to identify and characterise marker compounds for future quality control. One of the main differences is that many of these studies are characterising a plant and identifying markers for the first time which can then be found with a simple chromatography test, or where a forensic analysis of herbs was being undertaken without prior knowledge of what the researchers were searching for. However, the cost is high, approximately $500,000 initial layout with $250,000 yearly maintenance fees (El-Khoury, 2018) making them the domain of specialist laboratories dedicated to research and not for a cost-conscious quality control centre of a small herbal business.

MS is usually combined with a chromatography step to separate the compounds before analysis. 225 (94%) studies included in this paper combined it with some form of chromatography with LC being the most popular (204, or 91%), followed by GC (27, or 12%) and rarely with TLC (6, or 3%). These preparatory steps can have considerable influence on the results. A comparison of MS studies searching for mycotoxins in conventional tea (*C. sinensis*) demonstrates this: Reinholds et al. (2020) found that almost 97% of black teas, 88% of green teas and 100% of oolongs included in their study contained quantifiable levels of fungus with all Puerh samples containing mycotoxins despite having the lowest levels of fungal contamination, while Monbaliu et al. (2010) analysed 91 teas and found only one sample of Ceylon melange to be contaminated with no mycotoxins in the drinkable products despite also testing Puerh. The testing of Puerh is significant as this tea is fermented and known to contain high concentrations of mould (Sedova, Kiseleva, & Tutelyan, 2018; Pandey, Samota & Sanches Silva, 2022) showing that the sensitivity of MS makes it vulnerable to detecting differences in the preparatory steps as well as in the products themselves. In this example, Monbaliu et al.

(2010) used a UPLC separation technique, while Reinholds et al. (2020) used 2-dimensional LC. 2-dimensional LC has been developed to overcome the limitations of 1-dimensional techniques in the separation of compounds closely related in polarity and charge, such as may be found in food contaminants. This may explain how Reinholds et al. (2020) were able to find mycotoxins in Puerh, where Mobaliu et al. (2010) were not. Standardised methods of preparation can mitigate this, such as that proposed by Zhou, H. et al. (2021) who developed a method of extracting aflatoxins with acetonitrile and purifying with mixed fillers to develop a more sensitive method of detection with LC-MS. Hu, W. et al. (2023) also demonstrated that it may be possible to avoid this issue entirely by replacing chromatographic separation with paper spray ionisation combined with a portable mini-MS, allowing for direct analysis of complex samples.

2.3.2. Nuclear Magnetic Resonance

NMR is one of the preeminent techniques for determining the structure of organic compounds (Aryal, 2020). By placing molecules in a strong magnetic field the nuclear spin can be made to align with the magnetic force. The energy transfer required to make this happen occurs at a wavelength that corresponds to radio frequencies and when the spin is reversed it releases energy at this same frequency which can be detected as a radio signal and used to provide information about individual molecules and their functional groups (figure 12).

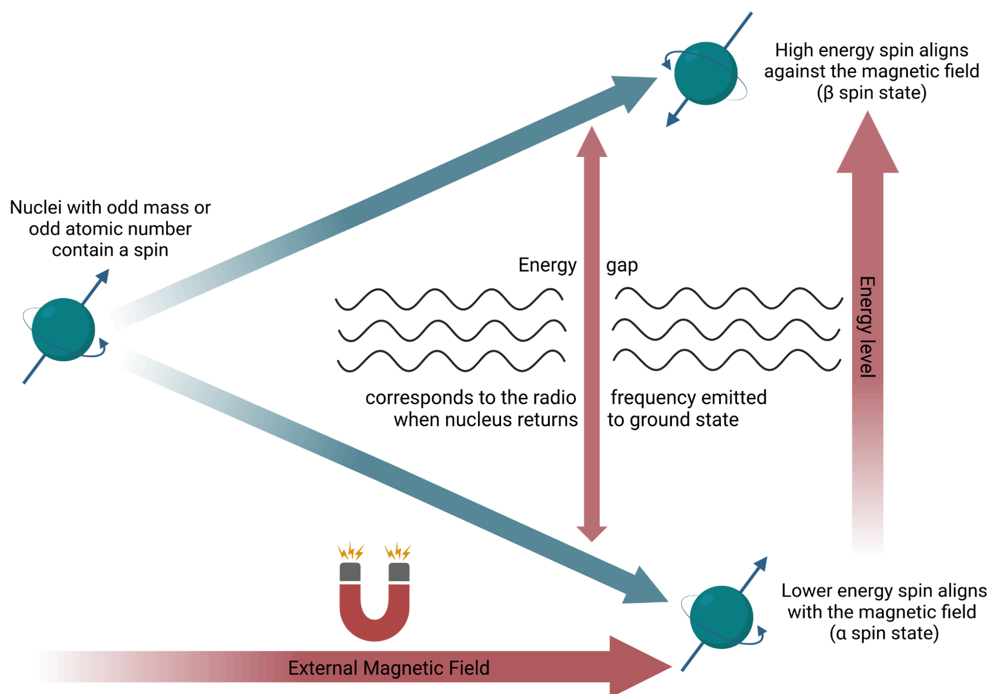


Figure 12: Principle of NMR Spectroscopy (created with BioRender.com).

It can provide information not just on the structure of the resonating atoms but their quantity, their neighbouring atoms and relative positions (Zinkel, 2019). By examining the peaks and cross-peaks of the readout which relate to the chemical shifts, multiplicity, coupling constants and integration characteristics of the molecules (figure 13), it is possible to build a complete 3D structure (Emery Pharma, 2018).

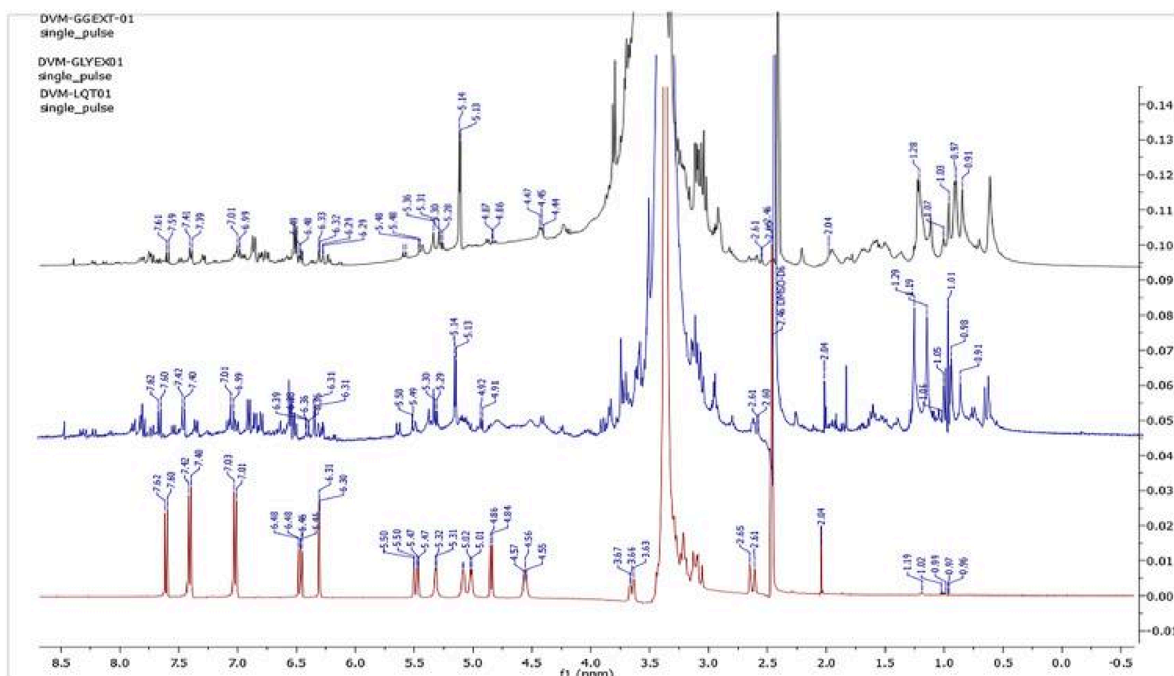


Figure 13: Example of an NMR readout of *G. glabra* (Danaboina et al., 2023; reproduced with permission).
Top line (black) shows *G. glabra* crude extract, middle line (blue) shows *G. glabra* extracted using a targeted enrichment method and the bottom line (red) shows liquiritin, the target compound, for comparison.

NMR is capable of determining the molecular structure of an unknown compound in even greater detail than MS but so is its expense with initial costs often exceeding $1 million although smaller, lower resolution benchtop versions are available for under $100, 000 (Reisch, 2015). NMR is also relatively insensitive requiring 10-100 times the amount of sample than MS, LC or GC to take a measurement and will still usually only return information on 50-200 compounds with concentrations >1 µM, compared with thousands that can be obtained by MS with concentrations as low as 10 - 100 nM, making it unsuitable for detection of trace metabolites (Emwas et al., 2019).

This is reflected in the literature with only 17 papers utilising NMR with more than half of these (10, or 59%) also using MS and often adding NMR to acquire additional information when MS was not specific enough. All but one study was searching for specific quality markers. The only paper that did not employ NMR to

identify structures was exploring a novel method to identify the composition of herbal mixtures without the use of prior separative techniques (Marchetti et al., 2020), building on the work of Booker et al. (2014) who used the same technique to analyse chemical variability of turmeric (*C. longa*) along value chains. Given the expense generally involved, it does not seem a likely method to become popular in the herbal tea industry but could if the results can be replicated with the cheaper benchtop NMRs.

2.3.3. Ultraviolet-Visible Spectrophotometry

UVS works by passing wavelengths of light in the UV (100 - 400 nm) and visible (400 - 700 nm) spectrums through a sample and measuring the light received by a sensor on the other side (Raja & Barron, 2020) (figure 14). The wavelengths not transmitted indicate the photons have exactly matched the energy band gap required to promote a molecule from its ground state to an excited state and have been absorbed. This provides an absorbance spectrum readout that can be compared to published literature for qualitative identification, or used quantitatively, measuring the concentration of a sample using Beer's Law if the absorptivity of a substance is known, or calculated with a calibration curve (Venton, 2020). The light source is usually a deuterium lamp that emits light in the 170–375 nm UV spectrum, and a tungsten filament lamp, which produces light from 350–2,500 nm, for the visible range which is then filtered for specific wavelengths. Alternatively a diode-array which can measure a whole spectrum of light in a single run can also be used. It is particularly useful in natural product research for determining the level of conjugation in organic molecules due to increased conjugation being associated with a lower energy gap, thus absorbing lower energy longer wavelengths of UV and visible light (Ashenhurst, 2022).

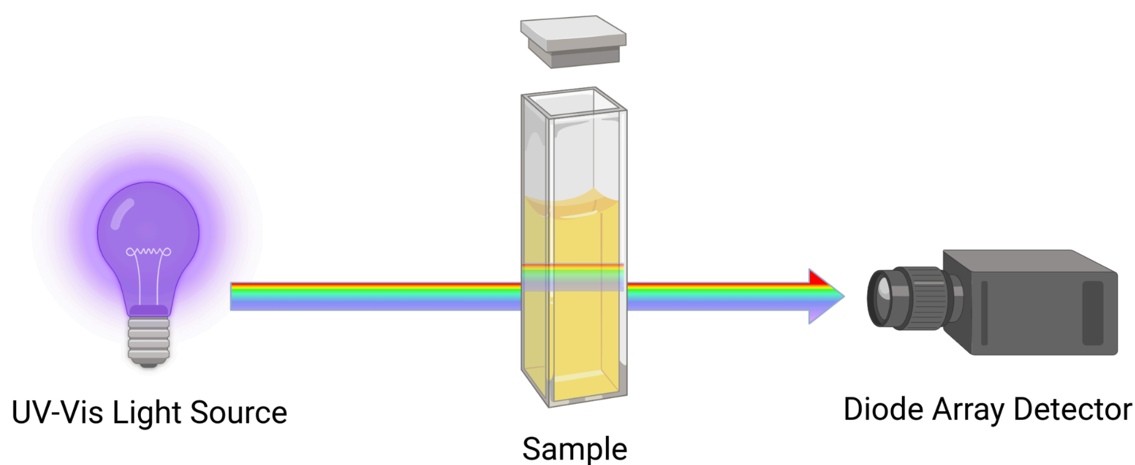


Figure 14: Principle of Ultraviolet-Visible Spectrophotometry (created with BioRender.com)

Of the 88 studies included in the review, 84 (95%) coupled UVS with HPLC. This is a common combination and most HPLC systems feature UVS as an integral part of the system to detect compounds not visible light and enable their quantification by comparison to a reference beam (Taylor, 2015). This means that the other HPLC studies will have also used UVS but did not discuss the results in their papers. It is also integrated into most HPTLC devices where it enables detection of analytes invisible under normal light (Camag, n.d.) (figure 15).

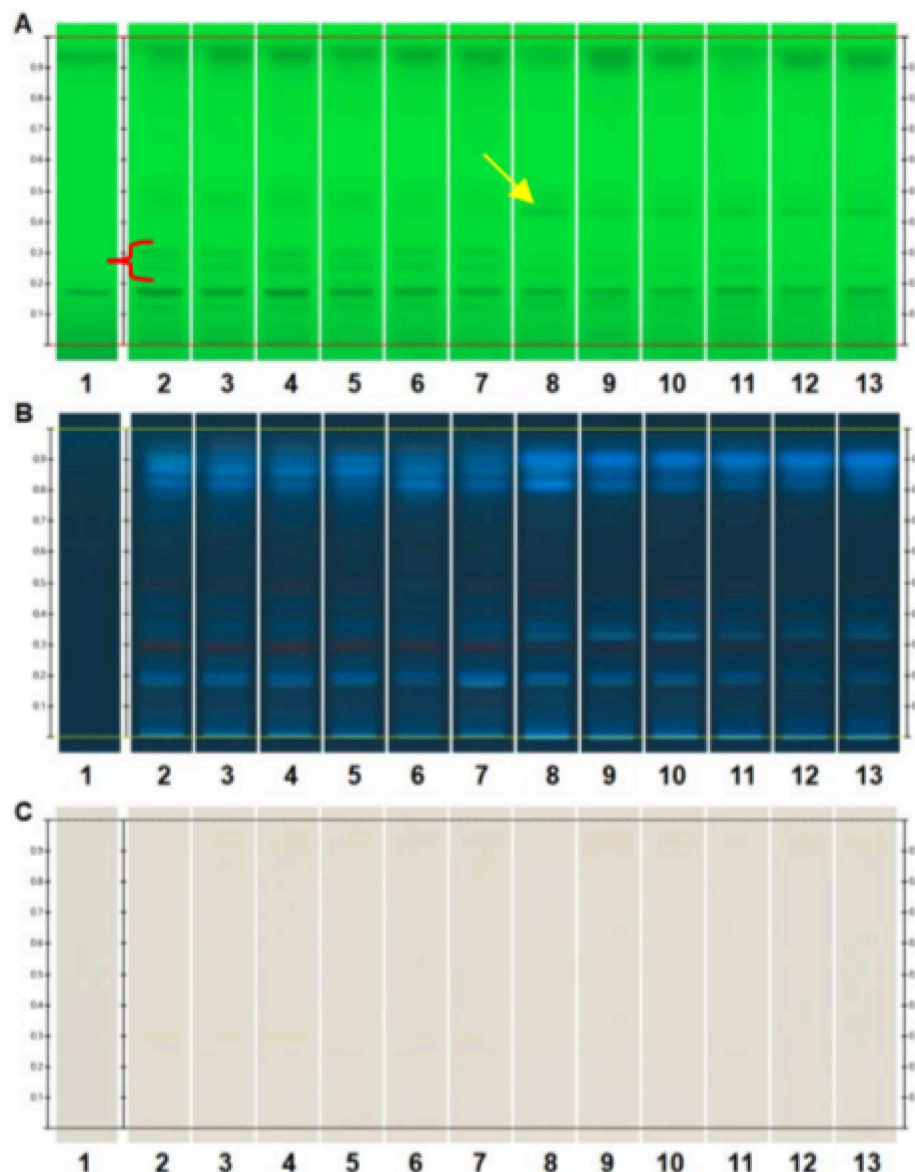


Figure 15: Comparison of Underivatized HPTLC Chromatograms of *Glycyrrhiza spp.* Under Normal (C) and Ultraviolet Light (A & B) (HPTLC Association, 2019: reproduced with permission).

The reference compound, glycyrrhizin (track 1) is only visible under UV 254 nm.

One of the major limitations of UVS is its reliance on published literature. Most studies (84 or 95%) compared their analytes against known constituents. Among other uses, Zhao et al. (2012) suggested UVS to detect adulteration of Chinese medicines with drugs. They successfully identified 11 antihypertensive drugs mixed into herbal medicines, but since they only used spiked samples, they had the references to hand. Herbal medicines are increasingly adulterated with

designer analogues of drugs made to avoid detection, meaning LC-MS and NMR are often necessary for their identification (Patel et al., 2014; Haneef et al., 2013). Zhang, R. et al. (2020) and Özzeybek (2020) employed UVS to detect pesticide residues, which constitutes an application more appropriate to real world situations since the pesticides the farmers use can be supplied to the quality control centres and used as reference standards. Zwerger et al. (2023) also used UVS to detect pyrrolizidine alkaloids from ragwort (*Senecio spp*. L., *Asteraceae*), also using MS to identify them initially, but which could then be analysed for their UV spectra and detected quickly and cheaply with a supercritical fluid UPLC method coupled with UVS.

Another limitation of UVS is that the wavelength of interest can be absorbed by non-target analytes resulting in an overestimation of quantities (Gafner et al., 2023). This can be taken advantage of by fraudulent sellers who may adulterate an extract with another substance that has a similar absorption spectrum, such as adding anthocyanin-rich extracts to bilberry (*Vaccinium myrtillus* L., *Ericaceae*) or elderberry (*Sambucus* spp.) extracts.

2.3.4. Infrared Spectroscopy

The setup of IRS is similar to UVS spectroscopy, passing specific wavelengths of radiation, this time in the infrared spectrum (780 nm - 1 mm), through a sample and measuring how much is transmitted to the other side. The effect of infrared light is very different, interacting with the bonds of molecules causing them to stretch or bend in symmetric or asymmetric ways when energy is absorbed from a particular wavelength depending on the functional groups within the molecule (Reusch, 2013) (figure 16).

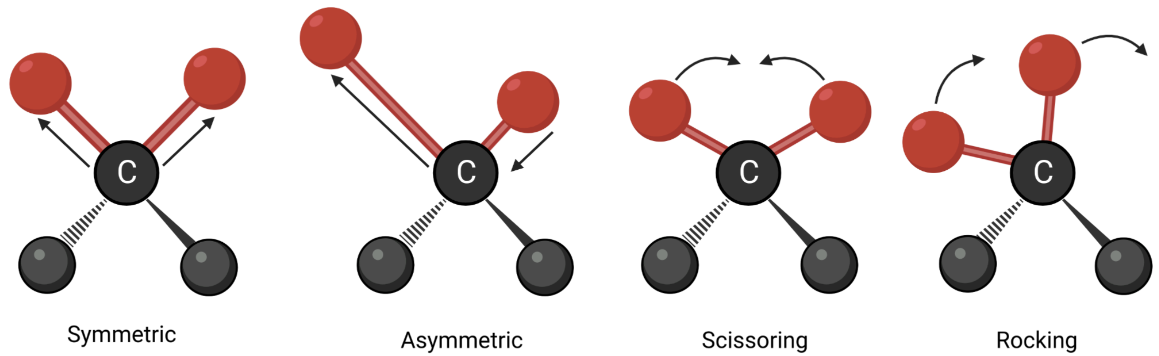


Figure 16: Effects of Infrared Radiation on Vibrational Modes of Molecules (created with BioRender.com, based on Soderberg, 2023)

Troughs in transmittance in the region above 6.5 μm gives information about functional groups, while the region below 6.5 μm is known as the fingerprint region and gives a very intricate pattern that can be used to determine a compound. IRS can take place in the near-, mid- or far-infrared regions, or use a Fourier Transform method to simultaneously beam many frequencies at once, repeated in bursts of different combinations over a short time and then use a computer to calculate the absorbance at each wavelength (figure 17). The near range, from 780 - 2526 nm is the most commonly used in quality control due to having higher energy and penetration capacity and producing less heat (Zeng et al., 2011, p.398) while the mid-range spectrum (2.5 - 25 μm) is superior for identifying structure and functional groups (Liang et al., 2011, p.153-158).

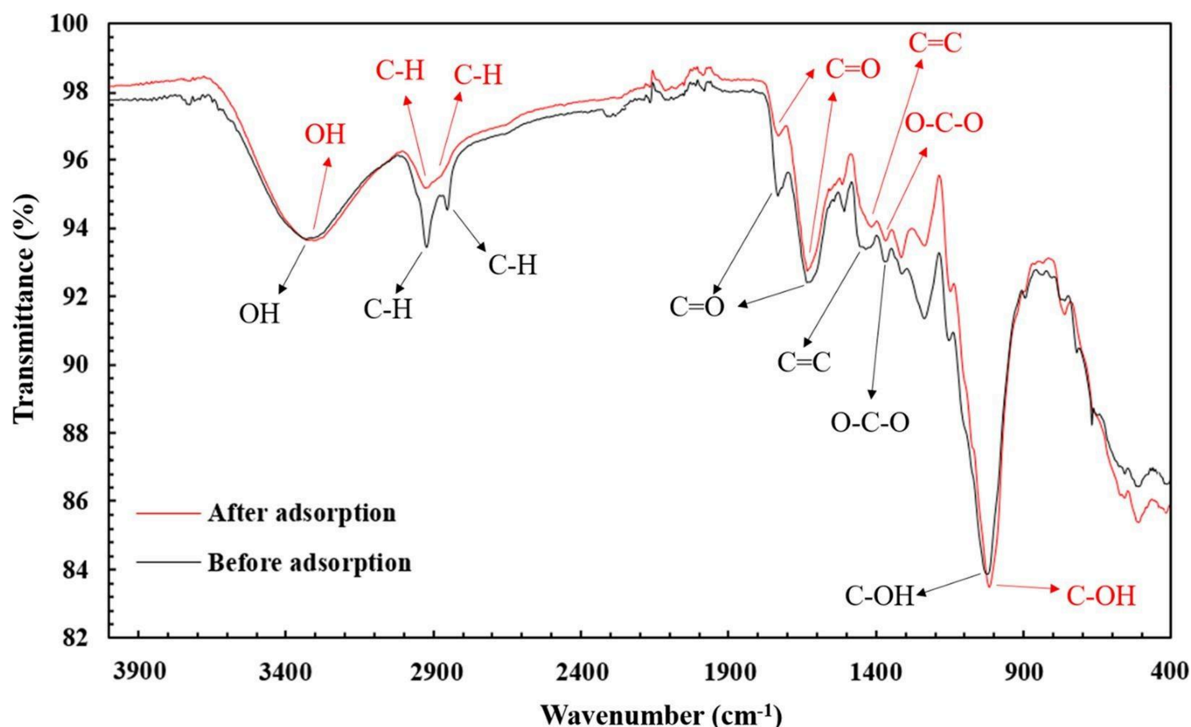


Figure 17: Fourier Transform Infrared Spectra of *G. glabra* (Pirsalami et al., 2021; reproduced with permission).
The two lines show how the transmittance of different infrared wavelengths as *G. glabra* is affected after interacting with copper ions in wastewater.

Only 16 papers included in the review used IRS but these reveal a wide range of potential uses. Kokalj, Štih, Kreft (2014) found it to be low cost, rapid and simple making it suitable for routine examination of single herbs and mixtures. Ma et al. (2017) were able to detect samples of Chinese yam (*Dioscorea polystachya* Turcz., *Dioscoreaceae*) powders that had been adulterated with cheaper corn starches. Yap, Chan & Lim (2007) used IRS to differentiate asian ginseng (*Panax ginseng* C.A. Meyer, *Araliaceae*) from its American counterpart (*P. quinquefolius* L., *Araliaceae*). Lee et al. (2014) found IRS to be superior to GC for identifying the caffeine and catechin content of tea (*C. sinensis*). Chen et al. (2019) found IRS equally effective to HPLC when evaluating decoction pieces of *Rhizoma Atractylodis* (*Atractylodes macrocephala* DC., *Asteraceae*). Qin et al. (2024) employed IRS along with GC to detect age fraud in sun dried, age stored mandarin peel (*Citrus reticulata* Blanco, 1837, *Rutaceae*). Cebi, Yilmaz & Sagdic (2017) used IRS to detect sibutramine in spiked tea samples, although real world

adulteration with designer analogues that lack reference standards might be more problematic. This wide range of uses demonstrates the potential versatility of IRS for a variety of applications ranging from species identification, constituent quantification, adulteration with drugs and even age fraud.

Despite this range of uses, the use of IRS remains low in Europe. This is due to most labs having UVS fitted as standard equipment on their chromatography devices meaning an extra expense to install an infrared device, train staff in its use and to develop the reference models, for which there are few available in English and whose development requires time and expertise in chemometrics (Zeng et al., 2011, p.399 & 412). The literature is instead dominated by MS or NMR for which the literature shows there is already a vast library of data from universities, pharmaceutical companies and the Asian herbal medicine market which is far more integrated into conventional healthcare and better funded than in Europe (Liu & Salmon, 2010).

2.3.5. Optical Emission & Atomic Absorption Spectroscopy

OES and AAS have a very specific use for detecting metal content. While OES achieves this by measuring the wavelengths of light emitted by a sample, AAS measures the wavelengths absorbed by a sample (CS Analytical, 2020). Due to the way they interact with light, it can only offer elemental information on metals and for chemical structure or chirality, MS is the preferred method (Measurlabs, 2024).

In OES, a spark is generated by an electrode and the sample in an high energy inductively coupled plasma state and a spectroscope detects the unique spectrum specific to each element (Shimadzu, n.d.) (figure 18).

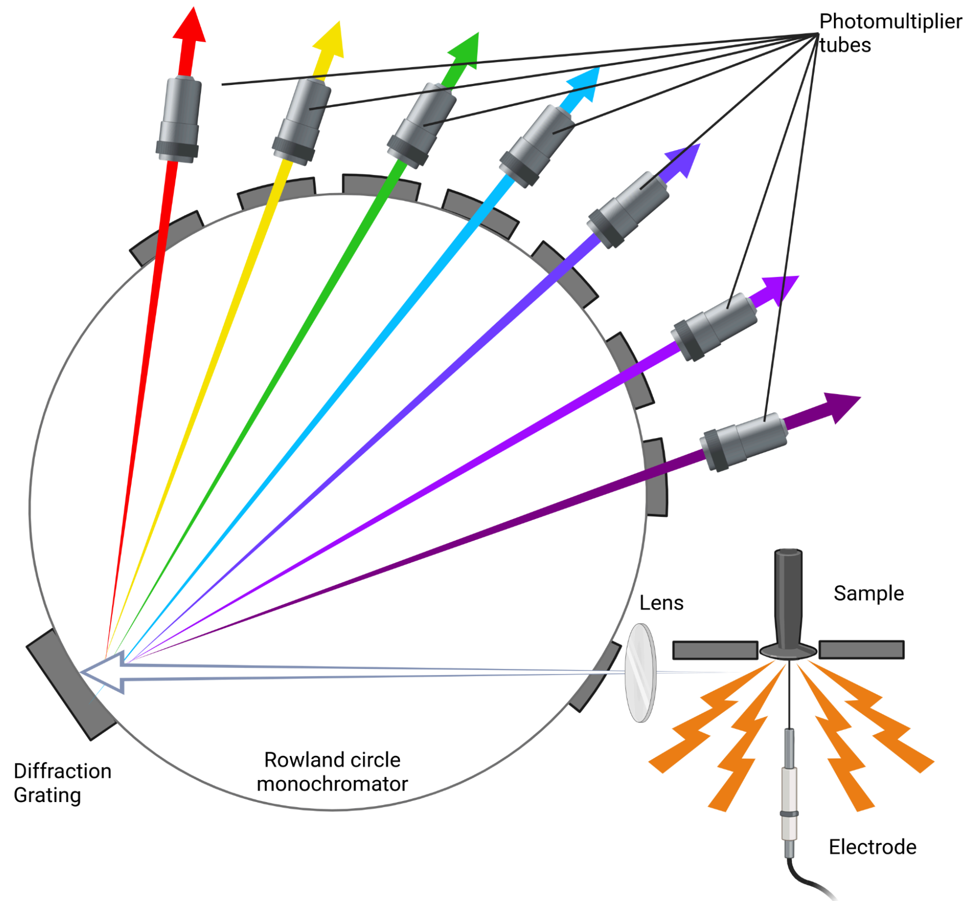


Figure 18: Principle of Optical Emission Spectroscopy (created with BioRender.com).

In AAS, the sample is nebulised and then atomised with a heat source, usually either a flame or a graphite furnace. Then, specific wavelengths of light that correspond to the atomic absorption spectrum of particular elements is projected through the sample and the amount of light absorbed to transition electrons from their ground state to an excited state can determine the concentration of that particular element (figure 19).

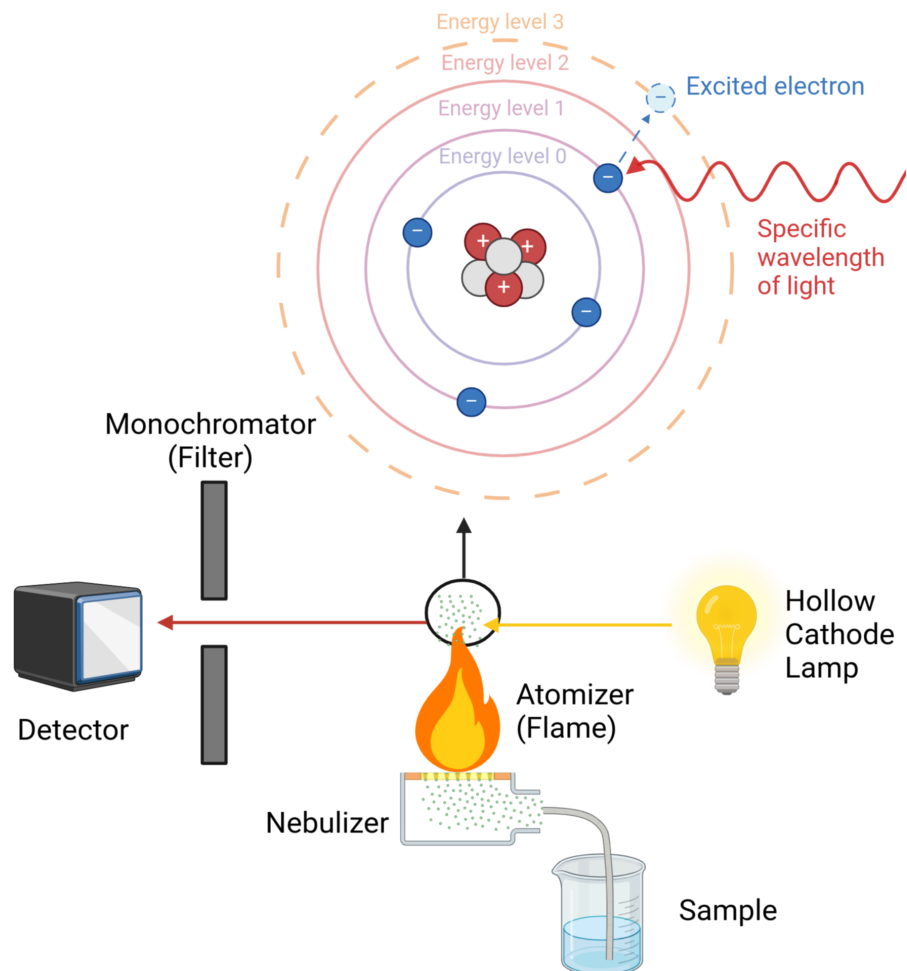


Figure 19: Principle of Atomic Absorption Spectroscopy (created with BioRender.com).

These techniques were the least common included in this review, totalling only 13 papers. 8 used OES and 7 used AAS (2 using both) with all employing this technique to evaluate toxic levels of metals in herbal teas. Rubio et al. (2012) used OES and found levels of aluminium in mint (*Mentha* sp. L., *Lamiaceae*) that warranted imposing consumption limits, while Popović et al. (2022) used AAS to find levels of manganese in *Betulae folium* (*Betula pendula* Roth and/or *Betula pubescens* Ehrh., *Betulaceae*) and *Frangulae cortex* (*Rhamnus frangula* L. syn. *Frangula alnus* Miller, *Rhamnaceae*) in Serbian markets that could potentially pose some health hazards to consumers. Malik et al. (2013) used both techniques to determine that hibiscus (*Hibiscus sabdariffa* L., *Malvaceae*) contained enough aluminium to warrant a consumption limit of no more than 1 L of hibiscus per day.

2.4. Biological Methods

Biological methods differ from those above in that they use some kind of biological material to test for authenticity and quality in herbs. The most widespread is certainly genetic testing but there were also several examples of using biological assays to assess the effects of herbs and extracts on cell cultures and even live animals to determine if the material was having the effect it was supposed to.

2.4.1. DNA Barcoding and Genetic Analysis

DNA barcoding takes a small section of genetic code from an unidentified organism and compares it to a reference library of DNA sections such as the Barcode of Life Data (BOLD) systems database (iBOL, 2020), Catalogue of Life database (Bánki et al., 2022) or the National Institute for Health's Genbank (Benson et al., 2012) (figure 20). It is often quicker and more precise than traditional taxonomic classification but the reliability of data is only as good as the database and, in the rush to categorise as many species as possible, there are many errors that may be present (Lathe, 2008). Countries where there is a strong tradition of herbal medicine have created large genetic databases dedicated to these materials, such as the Medicinal Materials DNA Barcode Database of Traditional Chinese Medicines (Lou et al., 2010; Wong et al., 2018), but where there is less of a vested interest in employing curators to error check and correct herbal plant materials, the number of vouchered species is likely to be less and the database of poorer quality.

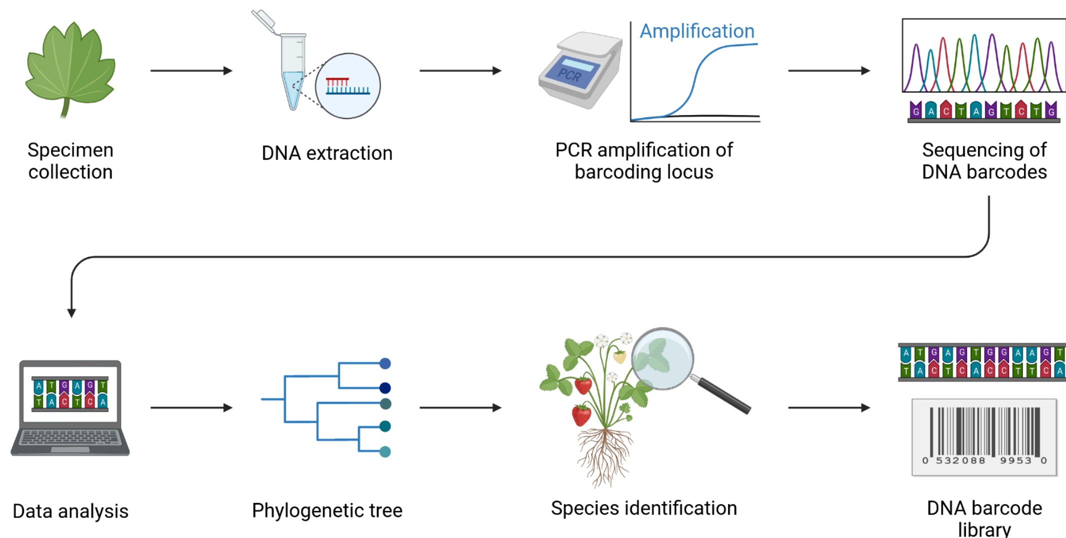


Figure 20: DNA Barcoding Workflow (created with BioRender.com)

26 studies included in the review used genetics. As expected, all were being used to identify the correct species, almost half (12, 46%) to check for adulterant species and identify them, the rest to confirm the species when using another technique for identification. 11 papers looked at adulteration in commercial supplies, revealing just how frequently adulterated tea samples can be:

Author	Aim	Result
Chan et al. (2024)	Investigate the quality of 33 commercial “five flowers tea” products, containing *Lonicera japonica* Thunb. *Caprifoliaceae*, *Plumeria rubra* L. *Apocynaceae*, *Pueraria lobata* (Willd.) Ohwi, *Fabaceae*, *Bombax ceiba* L. *Malvaceae* and *Sophora japonica* L., *Fabaceae*	Only 8 samples (24.2%) contained all of the five floral herbs. Acceptable substitutions for cheaper alternatives included 28 (84.8%) substituting *S. japonica* for *Agrostis capillaris* L., *Poaceae*, and 21 (63.6%) substituting *P. lobata* with *Prunella vulgaris*, L., *Lamiaceae*. 10 samples (29%) contained adulterants, including *Desmodium styracifolium* (Osbeck) Merr. *Fabaceae*, *Potenilla spp.* and *L. confusa* being used in place of *L. japonica*. Contamination with insects *Lasioderma serricorne,* (Fabricius, 1792), *Ptinidae* was also confirmed by macroscopic investigation and DNA barcoding.
De Castro et al. (2017)	Examine 32 herbal teas.	2 (6%) found to be adulterated.
Duan et al. (2017)	Identify species in *Radix Clerodendrum* tea samples used in the Dai ethnic group’s medicine.	Of 27 samples, only 1 (3.7%) was authentic *Clerodendrum japonicum (Thunb.) Lamiaceae*. Most were other medicinal species but 4 were potentially toxic *Lantana camara L. Verbenaceae*.

Lu et al. (2022)	Identify adulterants in 18 batches of commercial *Violae herba* (*Viola philippica* Cav., *Violaceae*) decoction pieces.	Most decoction pieces, including the reference material were identified as *Viola prionantha* Bunge, clashing with the accepted pharmacopoeia which should be updated.
Olivar et al. (2016)	5 *Vitex negundo L. Lamiaceae* samples, often used as herbal tea in the Philippines.	Only one satisfied the database criteria for genetic authenticity.
Omelchenko et al. (2019)	Examine 6 herbal teas, 6 herbal medicines & 6 spices for adulteration.	12 (67%) products contained different materials to those labelled. 6 likely to be economically motivated.
Osathanunkul (2018)	Find adulterants in Soursop (*Annona muricata L. Annonaceae*) teas.	3 out of 11 (27%) samples contained incorrect species.
Wang et al. (2016)	To find a DNA signature region which can be used to identify *Angelica sinensis (Oliv.) Diels. Apiaceae* in decoction powders.	Of 9 decoction powders, 7 (78%) were identified as *Angelica pubescens Maxim. Apiaceae*.
Xin et al. (2015)	Authenticate 90 commercial *Rhodiola crenulata (Hook.f. & Thomson)*	Only 36 (40%) contained the correct species. 35 (38.9%) contained *R. serrata H. Ohba* and 9 (10%)

	H.Ohba Crassulaceae products from hospitals and drug stores.	*R. rosea L*. Remaining 10 (11.1%) were 3 other *R.* species.
Xin et al. (2022)	To use DNA barcoding to identify adulteration along the entire industrial chain of *Rhei Radix et Rhizoma* (*Rheum palmatum* L., *Polygonaceae*)	25% of decoction piece samples collected midstream and downstream were identified as adulterants but exact species of adulterant could not be identified. All 8 patent medicines were identified as genuine.
Xiong et al. (2022)	Identify *Andrographis paniculata* (Burm.f.) Nees, *Acanthaceae* and differentiate species from adulterants *Mesembryanthemum cordifolium* L.f., *Aizoaceae* and *Rhinacanthus nasutus* (L.) Kurz, *Acanthaceae*.	No commercial products were found to be adulterated, but five samples which used alcohol extracts could not be amplified.

Table 5: Results of Genetic Analysis on Teas to Detect Adulteration

DNA barcoding has added much to the safety of herbal medicines by confirming the correct species is being used but also has certain limitations. When reports of liver damage induced by black cohosh supplements (*Actaea racemosa*, L., *Ranunculaceae*), began to circulate in 2002, but without a causal relationship being identified, it was presumed that it may be due to adulterant species (National Institute for Health, 2020). Its current use in the West is for menopausal symptoms of hot flushes and excessive sweating, for which it is approved by the European Medicines Agency (EMA, 2018), but it has a history of use in the Americas for conditions as varied as musculoskeletal pain, fever, cough and gynaecological issues. Similar cohosh plants *A. heracleifolia*, *A. dahurica* and *A. cimicifuga* are also used in Chinese medicine under the name *Sheng Ma* 升麻 and sometimes *Hei Sheng Ma* 黑升麻 (*Hei* 黑 = “black,” and therefore “black cohosh” in Chinese) for releasing “heat” trapped inside the body and manifesting as fever, rashes, sores and ulcers in the upper body (Bensky et al., 2004, p. 82). These overlapping indications and the complexity of the naming conventions of *Actaea* species, which all have several synonyms, including *Cimicifuga* being used interchangeably for *Actaea* in nearly all circumstances, *A. simplex* also being known as *A. cimicifuga*, and *Serratula chinensis* S. Moore, *Asteraceae* being referred to as *Guang Sheng Ma* 广升麻 or *Hei Sheng Ma* 黑升麻 (“wide” or “black cohosh”) in China and often exported to Hong Kong and the United States as black cohosh despite *A. racemosa* not growing in Asia, have caused much species confusion (Foster, 2013). DNA analysis provided a simple method which enabled species differentiation and was especially effective where HPTLC fingerprints produced similar results making chemical differentiation difficult, preventing a complete ban on black cohosh extract products for fear of safety concerns. However, no particular species was ever identified as causing the reports of liver toxicity and precautionary warning labels must be applied by law in Australia, Canada, the UK and US (Mohapatra et al., 2022). More recent studies has suggested that the *A. heracleifolia* used in Chinese medicine is therapeutically similar and a promising alternative to *A. racemosa* (Miao et al., 2019), while the hepatotoxicity reports could have been due to the fibrous roots of *A. dahurica*, which demonstrate subchronic hepatotoxicity not shown by the rest of the root, not being removed during processing (Yu et al., 2022). This raises a question of whether it was the incorrect species or an incorrect part which caused the reports of liver damage.

This is a major limitation of DNA barcoding (Blumenthal in Gustafson, 2015) and vigilant reporting practices will determine whether reports continue despite DNA barcoding having determined the correct species is being used.

A second limitation of DNA barcoding is it cannot be used on extracts and processed herbal material where the DNA will be degraded, incomplete or not present at all (Parveen et al., 2016). This problem affected Xin et al., (2022) when attempting to apply DNA barcoding to decoction pieces of rhubarb root (*R. palmatum*) which had been processed by steaming or charring and Xiong et al. (2022) who could not amplify the DNA from five samples of *A. paniculata* that had been alcohol extracted. This can have serious consequences, as demonstrated by the assessment of herbal supplements by the New York State Attorney General who attempted to cease sales of herbal supplements after testing revealed that only 21% contained verified DNA from the substances listed on the ingredients (James, 2015). It was soon criticised because many of the supplements sold were extracts and a good extract should only contain the compounds they are extracting and not any DNA from the original material (Morrell, 2015).

Another argument that can be levelled against DNA barcoding in the taxonomy of traditional medicines is that these methods were not the methods used by the original authors when describing the materials they intended to use. Therefore any number of species which can fit the description may have been meant and we are now forcing their morphological and ecological descriptions to fit a narrow DNA profile. This is evident in the way that many reference books of traditional herbal medicine have multiple species assigned a single entry (Bensky et al., 2004). Hence, when analysing the medicine of a traditional ethnic group for authenticity such as Duan et al. (2017) undertook, claiming only one of 27 samples tested was the official species, it assumes an elitist position of DNA barcoding over the people who actually developed and defined the medicinal material in the first place. Similarly, Lu et al. (2022) found so many samples of *Violae herba* did not correspond with the official pharmacopoeia’s designation of *V. philippica* as the official species, that even the reference samples were actually *V. prionantha*. This was also the only species which had sufficient index components (cichoriin,

esculin, esculetin, and prionanthoside) to meet the Chinese Pharmacopoeia's requirements, suggesting that the official species needed to be revised.

2.4.2. Bioassays

Bioassays present an interesting opportunity to determine the quality of herbs by measuring their effects on a living cell culture or organism. It could be argued that this is the most important factor of quality control since people are often drinking herbal teas and consuming medicinal plants for their effects with the correct species and levels of a principal component being only a means to accomplish that effect. With herbal medicines, the components responsible for an effect may often be complex or unknown (Panossian, 2023; Zhang, W. et al. 2019) and several genetically varied species may have the same effect and be acceptable equivalents or substitutes (see section 2.6.2), so measuring the effect of a herb would be the ideal quality control mechanism.

66 papers included in the review used some form of biological assay to assess the quality of herbal teas. Many different assays were used for different purposes which are outlined in table 6.

Author	Bioassays Used	Purpose
Abourashed et al. (2003)	Neutral red cytotoxicity assay.	Assess safety of *Clutia richardiana* Müll.Arg., *Peraceae* decoctions by evaluating cytotoxicity.
Arthur et al. (2011)	DPPH and Ferric Reducing Antioxidant Power (FRAP) antioxidant assays.	Assess effect of steam pasteurisation on antioxidant capacity of *Lippia multiflora* Moldenke, *Verbenaceae* to determine if it affects the quality of the herb.
Cheng et al. (2022)	Minimum inhibitory concentration assays for *Staphylococcus aureus* and *Moraxella catarrhalis*	Determine the compounds discovered by UPLC-MS that are most effective at inhibiting bacterial species responsible for upper respiratory tract infections in Artemisia and Soft-Shelled Turtle Shell Decoction (*Qinghao Biejia Tang*), for use as quality markers.
Chua et al. (2018)	Folin–Ciocalteu, formation of flavonoid-aluminium complex, DPPH, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Ferrous ion (Fe^{2+}) chelation assay and Ferric (III) reduction assay.	Compare the effects of storage duration on antioxidant capacity of *Orthosiphon aristatus* (Blume) Miq., *Lamiaceae*, on total phenolic content (TPC), total flavonoid content, antioxidant capacity, iron chelating and reducing capacity.
Dong et al. (2023)	*In vivo*: Hot plate and acetic acid writhing test in mice	Determine the constituents, discovered with HPLC-MS, with the most analgesic and antispasmodic effects in

		Peony and Liquorice (*Shaoyao-Gancao Tang*) decoction pieces and granules, for use as quality markers.
Fang et al. (2022)	Immunochromatographic strip.	Detection of various aminopyrine (a nonsteroidal anti-inflammatory drug) in adulterated herbal teas using a method of combining chromatography with antibody coated strips.
Félix-Silva et al. (2018)	Antimicrobial broth microdilution and agar dilution antimicrobial assays used to assess bacterial inhibition.	Compare antimicrobial activity of aqueous leaf extracts of *Jatropha gossypiifolia* L., *Euphorbiaceae* and *J. mollissima* (Pohl) Baill. used in Brazilian folk medicine and decide components to be used in quality evaluation, detected with HPLC and TLC.
Ferreres et al. (2014)	DPPH, Superoxide Anion ($O_2^{\bullet-}$) and Nitric Oxide (NO) scavenging antioxidant assays; acetylcholinesterase, butyrylcholinesterase and monoamine Oxidase-A inhibitory assays; lactate dehydrogenase release assay; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe	Assess antioxidant, cholinesterases and monoamine oxidase-A inhibition; cell injury, cell viability and protection from H_20_2 toxicity of *Grindelia robusta* Nutt., *Asteraceae* components, identified by HPLC, to select quality markers.

	nyltetrazolium bromide (MTT) cell viability assay; Hydrogen Peroxide (H_2O_2)-induced toxicity assay (MTT after exposure to herb and then H_2O_2).	
Guan et al. (2024)	Intracellular accumulation of 5(6)-Carboxyfluorescein diacetate to measure efflux by Multidrug resistance-associated protein (Mrp)-2 compared against MK-571 as positive control.	Identify compounds in Fresh Ginger Decoction to Drain the Heart (*Shengjiang Xiexin Decoction*) and its metabolites in rat plasma, detected with UPLC-MS, with Mrp-2 inhibitory activity for use as quality markers based on previous study that suggested this was its mechanism of action.
Guo et al. (2007)	Cell Counting Kit-8 (CCK-8) assay to measure cell viability and Nitrite Reporter gene for Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity assay.	Evaluate the best extraction method of Peony and Liquorice Decoction (*Shao Yao Gan Cao Tang*) by comparing their constituent profile using HPLC and biological activity to find the best components to optimise extracts for superior quality.
Hahm et al. (2021)	Folin–Ciocalteu and DPPH	Measure TPC and antioxidant capacity of 22 regional samples of ground ivy (*Glechoma hederacea* var. *Longituba*, L., *Lamiaceae*) to find consistent antioxidant

		compounds that can be used as quality markers for all regional specimens.
Han et al. (2023)	Total Flavonoids, Total Polysaccharides and TPC; DPPH; inhibition of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* assay.	Discover the best method of preserving *B. striata* by comparing preserved samples flavonoid, polysaccharide and phenolic content, antioxidant capacity and ability to inhibit bacterial growth.
He et al. (2022)	Pancreatic lipase inhibitory activity assay with Orlistat as positive control, α-Glucosidase inhibitory activity assay with Acarbose as positive control.	Correlate the 14 main peaks obtained by UHPLC-MS/MS with *in vitro* pancreatic lipase and α-glucosidase inhibitory activity to determine the main active ingredients to use as quality markers.
Hernández-Abreu et al. (2011)	Vasorelaxant assay (ex-vivo, on endothelium-intact aortic rat rings).	Measure vasorelaxant capacity of tilianin, a component in *Agastache mexicana* (Kunth) Lint & Epling *Lamiaceae*, to determine its use as a quality control marker.
Jansen et al. (2017)	Plasmodial lactate dehydrogenase activity assay and	Determine anti-plasmodial and cytotoxicity of *Mezoneuron benthamianum* Baill., *Fabaceae* leaf constituents, identified with HPLC, NMR, UV, IR and MS

	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) cell proliferation assay.	for quality control of traditional antimalarial teas.
Jiang et al. (2024)	Lipopolysaccharide (LPS) induced macrophage inflammation model.	Determine the anti-inflammatory capacity of selected quality marker compounds, identified with UPLC-MS in Astragalus and Cinnamon Twig Five Substance Decoction (*Huangqi Guizhi Wuwu Tang*).
Kan et al. (2022)	Dextran sulfate sodium-induced ulcerative colitis mouse model assayed with HE staining, immunohistochemistry analysis for occludin and ZO-1 antibodies stained with 3,3' Diaminobenzidine kit and counterstained with hematoxylin, enzyme-linked immunosorbent assay (ELISA) for serum levels of TNF-α, IL-1β, IL-6 and NO.	Compare the individual ginsenoside levels in The Four Gentlemen Decoction (*Si Jun Zi Tang*), measured with UPLC, and correlate them with the anti-inflammatory effects to decide which should be used as quality markers.
Lam et al. (2016)	DPPH antioxidant assay.	Evaluate the antioxidant capacity of 7 components of Snow Chrysanthemum (*C. tinctoria*), separated using

		HPTLC, to use as quality markers.
Li, J. et al. (2021)	Mouse myocardial infarction model with immunohistochemical analysis and HE staining.	Assays combined with HPLC analysis of components and molecular docking experiments to determine active components of “Compound *Longmaining*” decoction for use as quality markers.
Li, J. et al. (2022)	*In vitro*: CCK-8 assay and 3,3'-dioctadecylindocine-low density lipoprotein uptake assay; *in vivo*: total cholesterol, triglycerides, low density lipoprotein and high density lipoprotein levels of mice fed a high fructose diet and supplemented with either acacetin, diosmetin, rutin or nothing; *ex vivo* liver weight/body weight index and the alanine transaminase and aspartate transferase of the same mice.	Determine the lipid lowering constituents of citrus flowers (*Citrus aurantium* L. var. *amara* Engl., *Rutaceae*), determined by UPLC-MS, to decide constituents to be measured for quality control.
Li, T. et al. (2022)	Serum analysis of rats with adjuvant induced rheumatoid arthritis, before and after administration of Aconite	Serum analysed with UPLC-MS for metabolites of the herbal formula with potential anti-rheumatoid arthritis activity.

	Decoction (*Wutou Tang*) for 28 days.	
Li, Y. et al. (2021)	Everted intestinal sac model and *in vivo* portal vein blood samples of rats following oral administration.	Combined with HPLC-MS to determine the gut permeability and metabolisation of constituents in Angelica Decoction to Warm the Extremities (*Dang Gui Si Ni Tang*) to decide which compounds to use as potential quality standards.
Liang et al. (2021)	Cerulein induced acute pancreatitis mouse model.	Treatment of cerulein induced pancreatitis in mice used to validate the choice of quality markers, determined by HPLC-MS, selected for Beuplurum and Scutellaria Decoction to Order the Qi (*Chaiqin Chengqi Tang*).
Liang et al. (2022)	Alkaline phosphatase inhibition, thrombin inhibition and Na+/K+ -ATPase inhibition assays.	Determine the quality of rhubarb (*Rheum officinale* Baill., *R. tanguticum* Maxim. ex Balf., and *R. palmatum* Linn., *Polygonaceae*) based on bioactivity of major components identified with HPLC.
Liu et al. (2022)	Idiopathic pulmonary fibrosis rat model with histopathological analysis of cells using HE and Sirius red staining, and ELISA.	Detect pathological changes and levels levels of inflammatory cytokines after treatment with *Exocarpium Citri Grandis* (*Huajuhong*, *Citrus Grandis* (L.) Osbeck, *Rutaceae*), combined with network pharmacology and UPLC analysis of constituents to develop a method of determining grades of quality among decoction pieces.

Lu, Wang & Kong (2011)	*In vitro*: MTT assay; *in vivo*: mouse paw oedema model.	Measure anti-inflammatory effect of whole decoctions vs fractions, quantified with HPLC, and single herbs in Coptidis Decoction to Resolve Toxicity (*Huang Lian Jie Du Tang*). MTT is mentioned in methods but no results are discussed.
Mahamoud et al. (2024)	Total organic carbon concentration, Folin-Ciocaltreau assay to measure TPC and DPPH to measure ROS.	Determine the stability of polyphenols in red rooibos (*A. linearis*) tea, measured with LC-MS, to evaluate the degradation of teas used in rodent experiments which are often prepared in batches and stored.
Micheli et al. (2014)	DPPH and FRAP antioxidant assays.	Compare the traditional decoction method and modern extraction of *Teucrium chamaedrys* L., *Lamiaceae* on phenol content, measures with HPLC, and antioxidant activity.
Pinela et al. (2016)	Folin–Ciocalteu and DPPH assays.	Measure the effects of radiation on TPC and antioxidant capacity of Dwarf Mallow (*Malva neglecta* Wallr., *Malvaceae*) to determine if gamma radiation sterilisation of products affects their antioxidant capacity. UPLC-UV was also used to measure effects on constituents.
Qin et al. (2019)	Griess test assay and ELISA.	Compare Kudzu Decoction (Ge Gen Tang) dry herb pieces and its instant granules for their constituents,

		determined by UPLC-MS, and effects on NO production and modulation of interleukin(IL)-1β, IL-6 and Tumour Necrosis Factor-α (TNF-α) to determine anti-inflammatory effects.
Qu et al. (2023)	Allergic asthma rat model and zebrafish inflammation model.	Identify the anti-asthma and anti-inflammatory effect of major bioactive compounds found in rat serum and identified with UPLC-MS, after being fed raw and wine steamed *Schisandra chinensis* (Turcz.) Baill., *Schisandraceae*, to determine the main quality markers and how wine-steaming changes them.
Razmovski-Naumovski et al. (2020)	FRAP and DPPH antioxidant assays.	Compare *Angelicae sinensis Radix* (*A. sinensis)* decoction pieces and granules with UPLC and determine the superior antioxidant.
Schmeda-Hirschmann et al. (2003)	DPPH, xanthine oxidase & erythrocyte lipid peroxidation antioxidant assays; Folin–Ciocalteau and aluminium chloride assays.	Measure antioxidant capacity, TPC and total flavonoid content of components of Boldo (*Peumus boldus* Molina, *Monimiaceae*) to determine which should be used for quality control.
Schunk et al. (2016)	Micronucleus test assay for cytotoxicity.	Assess the cytotoxicity due to heavy metal content, determined by OES, in Brazilian herbal teas.
Seo et al. (2019)	Cytotoxicity assay, Oil Red O	Determine which compounds in Ginseng, Poria and

	staining, Triglyceride quantification assay & Leptin immunoassay.	Atractylodes powder (*Samryeongbaekchul-san*) decoctions, identified with HPLC, have an anti-adipogenic effect.
Shim et al. (2012)	ELISA	Detect aflatoxins in 70 Korean herbal medicines with ELISA and LC-MS to determine fungal contamination.
Simoes-Pires et al. (2014)	Antiplasmodial assay.	Discover active antimalarial alkaloids with NMR in *Argemone mexicana* L. *Papaveraceae* for quality markers of antimalarial teas.
Song et al. (2004)	Anti-platelet aggregation assay and oestrogenic effects measured by alkaline phosphatase activity with ELISA and cell count using MTT and protein count of MG-63 cells whose cell growth and alkaline phosphatase activity is modulated by 17β-estradiol (Lajeunesse, 1994).	Compare the effects of extraction time, extraction volume and number of repeats of the extraction on the anticoagulation and oestrogenic activity of Nourish Blood with Angelica Decoction (*Dang Gui Bu Xue Tang*) to determine the best extraction method.
Ting, Chow & Tan (2013)	Microbial plate assays.	Determine the effect of boiling on microbial concentrations of four herbal decoctions. Heavy metals were also evaluated with AAS.
Viegas et al. (2023)	Fungal cultures from tea samples	Determine the extent of fungal contamination in tea with

	grown on agar plates.	species and mycotoxins. Fungal cultures and specific toxins determined by DNA fingerprinting and HPLC-MS.
Viraragavan et al. (2020)	Glucose uptake assay by liquid scintillation in skeletal and liver cells, intracellular lipid content by modified Oil-Red-O assay, intestinal permeability of aspalathin measured by introducing aspalathin to apical compartment of caco-2 cells and measuring transport to basolateral compartment with HPLC-UVS.	Compare the effects of aspalathin, a component of green rooibos (*Aspalathus linearis* (Burm.f.) Dahlgren, *Fabaceae*) with known effects on metabolic disorders against the whole herb extract by measuring its effects of cellular glucose and lipid concentrations and its intestinal permeability, to decide if it can be used as a sole quality marker or if multiple compounds are required.
Walkowiak-Bródka et al. (2022)	DPPH antioxidant assay.	Evaluate the antioxidant capacity of 19 products containing *Morus alba* L. 1753, *Moraceae* and correlate this with levels of 1-deoxynojirimycin, an α-glucosidase inhibitor, detected and quantified by IRS and HPLC-UV
Wan et al. (2022)	Rat common cold infection model with analysis of blood and histopathological analysis of lung samples	Determine the effectiveness of Cinnamon with Kudzu Decoction (*Gui Zhi Jia Ge Gen Tang*) against a “Wind-Cold" type of common cold and find the constituents responsible for development of quality standards. The decoction was analysed with HPLC-MS

		for its constituents, and the rat blood serum levels for its metabolites, markers of immune response. Histopathological analysis of lung samples analysed for changes to lung tissue.
Wan et al. (2023) and Wan et al. (2024)	Rat observational study for toxicity markers (inactivity, lethargy, isolation, hair contamination, diarrhoea and weight loss), biochemical markers of rat serum for markers of liver and kidney injury (aspartate aminotransferase, alanine aminotransferase, total bilirubin, urea nitrogen, and creatinine and histopathological studies.	Develop toxicity quality markers for Gardenia and Magnolia Decoction (*Zhi Zi Hou Po Tang*) by correlating weight loss, biochemical indicators, cellular inflammation and damage with potential toxic compounds found in rat serum, detected with HPLC-MS.
Wang et al. (2017)	DPPH antioxidant assay.	Quality assessment of Big Red Robe Tea (*Da Hong Pao Cha*) by measuring antioxidant capacity and comparing to constituents measured by HPLC.
Wang et al. (2020)	High-fat diet / streptozotocin-induced diabetic mice tested with: serum and urine assays,	Determine the mechanism of compounds, determined UPLC-MS, responsible for antidiabetic effect of Tonify the Kidney and Invigorate Blood Circulation Decoction

	hematoxylin and eosin (HE) staining, periodic acid-Schiff staining, immunohistochemistry staining, Western Blot analysis.	(*Bu Shen Huo Xue Tang*) to define quality control markers.
Wang et al. (2022)	Immunochromatographic strip.	Detection of pain relieving and fever reducing drug acetophenetidin in herbal tea blends through coating a strip with antibodies that react to the presence of the drug.
Wang, Y. et al. (2023)	Bioluminescent bioautography.	Detect adulterant hypolipidemic drugs by using a bioluminescent bacterial solution as a derivatizing agent after HPTLC separation.
Wu et al. (2020)	DPPH antioxidant assay & Xylene-induced mouse ear oedema anti-inflammatory model (*in vivo*).	Compare two *Polygonum chinense* L.H.Gross, *Polygonaceae* varieties to find the best to use in “Cool Tea” (*Liang Cha*) for superior antioxidant and anti-inflammatory activity. Constituents measures with UPLC-MS.
Wu, Yang & Chiang (2018)	Total Anthocyanin Content (TAC), TPC and DPPH, ABTS & FRAP antioxidant assays.	Determine the effect of heat and pH on anthocyanins in roselle (*H. sabdariffa*) decoctions to determine the best methods of storage to preserve their antioxidant properties.

Xiao et al. (2022)	DPPH antioxidant assay.	Correlate antioxidant capacity of components with peaks obtained by HPLC in Three Whites Decoction (*San Bai Tang*).
Xie et al. (2022)	Immunochromatographic assay.	Detection of various sulfonylureas (anti-diabetic drugs) in adulterated multi-herbal teas using a method of combining chromatography with antibody coated strips.
Xu, N. et al. (2022)	DCFDA and ELISA.	Determine antioxidant and anti-inflammatory effect through C-Reactive Protein inhibition of individual components of Pinellia, Atractylodes and Gastrodia Decoction (*Banxia Baizhu Tianma Tang*), detected with UPLC, for use as quality control standards.
Xu, Y. et al. (2022)	*In vitro* smooth muscle contraction assay.	Determine the antispasmodic effects components of Peony and Liquorice Decoction (*Shaoyao Gancao Tang*), determined by HPLC, by measuring their effects on acetylcholine-induced contraction.
Yadav, Gupta & Aeri (2022)	Mouse forced swimming test, thiobarbituric acid reactive substances and GSH assay.	Evaluate antidepressant effect and antioxidant effects of Ashwagandha (*Withania somnifera* (L.) Dunal, *Solanaceae*) based formulations, compared using HTPLC, to determine a standardised formulation.
Yang, F. et al. (2022)	α-glucosidase inhibition assay.	Assess the difference between raw and fried cassia

		seeds (*Senna obtusifolia* (L.) H.S.Irwin & Barneby, *Fabaceae*) for their anti-diabetic effect and identify the components responsible with UPLC-MS, to use as quality indicators.
Zhang et al. (2014)	Antiviral assay	Assess antiviral activity of components of *Radix isatidis* (*Isatis tinctoria* L., *Brassicaceae* or *I. indigotica* Fort.), identified with LC, for use as quality markers.
Zhang et al. (2024)	*Ex vivo*: Rat model of heart failure by ligation of the left anterior descending branch of coronary artery and assayed with HE Masson and Sirius red staining and ROS detection (method not stated). *In vitro*: cell viability measured with CCK-8 assay after 6h treatment followed by anoxia model ($Na_2S_2O_4$ for 8h), protein analysis after anoxia model with western blotting	Determine the constituents, identified in decoctions and their metabolites in rat serum using UPLC-MS, and determine the mechanism responsible for the effect of Astragalus and Aconite Decoction (*Qi Fu Tang*) in heart failure to decide on quality markers.
Zhang, C. et al. (2021)	Bacteriostasis and α-glucosidase inhibition assays.	Compare *Coptidis Rhizoma* (*Coptis chinensis* Franch., *Ranunculaceae*) decoction pieces and granules to

		determine the superior antibacterial and antidiabetic effects in relation to constituents, quantified with HPLC.
Zhang, H. et al. (2020)	ELISA.	Discover compounds responsible for antidepressant effects in Lily Bulb and Rehmannia Decoction (*Bai He Di Huang Tang*), quantified with HPLC, to be used for quality control.
Zhang, M. et al. (2022)	Rat mifepristone induced abortion model, ELISA, reverse transcription polymerase chain reaction (RT-PCR) and western blotting.	Evaluate the best quality markers to use to evaluate Gelatin and Mugwort Decoction (*Jiao Ai Tang*). UPLC-MS identified main components which were tested with bioassays to determine: effect on uterine bleeding quantity and histopathological sections from rat study; effects on luteinizing hormone, follicular stimulating hormone, estradiol and progesterone using ELISA; gene and protein expression levels using RT-PCR and western blotting.
Zhang, X. et al. (2022)	Immunochromatographic strip.	Detection of morphine, codeine and thebaine from opium poppies (*Papaver somniferum* L., *Papaveraceae*) in herbal teas.
Zhao et al. (2022)	LPS-induced macrophage inflammation model, Griess assay for	Evaluate anti-inflammatory and antioxidant capacity of two species of yellow flowered tea (*Camellia nitidissima*

	NO production and DCFDA ROS assay.	C.W.Chi, *Theaceae*, and *C. euphlebia* Merr. ex Sealy) and a common adulterant (*C. insularis* Orel & Curry) to compare against UPLC-MS determined marker compounds.
Zhao et al. (2024)	Mifepristone and miso-prostol rat model of “Blood Stasis,” measured by volume of blood on intravaginal cotton ball and assayed with HE staining and ELISA to measure IL-6, TNF-α and IL-β in rat serum.	Identify the components of Four Substance Decoction with Peach Kernel and Safflower (*Tao Hong Si Wu Tang*) with UPLC-MS and evaluate which are most effective in a vaginal bleeding model to determine quality markers.
Zheng et al. (2020)	FRAP and DPPH antioxidant assays.	Find antioxidant components, quantified with HPLC, responsible for the effects of Clear Epidemics and Overcome Toxins Decoction (*Qing Wen Bai Du Tang*) to use as markers for quality control.
Zhou et al. (2019)	CCK-8 cell viability assay	Assess the antiproliferative effect of Four Gentlemen Decoction (*Si Jun Zi Tang*) and its components, detected with UPLC, on PC9 lung cancer cells to determine which compounds should be used for quality control when given to lung cancer patients.

Table 6: Biological Assays Used in Quality Assessment of Herbal Teas and their Purpose

One of the most notable points that emerge from the literature is that very few suggest the use of bioassays as a method of quality assessment in itself, but usually to find a biological mechanism of action behind certain compounds which can then be used for quality control with chromatographic or spectroscopic methods. Only one paper did not use a chemometric technique alongside the bioassays to identify compounds (Wu, Yang & Chiang, 2018) and their approach was less concerned with identifying marker compounds but assessing how processing methods affected the anthocyanin content and subsequent antioxidant activity to inform manufacturing processes. A spectroscopic technique was still used because anthocyanins were measured using a pH differential method that depends on anthocyanins reversibly changing colour under low pH which can be measured with a spectrophotometer (Lee, Durst & Wrolstad, 2005) but it does not identify the precise compound. Instead it compares this feature to the reference anthocyanin, cyanidin-3-glucoside. The overwhelming number of other studies that all attempted to find a specific active ingredient responsible for the effects observed in the bioassays reveals a distinct pharmaceutical bias in the fields of quality assessment of herbal teas, despite the fact that the isolation of active compounds frequently proves the isolated ingredients to be ineffective or less effective (eg. Lu, Wang & Kong, 2011), leading modern drug discovery to look at synergies between multiple compounds and complex effects using -omics analysis, network pharmacology and systems biology (Thomford et al., 2018; Panossian, 2023).

2.5. The Limitations of Existing Methods of Herbal Quality Control

The existing methods of quality control in herbal teas focuses heavily on chemometric testing for specific marker compounds or genetic sequences. When there is attention to biological activity it is largely to test novel compounds in order to determine which ones are biologically active and can potentially be developed into drugs. These often become the primary quality markers (eg. Qin et al., 2019),

followed by compounds unique to the species and then specific levels of others in relation to other species.

This reveals a distinct tendency towards the pharmaceuticalization of herbal medicine and presents a problem when dealing with complex mixtures and herbs with unknown mechanisms. This is demonstrated by Viraragavan et al. (2020) who compared the bioactive effects of green Rooibos tea (*A. linearis*) and aspalathin, the primary candidate for its bioactivity, on *in vitro* metabolic models, finding that it could not account for all effects and that up to 12 compounds showed metabolic bioactivity. When expanded to entire formulas, Fan et al. (2021), in attempting to develop a "holistic quality control" method using UPLC-MS/MS on a TCM formula (*Yangwei* Decoction, or "Nourish the Stomach" Decoction) consisting of 11 herbs , found 226 components, and in order to make a manageable shortlist focused on only 5 main quality markers from only 4 of the ingredients known to have an effect in gastric diseases, and still confessed to being unable to explain the effective components and pharmacological actions of the formula as a whole. Similarly, Feng et al. (2022) examined another TCM formula (*Danggui Jianzhong* Decoction, or "Construct the Middle with Angelica" Decoction), consisting of 6 herbs but found that all 7 peaks associated with quality were from just 2 herbs, *Paeoniae Radix Alba* (*Paeonia lactiflora* Pall., *Paeoniaceae*) and *Glycyrrhizae Radix et Rhizoma* (*G. glabra*), neither of which are in the chief herb named in the formula (*A. sinensis*) and both are common in many formulas, and even constitute a formula in themselves (*Shao Yao Gan Cao* Decoction, or "Peony and Liquorice" Decoction; Scheid et al., 2009, p. 344-345). In the most extreme case, Cheng et al. (2022) reduced a 5 herb formula (*Qinghao Biejia Tang*, or "Artemisia and Soft-shelled Turtle Decoction") to a single compound, mangiferin, deemed the main antibacterial constituent responsible for the formula's antibacterial action. This is despite fact that the use of artemisinin, another compound extracted from *artemisia annua* L., *Asteraceae*, as a monotherapy for malaria has contributed to the development of resistant *Plasmodium falciparum* parasites (Czechowski et al., 2020) and that the traditional method of combining *A. annua* with other herbs with different mechanisms of action that may be responsible for the formula's effects and the reason *A. annua* has remained effective for thousands of years.

Another instance where limitations in current methods are exposed is when the correct plant is used but has been incorrectly grown, handled or stored in a way that may reduce its efficacy. These may be best tested functionally, based on what they do to a complex biological system when tested as a whole blend rather than the sum of its parts.

2.6. Functional Adulteration and the Problem of Functional Testing

2.6.1. Functional Adulteration

Functional adulteration refers to forms of adulteration that affect the functioning of the herb or blend in question. This most often refers to adding drugs to herbal supplements in order to impart efficacy to an otherwise inactive blend (Choules et al., 2020), however another form of functional adulteration is to offer a poor quality sample of the correct herb coming from a poor growing region, herbs that have been harvested or handled incorrectly post-harvesting, or a blend that has been mixed incorrectly, as a high quality sample. The result is that the herb may be the correct species and so pass visual and genetic identification and contain a chemical profile that may not appear outside of the normal range of variation on chemometric analysis, but their clinical effects may be diminished. On occasion pre-extracted herbs can be dried again and resold by unscrupulous sellers if their appearance is unchanged, as in the case in weeping forsythia capsules (*Forsythia suspensa*, (Thunb.) Vahl, *Oleaceae*; Al-Khafaji, 2016), anise (*Pimpinella anisum*, L., *Apiaceae*; Ilyas, 1980) and cloves (*Syzygium aromaticum* (L.) Merr. & L.M.Perry, *Myrtaceae*; Centre for the Promotion of Imports from Developing Countries, 2018). While it is most common in these hard herbal materials that do not change much in appearance after extraction (figure 20), other herbs have been adulterated in this manner, with suspected spent material being present in herbal extracts detected through a complete but weak HPTLC fingerprint reported in milk thistle seed (*Silybum marianum* (L.) Gaertn., *Asteraceae*), ginkgo (*G. biloba*) leaf, echinacea (*Echinacea angustifolia* DC., *E. pallida* (Nutt.) Nutt., or *E. purpurea* (L.) Moench, *Asteraceae*) root or herb,

cranberry (*Vaccinium oxycoccos* L., *Ericaceae*) fruit and St. John's wort (*Hypericum perforatum* L., *Hypericaceae*) herb (Gafner et al., 2023). There has been also been one case of pre-extracted rhodiola root (*Rhodiola rosea* L., *Crassulaceae*) being resold as dried herbs by a major supplier for which suspicion was aroused by its lack of organoleptic properties and subsequent chemometric testing found it almost completely absent of active ingredients (Etheridge, 2023).

Figure 21: *Forsythia fructus* (left) and *Flos Syzygia* (right)
Both are unchanged in outward appearance by extraction and so commonly adulterated with exhausted products (photos by Hazeena Azeez, 2023)

This latter form of functional adulteration has received little attention in modern literature since it remains elusive to most modern techniques: being the correct species, it will appear the same under visual and microscopic inspection, and containing the same genetic and chemical markers. Quantities of an active ingredient may be less, but this is difficult to determine if no single active ingredient is known to be responsible for its therapeutic effect. This becomes an even greater challenge in formulas, blends, extracts and powders where the number of potential markers is increased and where marker compounds may be altered and DNA may be degraded from processing (Parveen et al., 2016). This has resulted in a publication bias (DeVito & Goldacre, 2019) against reporting this form of adulteration despite being of great concern to historical authors.

In 1st - 2nd century Rome, Pliny the Elder documented the use of smoking to make new wine appear older (Bush, 2002) and in 5th century China Tao Hongjing raised the issue of herbs grown in an incorrect terroir, or handled inappropriately in harvesting or storage (Liu, 2021), resulting in the correct plant being used but the quality and potency being negatively affected. Pliny the Elder even exclaimed that “By Hercules! - a thing truly astounding - that, in reality, a wine is more innoxious in its effects, in proportion as it enjoys a less extended renown” (Pliny the Elder, Bostock & Riley, 1857). The adulteration of wine presents a comparable issue to herbal medicine due to the issue of terroir (Kim, 2023) and there have been attempts to address this problem through chemometric means but it has met many problems (Merkytė et al., 2020). Even Pliny the Elder’s issue of detecting smoked wine can be difficult today due to the legitimate use of charred and toasted barrels to impart flavour to alcoholic beverages including wine (Archibald, 2020). This introduces a question of what is a legitimate level of foreign chemicals imparted from treatment or processing before adulteration can be suspected? Also, once an aged chemotype is discovered, the marker components can be artificially added or created. For example, trans-resveratrol has been shown to convert to cis-resveratrol during the ageing of wines (Naiker et al., 2020), but this conversion can be promoted by exposure to UV radiation (Pannu & Bhatnagar, 2019) or added from other plants that are abundant in resveratrol and grow much faster than grapes, such as Japanese Knotweed (*Polygonum cuspidatum* Sieb.et Zucc., *Polygonaceae*) that grows invasively throughout Europe (Peng et al., 2013). The cis- isomer is less valued for health supplementation than the trans- isomer, so trans-resveratrol could be extracted and sold for health supplements while the remaining cis-resveratrol could be added to wines to imitate the ageing process and raise their value. Similar adulteration has been reported with other herbs such as rutin from corn added to ginkgo (*G. biloba*) supplements (Booker et al., 2016) making this a realistic possibility. Although wine’s primary bioactive compound is alcohol which disrupts mitochondrial function, increasing ROS production and reducing antioxidant defences (Hoek, Cahill & Pastorino, 2002) and will mask the more subtle changes induced by other components making comparison between varieties difficult, research into functional adulteration may be of use to the wine industry since the samples will have to be dried in order

to add to a culture media which will remove the alcohol and allow the remaining compounds to be tested for their functional activities.

In Chinese herbal medicine, the importance of region has also long been recognised as an important factor affecting the potency. The oldest classics of Chinese herbal medicine, The Divine Farmer's Materia Medica (*Shen Nong Ben Cao Jing*), compiled 25–220 C.E., states that:

> *"Whether they are dried in the shade or in the sun, the time and month when they are gathered and prepared, whether they are used raw or cooked, the locale where they come from, and whether they are genuine or false, and old or fresh, these also have their precedents"* (Wilms 2017, p.23).

Even in this ancient text, the correct species is only one condition of several for ensuring the potency of herbs. Even after the attempts of Tao Honging to improve on functional quality evaluation with his commentaries, successive authors also raised the issue, providing the best growing regions along with other clinical information in their pharmacopoeias (Liu, X. et al., 2020). It was eventually given the term *Daodi* (道地, literally "the way of the earth) in the Ming dynasty (1368 - 1644 CE) and referred not only to different soil associated with specific regions but the weather conditions, hours of sunlight and the experience of the local people in cultivation, harvesting, processing and storage which lead to a superior product. Clinical and animal trials have found a superior effect to *Daodi* herbs compared to non-*Daodi* herbs in 83.3% of published animal trials and in one clinical trial on humans (Yang, X. et al., 2018). However, the problem which faced Tao Hongjing has resurfaced many times since. In one publicised example, the demand for ginseng (*Panax ginseng*, C.A.Mey., *Araliaceae*) has resulted in exhaustion from overharvesting in its native northern regions several times. During the Great Leap Forward, attempts were made to cultivate it on the southern island of Hainan but resulted in roots that were large but almost completely lacking in active ingredients (Zhao, Guo & Brand, 2012).

2.6.2. Adulteration and Substitution

To complicate matters further, when preparing formulas or blends based on a traditional prescription, it is an accepted practice to substitute herbs which are problematic due to scarcity, legality, cost or toxicity with another that has similar properties and actions (Kashari, 2021; Leon & Lin, 2017; Moorhouse et al., 2022). This can mean that there may be several potential variations of a traditional formula which are correct, each containing different ingredients but aimed at having a similar effect to the original.

For example, in the U.K., all aconite species (*Aconitum spp.*, L., *Ranunculaceae*) are banned from internal consumption (Medicines & Healthcare products Regulatory Agency, 2014) but in CHM this has been an essential herb since the earliest herbal formulary was written in the Han dynasty (Zhang & Liu, 2016). Prepared aconite root, known as *Fu Zi* (附子, *Radix Aconiti Lateralis Preparata* from the species *Acontium carmichaelii*, Debeaux, *Ranunculaceae*) is understood to powerfully invigorate *Yang* (the warm, energetic, virile aspects of the body under the Yin-Yang dualistic understanding of TCM), having indications such as weak pulse with cold limbs, shortness of breath and chest pain, or joint pain that is worse in cold, damp conditions, or impotence and infertility (Bensky, 2004, pp.673-4). While China has developed processing methods to alleviate the toxicity posed by the diterpene alkaloids present in this plant, to the point where it is even sometimes used as a vegetable in soups, and all medical products containing aconite are subjected to HPLC analysis to ensure safe levels are met (Singhuber et al., 2009), the U.K. has adopted a complete ban. This means that CHM practitioners in the U.K. have to find substitutions for this herb and the various products on the market have adopted different solutions for this issue.

The classic aconite containing formula *Jin Gui Shen Qi Wan* (Kidney Qi Pill from the *Essentials from the Golden Cabinet*) utilises aconite for all of these potential functions and in modern use different versions of this formula have opted for different substitutions. The indications of fatigue, weak pulse and cold limbs suggest imminent heart failure are referred to hospitals in the U.K. but the formula

is still commonly used for the remaining indications of cold-type joint pain and impotence or infertility. Hence, Aura Herbs (2023) have chosen to increase the amount of cassia bark (*Cinnamomum cassia,* (L.) J.Presl, *Lauraceae*) already present in the formula for its similarity to aconite in terms of its hot taste and warming nature with similar indications for alleviating joint pain. Meanwhile, GinSen (n.d.) have chosen to add fo-ti (*Polygonum multiflorum*, Thunb., *Polygonaceae*), goji berries (*Lycium barbarum* L. *seu chinense* Mill., *Solanaceae*) and schisandra berries (*Schisandra chinensis* (Turcz.) Baill., *Schisandraceae*) which have few warming effects and offer little assistance with joint pain but instead share aconite's indication for fertility issues which their clinic specialises in.

These will obviously have vastly different chemical profiles from both each other and from the formulas produced in China but all are acceptable variations of the same formula aimed at treating similar patterns of disease. The ideal method to assess whether they are equivalent or should be considered functionally distinct formulas would be to compare their effects on a living system to find out if they actually achieve the same desired effect.

2.6.3. Functional Testing

The methods for functionally evaluating the quality of herbs have not evolved much since 2nd century Rome when Galen proposed testing the effectiveness of antivenom purchased at the markets by separating two groups of chickens and giving them an equal dose of poison, then administering the antivenom to the active arm and comparing the death rates to the control group (Karaberopoulos, Karamanou & Androutsos, 2012). A similar method is described in 11th century Chinese literature, using human volunteers to test the quality of ginseng (*Panax ginseng* C.A.Mey., *Araliaceae*) by pitting two runners against each other, one who has taken the ginseng while the other did not, and comparing their breathing after a race (Shang & Gong, 1994).

This method of observing an effect on a living organism may be more sensitive than chemical analysis for detecting the functional potency of herbs. A

review of studies comparing *Daodi* to non-*Daodi* herbs found when chemical analysis was used to assess quality by equating higher levels of active ingredients to efficacy, 64.5% found *Daodi* herbs to be superior. However, when using animal models to assess direct effects on living systems, 83.3% of papers found *Daodi* herbs to be more effective (Yang, X. et al., 2018). Animal studies are ethically problematic (Ferdowsian & Gluck, 2015) and provide limited information (Bracken, 2009) making them unsuitable for routine investigation into the functional potency of herbal products. Meanwhile, formal human testing through clinical trials is even less ethically appropriate for routine examination of herbal supplies, while also being too costly and time consuming to be practical for batch testing. Unofficially though, retrospectively assessing the efficacy of each batch is often the only method herbal clinicians have to evaluate the quality of their supplies. This means that they are using herbs of unknown potency every time they purchase a new batch, potentially putting patients at risk of adverse effects through a sudden increase in potency, or reduced therapeutic efficacy from reduced potency. A method of functionally evaluating herbs that does not put human or animal lives at risk from poor quality herbal medicines would clearly be advantageous to the herbal industry, clinicians and patients.

Chapter III

3. Mitochondria as a Potential Focal Point for Functional Testing

Mitochondrial analysis presents a unique opportunity to solve the problem of functional testing. Mitochondria are integral to most biological functions, providing the energy necessary to perform most tasks as well as generating important signalling mechanisms both within (Quirós, Mottis & Auwerx, 2016) and between cells (Liu & Ho, 2018; Picard et al., 2018) through DNA, redox, hormonal, neurological and immune mediated pathways. Their functioning is altered in both normal physiological states and disease (Annesley & Fisher, 2019; Nunnari & Suomalainen, 2012) which can be measured and used to assess the functional effects of a herb or blend on a complex living system.

3.1. The Role of Mitochondria in Health and Disease

3.1.1. Respiration

Mitochondria were first identified as the site of respiration in 1949 (Kennedy & Lehninger, 1949) who observed fatty acid oxidation occurring in isolated rat liver mitochondria. This process had already been described by Hans Krebs in the 1930s who developed the model which now bears his name, also known as the citric acid cycle or tricarboxylic acid cycle, after the compound created from acetyl coenzyme A in its first step (figure 22). In this model, the pyruvate generated from glycolysis enters the mitochondria and is converted to acetyl coenzyme A, which then acts to renew a cyclical series of reactions by converting oxaloacetate into citric acid with the

addition of two carbon atoms (C), four hydrogen atoms (H) and two oxygen atom (O) units (Alberts et al., 2002). As the cycle progresses, carbon and O units are lost giving off carbon dioxide (CO_2), 2 units of adenosine triphosphate (ATP) are generated which can be used as energy, and H is used to convert nicotinamide adenine dinucleotide (NAD)+ to NAD + H (NADH) and flavin adenine dinucleotide (FAD) to FAD + H ($FADH_2$). These activated carrier molecules provide high energy electrons that can enter the electron transport chain (ETC) to generate further ATP through oxidative phosphorylation (OXPHOS) (Cooper, 2000).

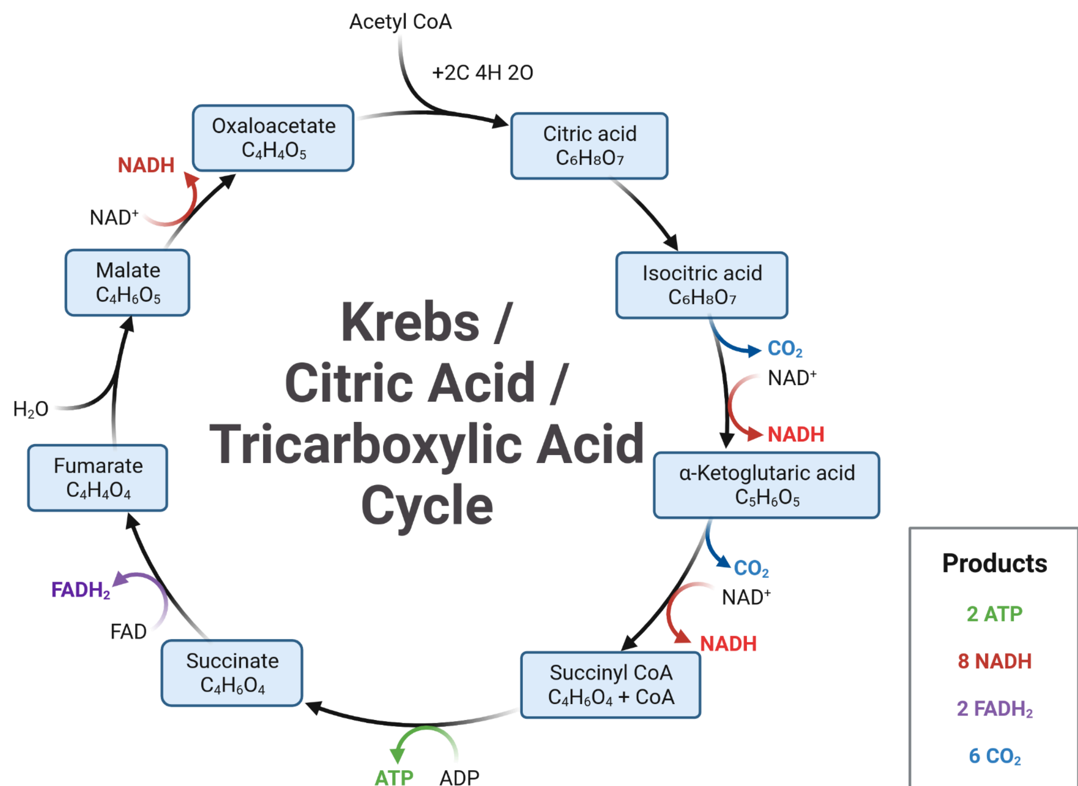


Figure 22: The Krebs Cycle, Citric Acid Cycle or Tricarboxylic Acid Cycle (created with BioRender.com)

The process of OXPHOS to generate chemical energy was then completely described by Mitchell in 1961. In this model a series of proton pumps located in the inner mitochondrial membrane (IMM) utilise the energy from the electrons donated by NADH and $FADH_2$ to pump protons across

the IMM to the intermembrane space creating a chemiosmotic gradient which drives ATP synthase to generate ATP from adenosine diphosphate (ADP) and phosphate (P) (figure 23). Molecular oxygen (O_2) acts as the final electron acceptor in this chain resulting in the creation of water (H_2O).

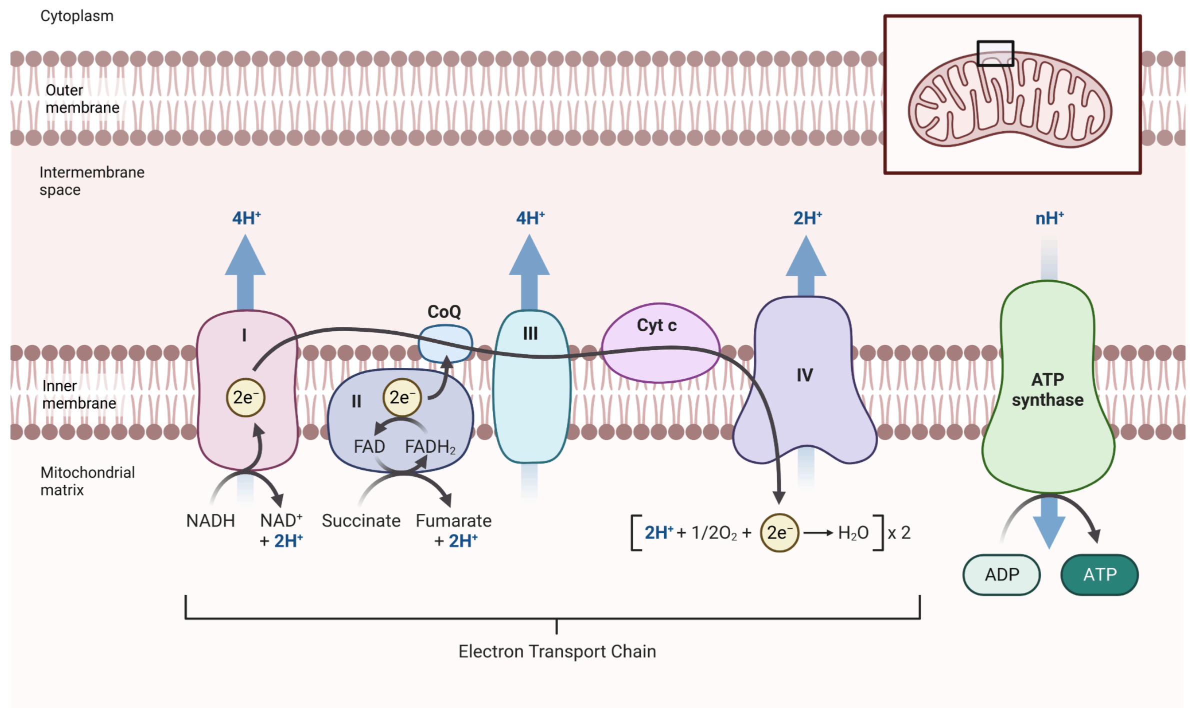


Figure 23: Oxidative Phosphorylation (created with BioRender.com)

The advantage of OXPHOS is that it is much more efficient than glycolysis, producing approximately 30 ATP from a single molecule of glucose, compared to 2 ATP generated by glycolysis or fatty acid oxidation alone with its end product being water (Alberts et al. 2002). The disadvantage is that it is relatively slow compared to glycolysis (Zheng, 2012) so the glycolytic pathway is utilised when energy needs exceed that which the mitochondria can provide, either due to excessive demand or lack of O_2. When mitochondria are unable to use the pyruvate generated from glycolysis, it is fermented using NADH to become lactate. This was traditionally seen as a waste product which is excreted from the cells but emerging evidence suggests this may actually be an alternative fuel source

which can then be taken back up by cells which reverse the reaction to generate NADH for OXPHOS (Rabinowitz & Enerbäck, 2021).

3.1.2. Reactive Oxygen Species

Reactive Oxygen Species (ROS) are a by-product of aerobic respiration. In an ideal situation, electrons transported through the ETC combine with O_2 and 2 hydrogen ions (H^+) to form H_2O (Figure 23) but under normal physiological conditions, an estimated 0.2-2% electrons leak from up to eleven potential sites in the mitochondria to combine with O_2 to form $O_2^{\bullet-}$ (Zhao et al., 2019; Zhang, B. et al., 2022). In order to prevent this reactive molecule from damaging the mitochondria, it is converted by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2) which can diffuse out of the mitochondria and act as a redox signalling molecule (Zhao et al. 2019). H_2O_2 is then broken down further by catalase (CAT) or glutathione peroxidase (GPx) to become H_2O and O_2 again (figure 24). GPx can then subsequently be restored through the action of glutathione (GSH) and glutathione reductase (GR). Peroxiredoxins (PRX) and thioredoxin (TRX) are other enzymes that can catalyse the reaction of H_2O_2 to H_2O in a similar fashion. If the amount of ROS exceeds that which the cell can dismutate, it may react to form several other highly reactive species (Phaniendra, Jestadi & Periyasamy, 2015). If NO reacts with $O_2^{\bullet-}$ then Reactive Nitrogen Species (RNS) may be formed, while excessive H_2O_2 can transform into hydroxyl radicals ($^{\bullet}OH$) through the Fenton reaction with ferrous ions (Fe^{2+}), or the Haber-Weiss reaction with $O_2^{\bullet-}$.

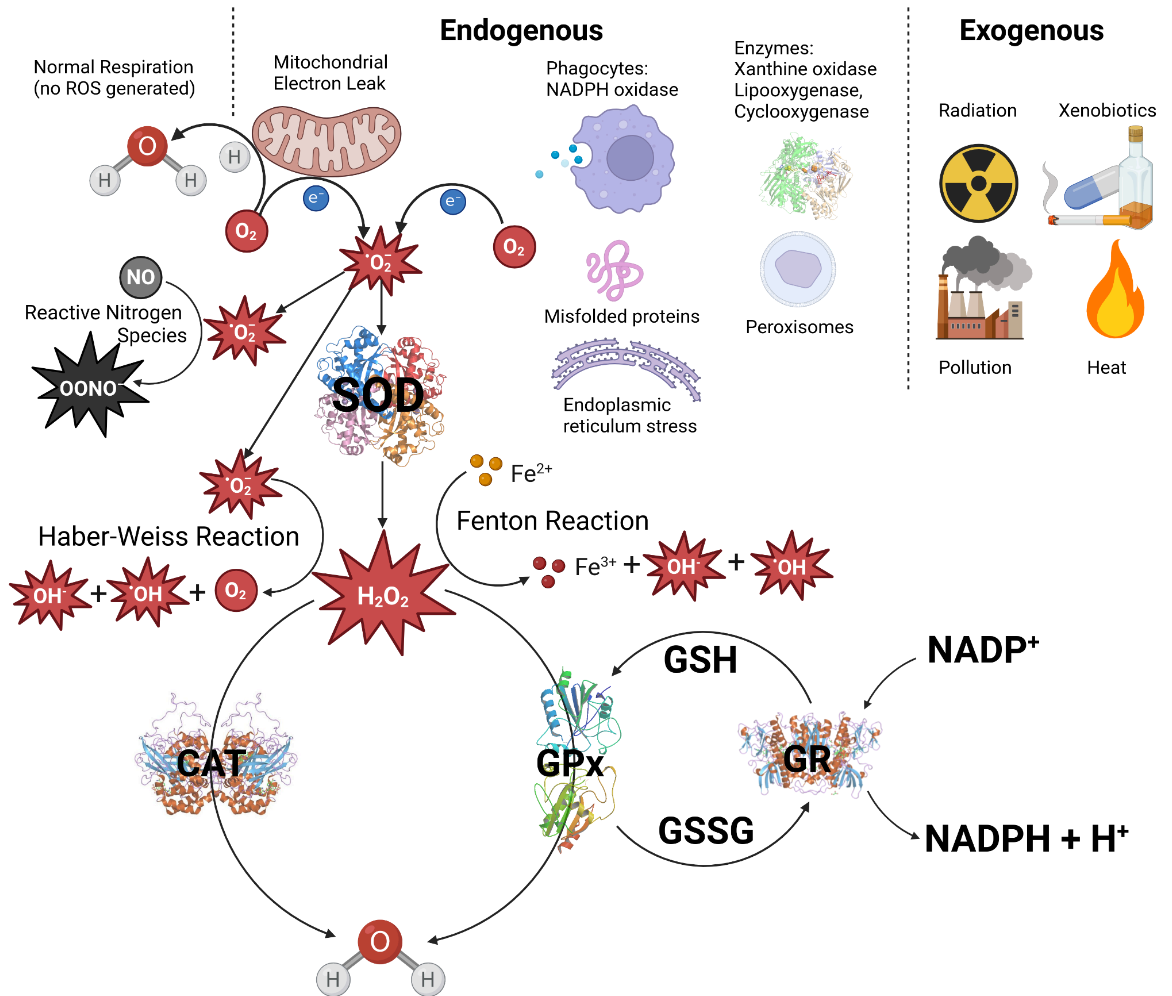


Figure 24: Sources of ROS and antioxidant defence mechanisms (created with BioRender.com).

Abbreviations: SOD: superoxide dismutase; CAT: catalase; GPx: Glutathione Peroxidase; GR: Glutathione Reductase; GSH: Glutathione; GSSG: Glutathione disulphide, oxidised GSH

Although mitochondria are some of the main sources of intracellular ROS, other sources such as NADPH oxidases (NOX), xanthine oxidase and other oxidative enzymes present in the cell are also important contributors, possibly more so than mitochondria (Zhang & Wong, 2021). Endoplasmic reticulum (ER) stress can also stimulate the expression of NOXs and the pathological accumulation of misfolded proteins such as β-amyloids and oligomeric α-synuclein can also produce ROS independent of mitochondria,

while tau and huntingtin can interfere with the ETC to cause the mitochondria to produce more ROS (Abramov, et al., 2020). In addition, exogenous sources such as radiation, pollution, heavy metals, pesticides and drugs can be important contributors of ROS (Phaniendra, Jestadi & Periyasamy, 2015). Regardless of the sources of ROS, mitochondria still play an important role in acting as ROS sinks due to the high level of antioxidative enzymes they contain to manage their own ROS production.

Excessive levels of ROS result in oxidative stress that have been associated with poor health outcomes. This is due to the cumulative damage that it causes over many years and has been posited as a potential mechanism for ageing since Harman first presented the "free-radical theory of ageing" in 1956. More recent versions of this theory suggest that ROS from mitochondrial dysfunction can damage telomeres which, in turn, inhibits the biogenesis of mitochondria leading to a cycle of disruption and senescence (Gao et al., 2022).

ROS are not exclusively associated with cellular damage and degenerative disease but have recently been implicated in a number of important signalling processes that are essential for cellular health (Zhang et al., 2016). ROS influences the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) through both canonical and non-canonical pathways, the extracellular signal-regulated kinase (ERK) pathways of the mitogen-activated protein kinase (MAPK), it promotes the dissociation of nuclear factor erythroid 2-related factor 2 (Nrf2) and kelch-like ECH-associated protein 1 (Keap1), activates phosphoinositide-3-kinase while inhibiting phosphatase and tensin homolog that negatively regulates protein kinase B, has a bidirectional relationship with calcium ion (Ca^{2+}) signalling, acts upon the mitochondrial permeability transition pore (mPTP) both directly and in conjunction with Ca^{2+}, interacts with protein kinases and modulates the ubiquitination/proteasome system.

These pathways are involved in many cellular processes including immune, inflammatory, proliferation, differentiation, autophagy, senescence, apoptosis and stress responses.

Some of these reactions are of particular importance in the redox homeostasis of ROS. The first of these is Nrf2 which is bound to Keap1 under normal conditions, targeting it for ubiquitination. In the presence of ROS, Keap1 dissociates from Nrf2, enabling its activation which leads to enhanced transcription of multiple antioxidant enzymes (Baird & Yamamoto, 2020), creating a negative feedback loop assisting the cell to maintain optimal levels of ROS. Exercise generated ROS also activates Peroxisome Proliferator Activated Receptor-gamma (PPARγ) which can sensitise tissues to insulin, and Adenosine monophosphate (AMP)-activated protein kinase (AMPK) which acts as a gatekeeper to the master regulator of mitochondrial biogenesis, PPARγ Coactivator-1α (PGC-1α), leading to increased numbers of mitochondria and increased mitochondrial fusion (Webb et al., 2017; Cantó & Auwerx, 2009).

It has also been suggested that, since mitochondria share a common ancestry with bacteria, that they may possess signalling pathways to communicate with our microbiome. Ballard & Towarnicki (2020) have proposed that ROS may act as a bi-directional signalling molecule, with gut dysbiosis being correlated with chronic inflammation marked by excessive levels of ROS and lower levels, within the stimulatory cell signalling range, being correlated with greater diversity of the microbiome, maintaining intestinal barrier integrity and appropriate mucosal immune responses. Additionally, many toxins released by pathogenic gut microbiota are able to affect mitochondria directly and can even cross the blood-brain barrier to affect the mitochondria of neuronal cells in the brain providing a chemical link for the postulated gut-brain axis (Zhu et al. 2022). Exercise has also

been shown to have positive effects on both mitochondria and gut microbial health through shared signalling pathways (Clark & Mach, 2017).

3.1.3. Immune Responses

Mitochondria regulate the immune system through multiple mechanisms. In response to stress, mitochondria release a number of Damage Associated Molecular Patterns, including cardiolipin, n-formyl peptides, ROS and mitochondrial DNA (mtDNA), into the cytosol or extracellularly which are detected by pathogen recognition receptors that trigger the release of cytokines which recruit and activate immune cells and regulate responses to the insult (Banoth & Cassel. 2018). In addition, pro-inflammatory (M1) macrophages utilise a “broken” TCA cycle by downregulating isocitrate dehydrogenase, inhibiting the conversion of citrate into α-ketoglutarate resulting in citrate accumulation and its conversion into itaconate. In addition to some direct antimicrobial effects, itaconate also inhibits succinate dehydrogenase, complex II of the ETC, resulting in increased ROS formation that, combined with additional ROS generated from NOX2, inactivates phagocytosed microbes and stabilises Hypoxia Inducible Factor 1α (HIF-1α) leading to upregulation of pro-inflammatory cytokine IL-1β (Herb & Schramm, 2021). Mitochondria are also involved in antiviral defences through the activation of Mitochondrial Antiviral Signalling proteins located on the outer mitochondrial membrane (OMM) (Mills, Kelly & O'Neill, 2017). Viral ribonucleic acid within the cell activates this system leading to activation of the Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP) 3 and NF-κB inflammasome responses resulting in pyroptosis of the infected cell.

Besides driving inflammatory responses, mitochondria have also been suggested as the drivers behind stopping the inflammatory process and enabling the body to begin healing. Naviaux (2023) proposes that the

inflammatory response is only part of the healing cycle and, to fully recover, the organism must also undergo phases of proliferation to regrow the damaged tissue and differentiation in order to ensure the cells are fit for purpose. Changing levels of extracellular ATP (eATP) from different mitochondrial phenotypes drive the transition between each of these phases. Inability to successfully transition from one stage to the next results in the organism becoming stuck in a loop involving one or more of the phases of the healing cycle, driven by abnormal eATP signalling (figure 25). This results in chronic inflammation, disorders of excessive proliferation or incorrect differentiation, or a combination of these, which account for many health conditions prevalent today. Unfortunately there is little mention of what therapies might be able to facilitate this healing response, only that they should work in tandem with behavioural and device-based methods to shift the whole body from one phase to the next, meaning that there is little concrete evidence for this model's therapeutic efficacy. However, it may help to explain how some traditional healing methods that appear to be triggering fresh trauma could operate (eg. vigorous forms of manipulation, acupuncture, cupping or bloodletting), by restarting the healing cycle with an artificial stressor and allowing an opportunity for it to continue to completion.

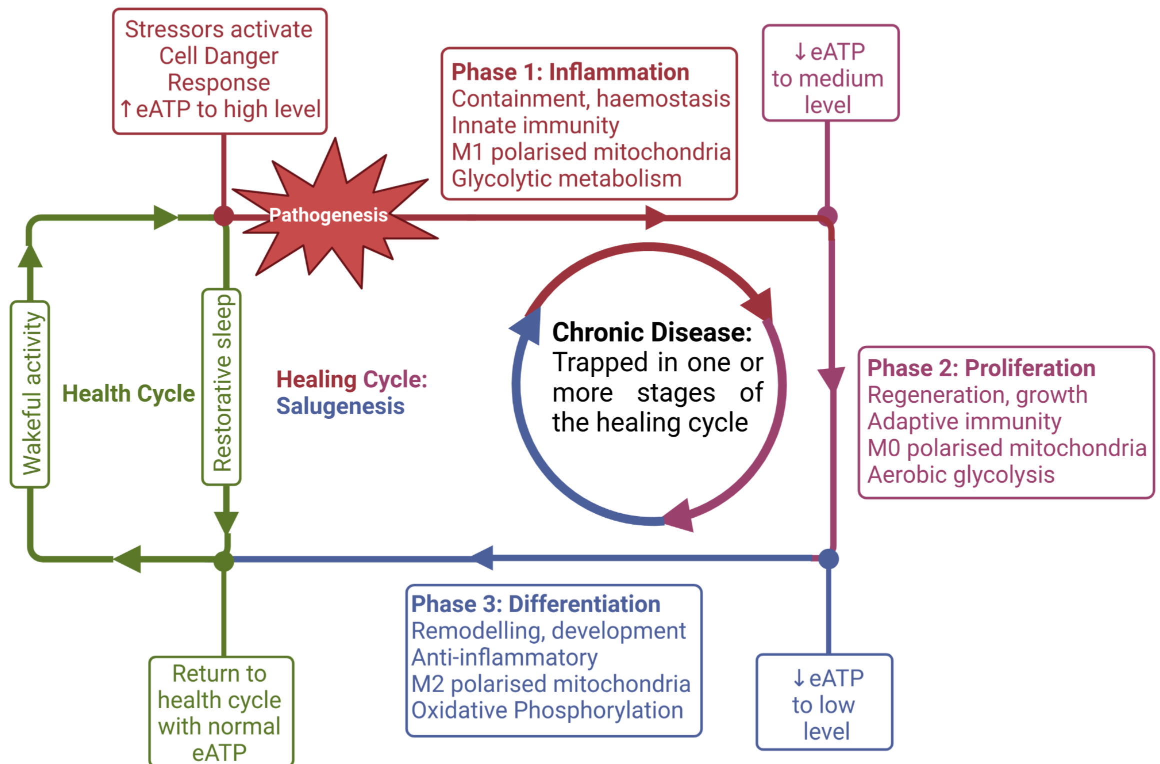


Figure 25: Simplified Diagram of the Healing Cycle, *Salugenesis* (created with BioRender; adapted from Naviaux, 2023).

3.1.4. Apoptosis

Besides being central to cellular life, mitochondria are also pivotal to initiating apoptosis, the process of programmed cell death which ensures that defective cells are destroyed in a way that does not harm the organism (Bock & Tait, 2020). Failure to initiate apoptosis can result in cancer or autoimmunity whereas excessive cell death results in degeneration.

There are two pathways to initiate apoptosis, both of which involve mitochondria (figure 19). The intrinsic pathway, also known as the mitochondrial pathway, is initiated when cellular stresses exceed the mitochondria's threshold triggering members of the B cell lymphoma 2 (Bcl-2) protein family activate the apoptosis regulator proteins Bcl-2

associated X (Bax) and Bcl-2 antagonist killer (Bak) which cause the mPTP on the outer mitochondrial membrane to open and release cytochrome c from the ETC into the cytoplasm. Cytochrome c then binds with apoptotic peptidase activating factor 1 to form a complex called the apoptosome. This binds to caspase 9 which activates the executioner caspases 3 and 7 starting an irreversible process towards cell death. The various stimuli which can initiate this process include withdrawal of negative feedback such as growth factors that lead to failure of suppression of cell death programs, or positive stimulation from radiation, toxins, hypoxia, hyperthermia, viral infections or ROS (Elmore, 2007). Mitochondria are also involved in the extrinsic apoptotic pathway through the activation of Bax/Bak although it also directly activates the executioner caspases through the activity of caspases 8 and 10.

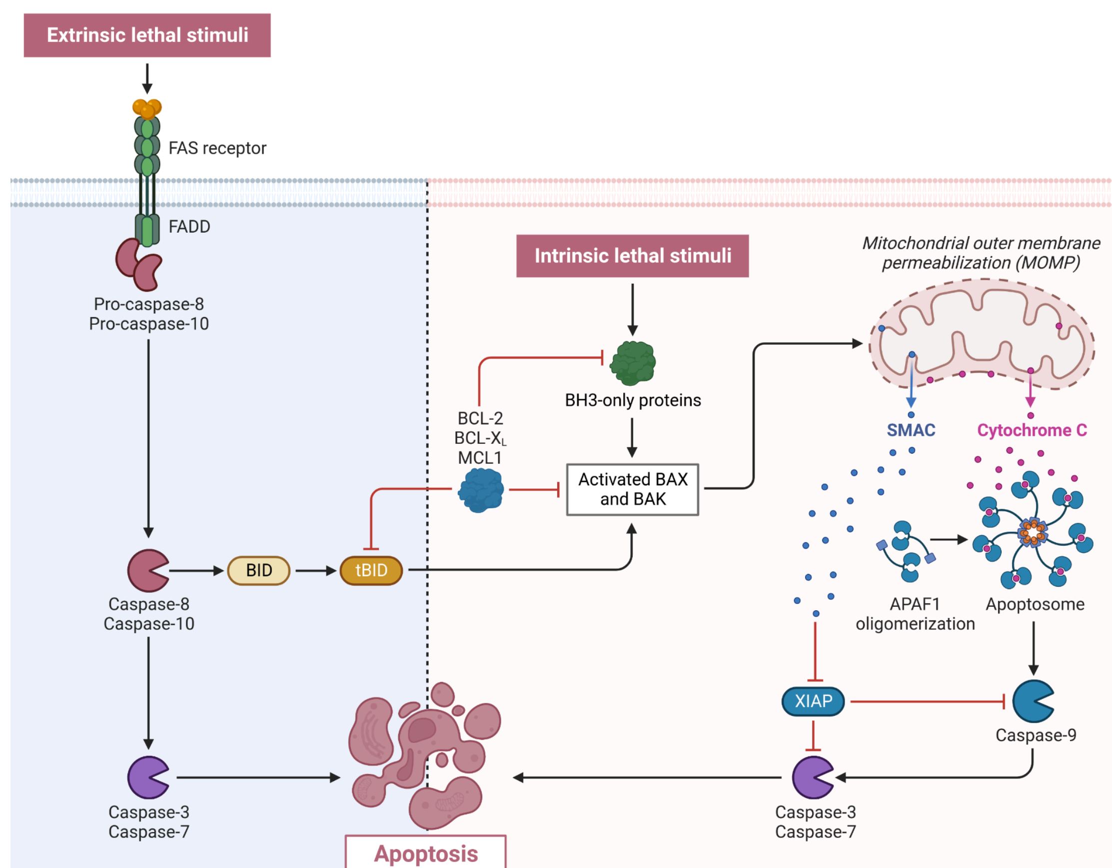


Figure 26: The Role of Mitochondria in Apoptosis (created with BioRender.com).

3.1.5. Hormesis and Adaptation to Stress

Besides prompting the cell to die in the presence of excessive stress, mitochondria also facilitate adaptive responses to stress. The traditional understanding of oxidative stress runs through a simple scale of low levels being healthy as the cell can easily maintain homeostasis, through to high levels causing apoptosis as described in section 3.1.4 above. A middle level is also proposed where the conditions required for cancerous mutations and tumorigenesis are met, where ROS is enough to cause damage to DNA while remaining below the apoptotic threshold (Moloney & Cotter, 2018) (figure 27).

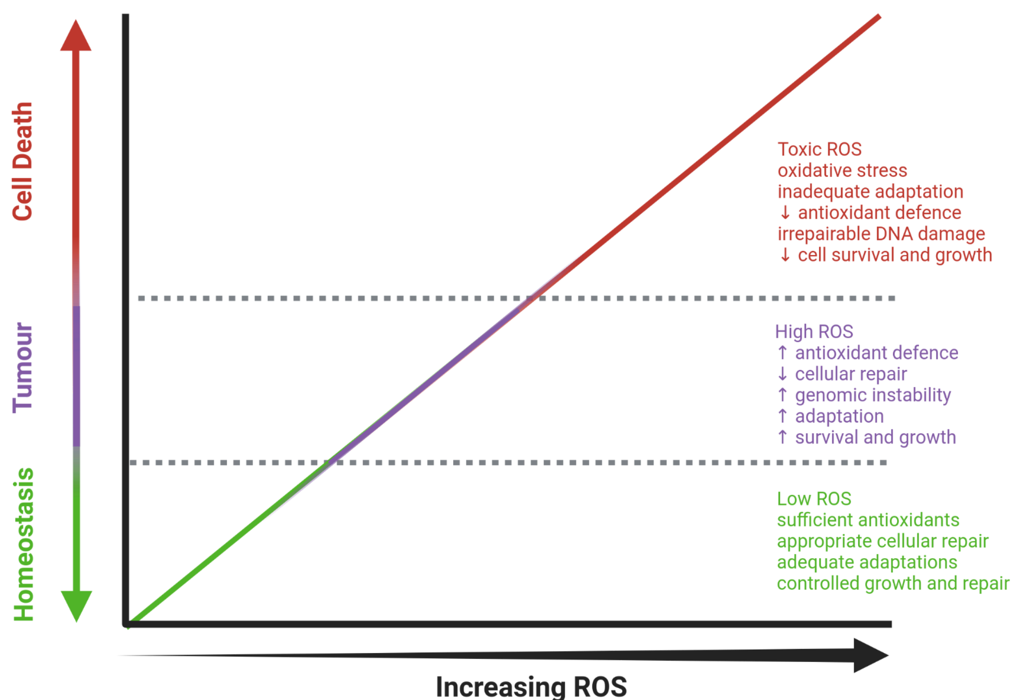


Figure 27: Traditional Model of ROS in Cells
(created with BioRender.com, adapted from Moloney & Cotter, 2018).

In contrast, the hormetic model proposes that small amounts of a potentially harmful stimulus promote cellular adaptations that lead to greater resilience against future exposure, explaining the health benefits seen in activities such as exercise and fasting (Pinches et al., 2023). Too little oxidative stimuli is also becoming recognised to cause an impaired state of cellular functioning, known as reductive stress (Xiao & Loscalzo, 2020) (figure 28).

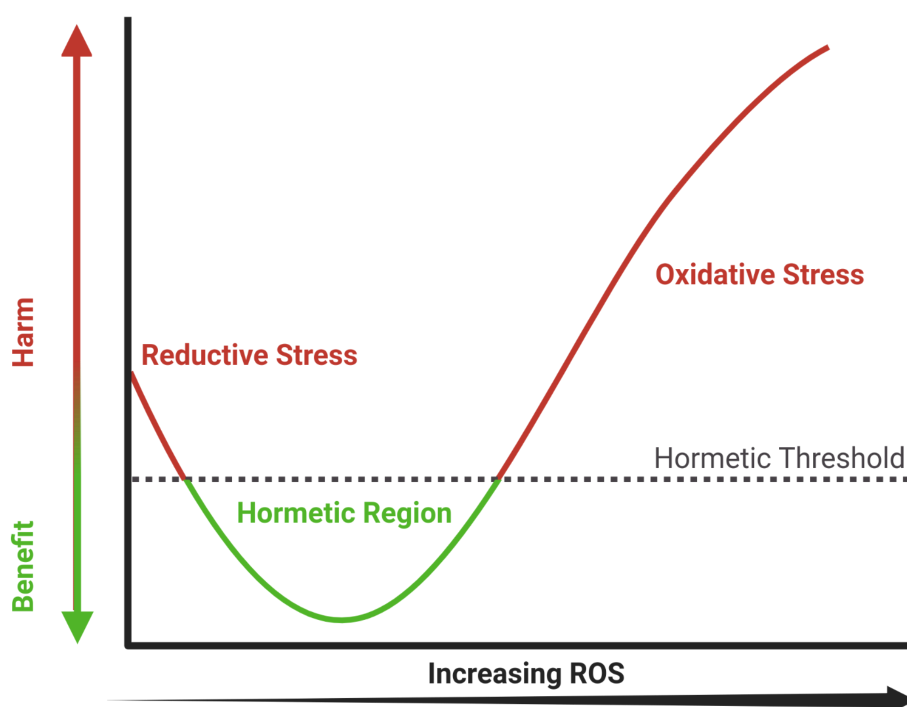


Figure 28: Hormesis Dose-Response Curve (created with BioRender.com).

Hormesis has a long observational history, with Mithrades (135 - 63 BC) being reported to have taken low doses of poison so that he could survive a larger dose if administered by conspirators, through to the observation of Paracelsus in the 16th century that: “All things are poison and nothing is without poison; only the dose makes a thing not a poison”

(Stringer & Rossiter, 2017). In the 19th century, this biphasic dose-response was coined the “Arndt-Schultz law” and suggested to be the mechanism behind homoeopathy until homoeopathy fell out of favour with the medical establishment (Calabrese et al. 2013). It was then forgotten until 1943, when Southam and Erlich noted that small doses of antifungal red cedar extracts promoted fungal growth at low doses and coined the term “hormesis”. Since then it has been proposed as a hypothesis which states that a low dose of toxic stimulation applied to a living organism can increase growth and respiration while a high dose of the same stimuli can have an inhibitory effect (Calabrese & Baldwin, 1998). Two main mechanisms have been proposed for how hormesis may work: overcompensation and direct stimulation (Calabrese et al., 2016). Overcompensation happens when an organism experiences a disruption in homeostasis and responds with an overcompensated response that ultimately leads to greater resistance to the stressor in future. Direct stimulation refers to a situation where the same drug may interact with different receptors or pathways depending on its dose. Three potential mechanisms have been proposed for this (Calabrese, 2013): either the same receptors are stimulated or inhibited by the same substance at different doses, or different receptors mediate the response to the same substance at different doses, or a low dose may activate a receptor or signalling pathway, but a high dose does not.

When applied to mitochondria it is termed mitohormesis and states that mild mitochondrial stress activates a cascade of signalling pathways that render the cell less susceptible to future perturbations (Yun & Finkel, 2014). The most relevant of these signalling pathways are ROS which are generated from any number of situations in the mitochondria including increased energy demand from exercise (Merry & Ristow, 2016), or reduced supply from calorie restriction (Schulz et al., 2007), or an increase or perturbations in O_2 supply (Burtscher et al., 2022). Exercise places excess demand upon the mitochondria, beyond their normal capacity, while fasting

or hypoxia affects the supply of substrates necessary for efficient functioning of the ETC and results in an increase in ROS. Hyperoxia also results in increased ROS because there is extra O_2 present which bonds to free electrons, creating more ROS. Although the pathways for each are different, all of these stressors function as a signal to initiate improved mitochondrial function through the upregulation of antioxidant defences, increased fission and mitophagy to remove defective mitochondria, mitochondrial biogenesis to increase mitochondrial numbers, and the fusion of healthy mitochondria into a network that can share resources and improve the functioning of existing mitochondria.

The ER is an important piece of cellular infrastructure that allows the cell to communicate stress to the mitochondria which can then respond to the increased energy demand (Rossi, Pizzo & Filadi, 2019). In times of stress, Ca^{2+} reserves that are stored in the ER are released through inositol 1,4,5-trisphosphate receptors into the cytosol near to the mitochondria that are bound in close proximity to the ER with mitochondria associated membranes (Marchi et al., 2018) (figure 29). This enables them to be exposed to much higher levels of Ca^{2+} than are present in the general cytosol, and which are taken up through voltage dependent anion channels into the intermembrane space, to be transported through a specialised mitochondrial calcium uniporter (MCU) into the mitochondrial matrix.

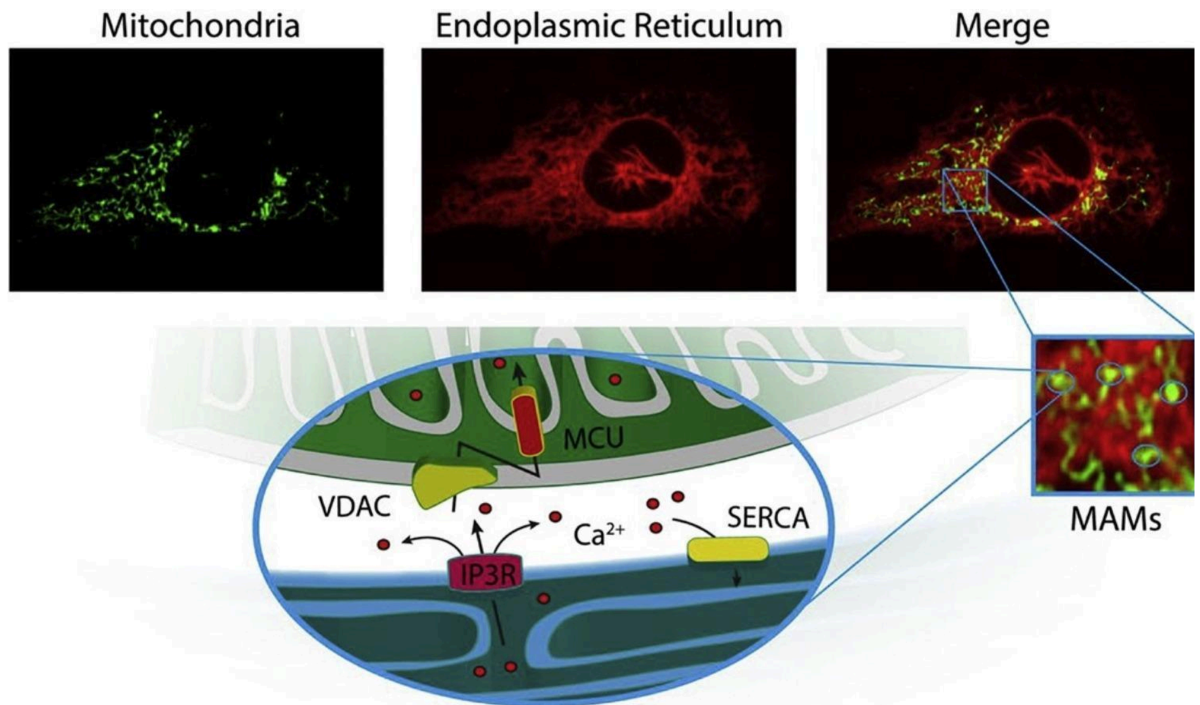


Figure 29: Mitochondria, the Endoplasmic Reticulum and Ca^{2+} (Marchi et al., 2018; reproduced with permission)
Abbreviations: MAMs: Mitochondria Associated Membranes; MCU: Mitochondrial Calcium Uniporter; SERCA: Sarco-Endoplasmic Reticulum Calcium ATPase; VDAC: Voltage Dependent Anion Channels;

Moderate levels enable mitochondrial adaptation to the cell's metabolic needs by boosting ATP production through the activation of several enzymes in the citric acid cycle, while high levels sensitise the mitochondria to the opening of mPTP, initiating apoptosis (Bravo-Sagua et al., 2017). Several diseases involve this calcium dependent apoptotic mechanism including ischaemia-reperfusion injury (IRI), cardiomyopathy, liver and muscle diseases, cancer and neurodegenerative disorders (Finkel et al., 2015; Britti et al., 2018; Romero-Garcia & Prado-Garcia, 2019).

3.1.6. Ageing and Degeneration

The damaging effects of ROS on cellular constituents and connective tissues led to the “Free Radical Theory of Ageing” being proposed in the 1950s (Harman, 1956). Under this theory it is the gradual, cumulative damage to DNA, lipids, proteins and polysaccharides caused by ROS which leads to ageing and the various disorders that increase with age, especially in tissues with a high energy demand such as neurons and cardiovascular cells, and those with a link to damaged to DNA, such as cancer.

3.1.6.1. Neurodegeneration

Neurons have a high energy demand and an unusual shape, sometimes as much as 10, 000 times the size of an average cell, with a strict aerobic metabolism (Vergara et al., 2019), which has led them to develop unique methods to achieve mitochondrial homeostasis. Neurons have to last a lifetime, and require high amounts of energy to maintain resting potentials, generate action potentials and achieve synaptic neurotransmission, with these functions often needing to be maintained a long distance from the soma of the cell. Most of this energy is derived from mitochondrial ATP so mitochondria must be continually renewed and consumed. Canonically, this has been thought to take place in the soma but emerging evidence suggests that both biosynthesis and mitophagy may happen locally, and mitochondria may even be imported from adjacent cells (Misgeld & Schwartz, 2017). Regardless of their origin, they are docked at high energy demand areas of the cells through a variety of tightly regulated docking mechanisms in the cytoskeleton (Cardanho-Ramos, Faria-Pereira & Morais, 2017). This high energy demand will result in the generation of high levels of ROS, which must be managed effectively. A certain level is necessary for optimal function (Cobley, Fiorello & Bailey, 2018) but excessive levels lead to

dysfunction, apoptosis and neurodegenerative disorders (Abdelhamid & Nagano, 2023).

Closely related to neurological health is sleep. Loss of sleep with age appears to have a bi-directional relationship with neurodegenerative disorders (Mander, Winer & Walker, 2017), resulting in elevated levels of oxidative stress (Singh et al. 2019). Reimund's (1994) "Free Radical Flux Theory of Sleep" attempts to explain the fundamental reason for sleep. While many theories of sleep consider it useful for cognitive functions, such as consolidation and organisation of memory (Cousins & Fernández, 2019), Reimund was interested in why animals, whose great advantage over plants are that they can move in order to seek food and evade predators, spend almost a third of their life unconscious and motionless. His hypothesis was that the high energy demand of the brain, which consumes around 20% of the total O_2 supply of the body, results in high levels of ROS. This is managed by periodically decreasing the cerebral metabolic load through sleep while ROS are removed. Melatonin, which is an important regulator of nocturnal physiology, whose synthesis is inhibited by daylight (Zisapel, 2018), is also a potent antioxidant found in high concentrations within mitochondria (Rieter et al., 2018) and protects the ETC from neuronal nitric oxide synthases which cause excessive levels of highly damaging peroxynitrites (see section 3.1.2), improving mitochondrial antioxidant defences and preventing apoptosis by inhibiting the opening of the mPTP (Hardeland, 2017). Sleep also results in a short glucose fast for the brain mitochondria, providing a hormesis effect while they must rely on ketone metabolism, and allows the clearing of accumulated β-amyloids that are generated during wakefulness (Kramer & Bressan, 2018). Sleep deprivation studies in animals and autopsies of humans with familial fatal insomnia reveal signs of mitochondrial dysfunction from oxidative stress with decreased cristae density and alteration in morphology leading to increased apoptosis (Melhuish Beaupre et al., 2021) (figure 30).

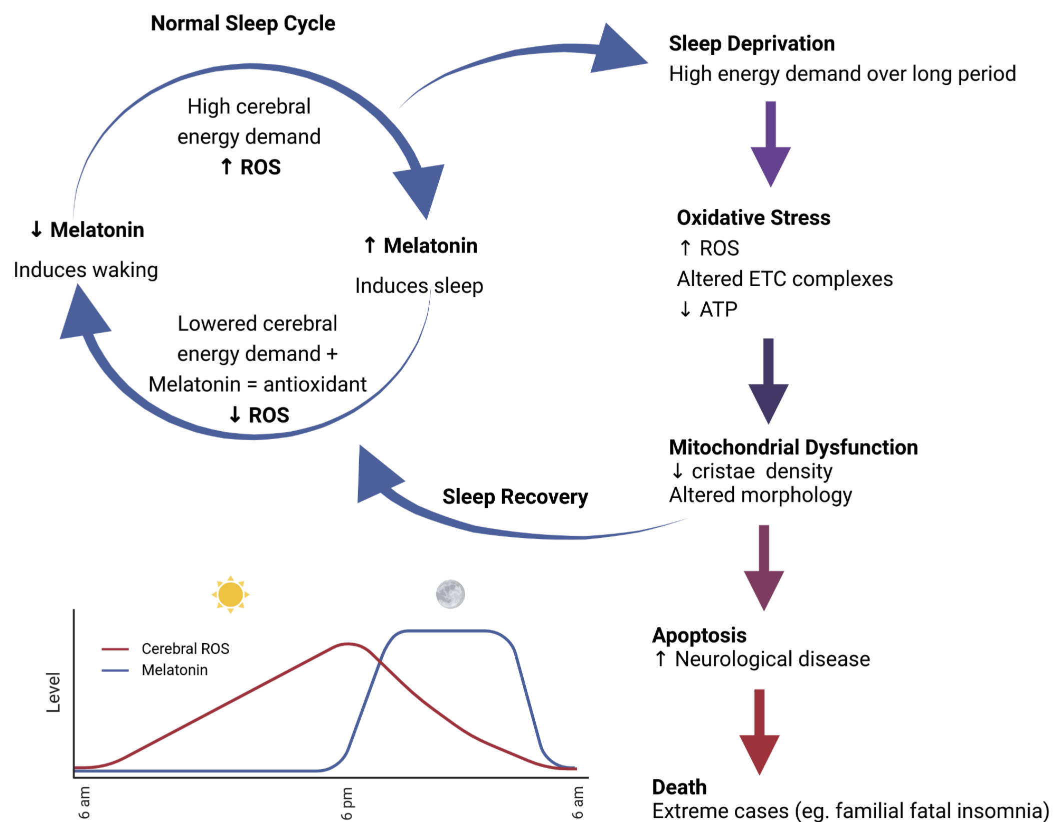


Figure 30: The Role of ROS and Melatonin in Sleep / Wake Cycles and the Oxidative Effects of Sleep Deprivation
(created with BioRender.com, adapted from Melhuish Beaupre et al. 2021).

3.1.6.2. Cardiovascular Disease

The cardiovascular system also has a very high energy demand and is subject to degenerative changes with age. Cardiomyocytes are some of the most mitochondria rich cells in the body, consisting of ~30-40% mitochondria (Murphy & Liu, 2023). In cardiovascular disease there is often altered mitochondrial morphology, reduced ATP production with excessive ROS production, increased apoptosis and deregulated autophagy (Chistiakov et al., 2019). In atherosclerosis, the oxidation of low density lipoproteins promotes atherogenesis by increasing leukocyte adhesion, inducing endothelial dysfunction and apoptosis, activating platelets and

increasing plaque instability which may trigger a cardiovascular event (Kattoor et al., 2017). The high reliance of cardiomyocytes on OXPHOS makes them extremely vulnerable to hypoxia and subsequent IRI during ischaemic heart disease (Ramachandra et al., 2020). In IRI, the initial ischaemia leads to hypoxia which forces the cell to rely on glycolysis, reducing the ATP required for effective contraction, while resulting in the production of lactate and accumulation of protons, which increases acidity. This acidification inhibits mPTP opening, disrupting its role in maintaining ion homeostasis causing the cell to overload with sodium ions (Na^+) and Ca^{2+}. Upon reperfusion, the influx of O_2 and nutrients causes the mitochondria to reinitiate OXPHOS, resulting in a burst of ROS. The MCU opens absorbing the high levels of Ca^{2+} that have accumulated during ischaemia, overloading the mitochondria with Ca^{2+} leading to more ROS. The normalisation of pH disinhibits the mPTP, which is opened by the high levels of Ca^{2+} and ROS leading to collapse of the mitochondrial membrane potential (ΔΨm), inducing rigour hypercontracture and cell death (figure 31).

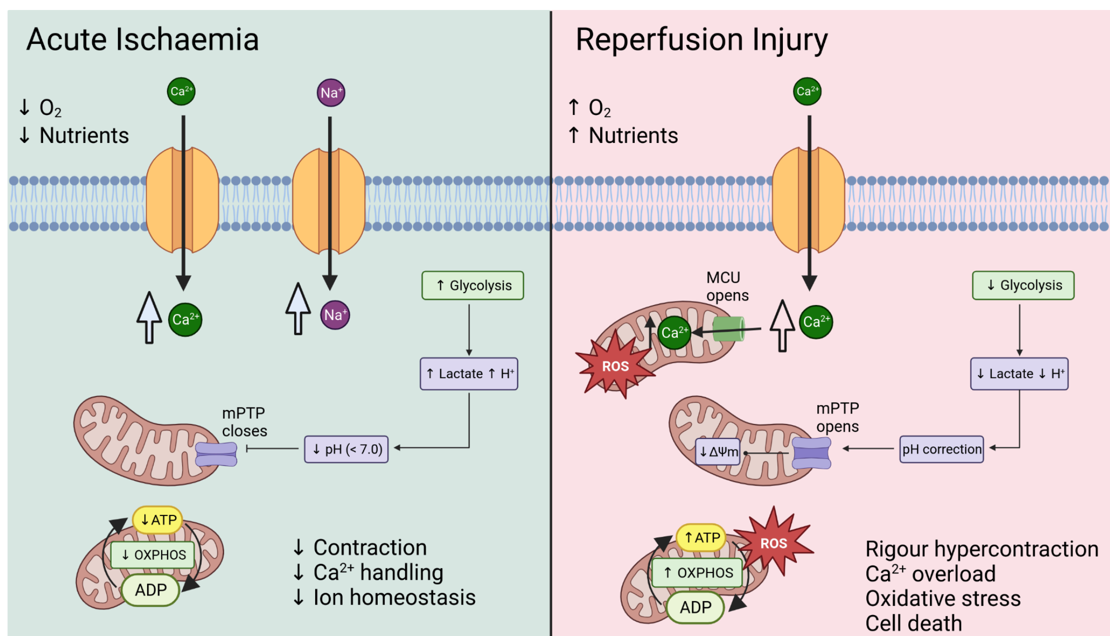


Figure 31: Mitochondrial mechanisms in ischaemia-reperfusion injury (created with BioRender.com).

Other forms of heart disease that involve mitochondrial dysfunction include cardiac hypertrophy and chronic heart failure. The precise mechanisms vary depending on the types and causes of heart failure and other comorbidities but they always involve a metabolic shift away from OXPHOS and a change in the substrates used by the mitochondria which leads to alterations in ROS, Ca^{2+}, morphology and biogenesis, resulting in an energy deficit that is compensated for with glycolysis that is unable to restore normal cardiac function due to its relative inefficiency (Lopaschuk et al., 2021).

3.1.6.3. Cancer

Another disease that becomes more common with age is cancer. Alterations in the metabolism of cancer cells to favour glycolysis, even in the presence of O_2 (aerobic glycolysis) were observed by Otto Warburg in 1927 who noticed that O_2 did not suppress glycolysis in tumour cultures (Otto, 2016) and reached acceptance with his famous paper in 1956 (Warburg, 1956), subsequently becoming known as the “Warburg Effect”. Since then, many more hallmarks of cancer that differentiate them from healthy cells have been identified with the Warburg Effect coming under a broader category of deregulated metabolism (Hanahan, 2022; Hanahan & Weinberg, 2011; Hanahan & Weinberg, 2000) (figure 32).

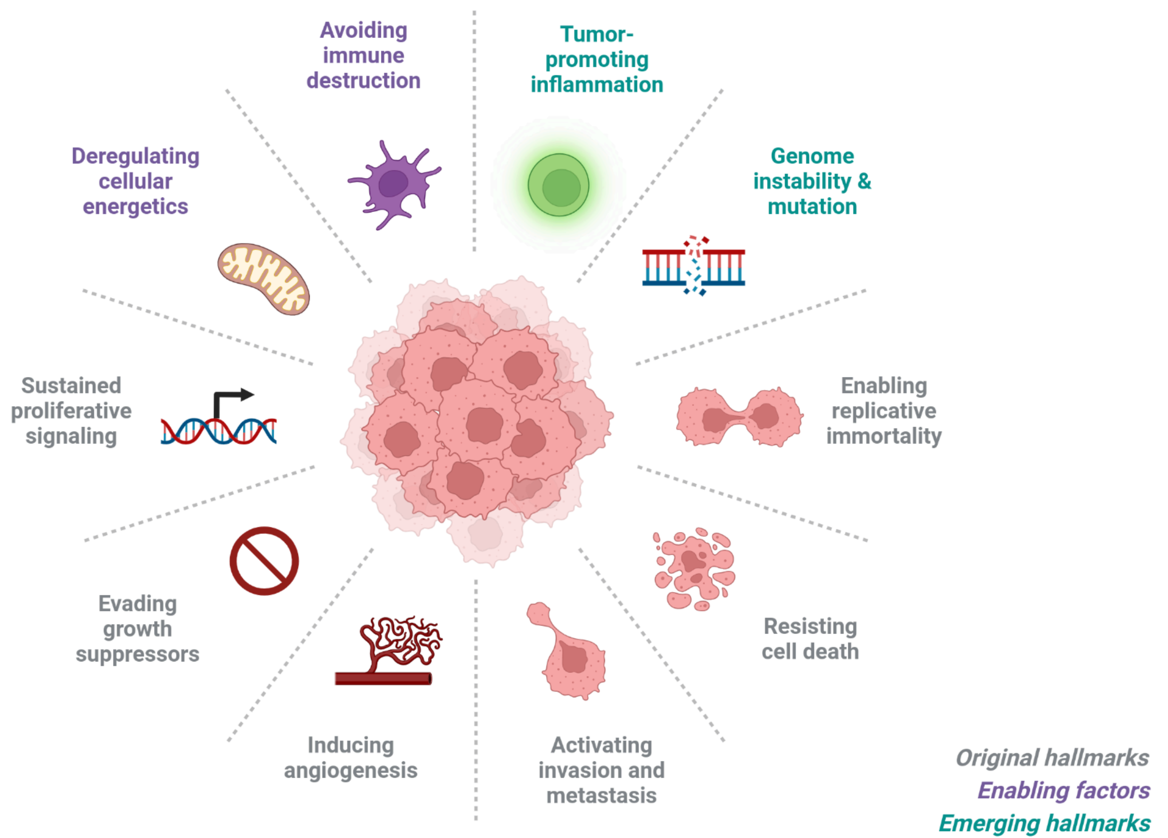


Figure 32: Hallmarks of Cancer
(created with BioRender.com, based on Hannehan, 2022)

Despite this early recognition of metabolic differences in cancer cells, the predominant theory on its origins has been that cancer is a disease triggered by genetic mutation, but with many of these hallmark processes being controlled by mitochondria, including the generation of ROS which causes genetic mutation, the resistance of apoptosis and deregulated metabolism, and the role of mitochondria in inflammation and immune function, cancer is rapidly being reframed as primarily a disorder of mitochondrial dysfunction (Seyfried & Chinopoulos, 2021).

3.1.7. Drug Resistance

As organelles that enable the cell to adapt to stress, mitochondria have also been identified as playing a potential role in drug resistance. The hormetic mechanisms of biogenesis and mitophagy, fusion and fission, which have been outlined in section 3.1.5. above, have also been found to be involved in chemoresistance (Jin, P. et al., 2022) and chemoresistant cancer cells have been found to have altered metabolism with a greater reliance on mitochondrial ATP (Bokil & Sancho, 2019). Sublethal release of cytochrome c combined with sublethal mPTP opening have been identified as key mechanisms that create drug-tolerant persister cells (Kalkavan et al., 2022). Chemoresistant cell phenotypes have also been identified to have reduced MAMs connecting to the mitochondria to the ER (Çoku et al., 2022), described in 1.5.1.5. making them less sensitive to Ca^{2+} and less likely to initiate apoptosis when confronted with the stress of chemotherapeutics.

Another mechanism of drug resistance that may be mitochondrial dependent is through the expression of efflux pumps. One of the largest families of these transporter proteins are the ATP-Binding Cassette (ABC) transporters which use mitochondrial generated ATP as their main source of energy (Giddings et al., 2021). Two of the most significant, Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance-associated Protein (MRP) 1, have been observed to be localised to mitochondria in doxorubicin resistant breast cancer cells, and the accumulation of doxorubicin is decreased in proportion to mitochondrial ATP production, an effect that can be partially reversed by oligomycin, a mitochondrial ATP synthase inhibitor (Dartier et al., 2017).

Cis-diamminedichloroplatinum(II) (cisplatin) is another chemotherapy agent widely used in the treatment of several solid tumours but which also

has encountered problems of chemoresistance (Cocetta, Ragazzi & Montopoli, 2020). Cisplatin acts through the induction of DNA lesions, with a greater affinity for mtDNA than nuclear DNA (Oliveira et al., 1995), inducing oxidative stress and apoptosis primarily via the intrinsic pathway, disrupting DNA repair and inducing cell cycle arrest (Ghosh, 2019). Common side effects include nephrotoxicity, hepatotoxicity and vomiting through the disruption of redox homeostasis and apoptosis in healthy cells, evidenced by the reduction of these side-effects by natural antioxidants (Zhou et al., 2022; Abd Rashid et al., 2021; Alam et al., 2017). Antioxidants such as melatonin (Baghal-Sadriforoush et al., 2022) and quercetin (Hasan et al., 2023) which have demonstrated conditional pro-oxidative effects on cancer cells, discussed further in section 3.2.5.1, increase the apoptotic effect of cisplatin, also indicating a redox pathway. These suggest that the problem of cisplatin chemoresistance is also one of mitochondrial adaptation and this has been observed in cisplatin resistant ovarian cancer cells which undergo a shift to enhanced oxidative metabolism, a reorganisation of the mitochondrial network and an increased mitochondrial turnover rate (Zampieri et al., 2020).

3.2. The Role of Mitochondria in Traditional Medical Systems

People seek traditional, complementary and alternative medicine for a variety of conditions for which mitochondria may hold a central role. Lists of conditions treated are difficult to obtain due to the Advertising Standards Agency requiring that medical claims be supported by strong and robust evidence (Advertising Standards Agency, 2021) which is often lacking in complementary and alternative therapies (Ernst, 2011). However, the Professional Register of TCM based in Ireland list the common complaints as described by patients frequently seen within TCM clinics and they include

a number of conditions mediated by mitochondria including infections and immune disorders, metabolic, cardiac and neurological conditions, stress and chemo- and radiotherapy side effects (Professional Register of TCM, 2022) whose connection to mitochondrial function have all been described above in section 3.1. Biomolecular texts of herbal medicines also focus on disorders of ageing, redox balance, metabolic regulation, immunomodulatory functions, and cancer prevention (Benzie & Wachtel-Galor, 2011) which all involve mitochondrial function. The multiple, non-specific effects of herbal treatments, said to guide the body in a general therapeutic direction, as opposed to the specific targeting of synthetic drugs (Karimi et al., 2015) may also indicate that mitochondrial modulation could be a common factor. Wallace (2008) even speculated that mitochondria may be behind fundamental traditional medical concepts such as "*Qi*" (气 or 氣, pronounced "Chee").

Some experimental evidence has supported this connection between mitochondria and *Qi* (Wong et al., 2012; Leong et al., 2018; Chen, Y., Li et al., 2020; Chen,Y., Feng et al., 2020) but a straightforward equation of the two concepts can be problematic. An update on the popular definition "vital energy" is well overdue, a definition that reflects more the vitalist doctrines of early translators of Chinese medical texts (Kendall, 2008) than the multi-valent concept that it is, which can be used to draw together many different ways of understanding the world, including cosmological, political, philosophical and physiological models and used in reference to phenomena in fields as diverse as diverse as astronomy, meteorology and other natural sciences; political relations; religious ritual; and internal or tactile sensations felt during emotion, disease states, medical and self-cultivation practices, in often overlapping metaphors (Ingraham, 2019; Stanley-Baker, 2022). Its use is deeply embedded in the Chinese language with meanings as diverse as "steam, vapour, air, anger, gas"

(Archchinese.com, 2023) and featured in hundreds of compound terms with highly diverse meanings (table 7).

Chinese	Pinyin	Literal Meaning	Interpreted Meaning
氣象	*Qixiang*	Qi image	Weather pattern
人氣	*Renqi*	Person Qi	Personality or character
生氣	*Shengqi*	Growing Qi	Anger
大氣	*Daqi*	Big Qi	Atmosphere, heavy breathing or magnanimity depending on context
名氣	*Mingqi*	Status Qi	Reputation or fame
士氣	*Shiqi*	Soldier Qi	Morale
運氣	*Yunqi*	Moving Qi	Luck
正氣	*Zhengqi*	Righteous Qi	Healthy Environment
氣味	*Qiwei*	Qi flavour	Odour or Scent
氣色	*Qise*	Qi colour or Qi Appearance	Complexion

Table 7: Multivalency of the Word *Qi* 氣 in Chinese
Translations obtained from ArchChinese.com (2023)

Narrowly defining *Qi* as “vital energy” has led to it becoming tied to the fate of vitalism that has long been disproven and discarded by the scientific and medical community and remains popular only in some naturopathic and chiropractic circles (Coulter, Snider & Neil, 2019). If *Qi* is tied too closely to mitochondrial function, we run the risk of repeating that mistake and leaving *Qi* at the mercy of another scientific trend. Alternatively, we may create too much of a narrow definition that strips *Qi* of any aspects

not demonstrated to be due to mitochondrial function, thereby losing its phenomenological and poetic uses which are arguably some of its most powerful (Wegmüller, 2015). However, these similarities do suggest that traditional indications of the effects of herbs on *Qi* could be a starting point for generating hypotheses to test with a scientific method, providing we remain mindful that *Qi* encompasses more than mitochondrial function alone.

3.2.1. Hormesis in Traditional Medicine

The concept of hormesis can be used to explain the beneficial effects of many modes of traditional medicine and health promoting systems. Hormesis has been noted to have particular similarity to the Chinese medical concepts of *Yin* and *Yang* (Sun et al., 2020) making this system of medicine, which comprises physical, psychological, dietary and herbal interventions, a good example of how hormesis is at work in traditional therapies.

3.2.2. Exercises

In exercise an increase in the energy demand of the cell results in increased ROS production that ought to cause an increase in oxidative stress damaging the system but actually produces the opposite effect, resulting in increased efficiency of the mitochondria and improvements in overall health (Merry & Ristow, 2016a). This is an example of the overcompensation mechanism of hormesis whereby an organism overcompensates for a disruption to homeostasis (Calabrese et al., 2016), but due to the number of different pathways that ROS activates (see 1.5.1.2), it is also an example of a single substrate activating different pathways depending on the dose. More extreme forms of exercise may push mitochondria above the hormetic threshold and result in a less efficient

network (Flockhart et al., 2021) and may explain the development of Long Covid among 4% of college athletes (Massey et al., 2022) whose activities should provide protection from a condition associated with mitochondrial health (Srinivasan et al., 2021; Cox, 2022).

Traditional medical systems do not only use physical exercises to promote health. Many also use mental exercises and breathing exercises in the form of meditation. The slowed breathing during meditation has demonstrated an acute decrease followed by a sustained increase in O_2 saturation (Bernardi et al., 2017) and mild, acute O_2 deprivation has also demonstrated a protective capacity against IRI through mitochondrial mechanisms (Boengler, Lochnit & Schulz, 2018). Meditation also increases resting levels of melatonin (Nagendra, Maruthai & Kutty, 2012), a powerful antioxidant (Reiter et al., 2016), and may help to replace sleep or pay off sleep debt (Kaul et al., 2010), suggesting a hormetic antioxidant mechanism.

3.2.3. Dietary Interventions

Fasting and ketogenic diets which limit carbohydrates and thus glucose availability, forces greater reliance on β-oxidation of fatty acids in mitochondria for fuel, and have demonstrated improvements in health outcomes from various metabolic disorders (Miller, Villamena & Volek, 2018). Both exercise and calorie restrictions increase the fusion of healthy mitochondria into networks and the fission and autophagy of defective mitochondria resulting in a more efficient system. Fasting is an important aspect of Ayurvedic medicine (Gangele & Paliwal, 2020) and intermittent fasting is practised in Unani medicine as part of the religious obligations required of all muslims during Ramadan (Visioli et al., 2022), both with many perceived health benefits (Trabelsi et al., 2022). Chinese medicine does not emphasise complete fasting but the early medical text Eliminating Grain and

Eating Qi (卻穀食氣 *Quegu shiqi*) from 168 B.C. encourages the avoidance of grain (辟穀 *Bigu*) as a technique for longevity (Harper, 1998, pp.305-9). It was later adopted as a method of eliminating malevolent ghostly parasites (三尸 *sanshi* or 三蟲 *Sanchong*) that were understood to feed upon grains and shorten the lifespan of their host (Dannaway, 2009) suggesting an early version of a ketogenic diet.

3.2.4. Manual Therapies

Even most manual systems of medicine such as acupuncture, massage and heat therapies often involve stimulating inflammatory reactions which suggest a temporary and controlled increase in oxidative stress (Checa & Aran, 2020) which are considered to be indicators of a positive outcome (Zhu, 2014) suggesting a hormetic intention. In addition, deformation of the skin through the use of needles, heat and electrical stimulation releases large amounts of eATP from keratinocytes which circulate, acting as signalling molecules (Burnstock, 2009). These are suggested to be a potential mechanism behind the reported effects of acupuncture on metabolism (Wang & Zhou, 2023), preconditioning against IRI (Wang, L. et al., 2023), circadian cycles (Wang, X. et al., 2023), pain modulation (He, J-R. et al., 2020), recovery from injury (Wang, M-J. et al., 2023), moderation of hypertension (Li et al, 2023), atherosclerosis (Wu et al., 2023) and depression (Yu et al., 2023; Zou et al., 2023). Besides mitochondria being the main source of eATP, this eATP also triggers Ca^{2+} entry into T-cells which enhances mitochondrial ATP production (Trebak & Kinet, 2019) making purinergic ionotropic receptors a means of communication between the keratinocytes deformed by needle manipulation and the subsequent visible immune response. In addition, the importance of mitochondria to maintain the high energy demand of neurological signalling (Misgeld & Schwarz, 2017; Datta & Jaiswal, 2021) might suggest that the

deliberate stimulation of nerve fibres in acupuncture which leads to adaptive neuroplasticity (Xiao et al., 2018) is also driven by mitohormetic changes.

Moxibustion, the application of heat on acupuncture points through the use of burning mugwort (*Artemisia vulgaris*, L., *Asteraceae*) on, or near, acupuncture points, or by fixing it to the top of needles, is another method used in TCM that most likely utilises hormetic mechanisms. The temperature achieved from indirect moxibustion can reach 65℃ on the outer skin and 45℃ on the subcutaneous layer and sometimes higher in methods involving direct skin contact (Deng & Shen, 2013). The body functions within a narrow temperature range and deviations from this induce the denaturation of proteins, including those involved in the ETC, antioxidant defences and cellular repair (Slimen et al., 2014). This will result in reduced ATP production, increased ROS, cellular damage that goes unrepaired, and ultimately apoptosis. To protect the cell from excessive heat, Heat Shock Proteins are activated by raised temperatures which act as chaperones to prevent the misfolding of proteins and refold those which have been denatured (Kurop et al., 2021). Since misfolded protein aggregation and a reduced Heat Shock Protein response is associated in many disease states (Hu et al., 2020), there is the possibility that they could be employed therapeutically and that moxibustion and similar energetic stresses like electroacupuncture may be stimulating this mechanism (Cakmak, 2009).

In cupping and scraping therapies, the haematomas produced imply the release of heme, a strongly pro-oxidant complex, which then breaks down into biliverdin and then bilirubin (Jeney et al., 2013), both potent antioxidants (Lowe, 2017). Experimental evidence has also shown an upregulation of antioxidant enzyme heme oxygenase-1 (HO-1) in internal sites distant to the bruising after the application of scraping (Kwong et al., 2009). These suggest a hormetic response to an artificially induced oxidative stress, which is followed by a decrease through both hormetic

mechanisms and the direct actions of biliverdin, bilirubin and HO-1. This may explain some of the findings of reviews that report it may be effective at reducing pain and inflammation (Wood et al., 2020; Mohamed et al., 2023), and others that suggest cupping has a potential use in controlling obesity (Kang et al., 2023), implying a mitohormetic mechanism, although all call for more higher quality research.

3.2.5. Herbal Medicine

Herbal medicines have also been suggested to follow a hormetic paradigm by promoting adaptive responses at low doses but having inhibitory effects at higher doses, corresponding to "regulating" and "curative" effects (Wang et al., 2018; Sun et al., 2020). These may be present in the same formula through the use of the "Monarch, Minister, Assistant, and Guide" system used in CHM whereby the herbs in a formula are ranked in order of descending dosage, promoting stimulation of some systems through low dose administration and the inhibition of others through a high dosage.

One example of this is the CHM use of cinnamon (*C. cassia*, (L.) J.Presl syn. *C. aromaticum* Nees et *Neolitsea cassia* (L.) Kosterm., *Lauraceae*) where it has a dual function: in small doses (1.5 - 4.5 g) the bark is said to "warm the *Yang*" (Bensky et al., 2004, pp.684), providing improved energy, mobility and bodily warmth but the branchlets are also used in higher doses to treat colds and flu (ibid., pp.8-10), typically around 9 g but sometimes gradually climbing as high as 30 g. Historically it may have been standard to use as much as 42 g[1] from the start and unlike modern texts, *Gui Zhi* (桂枝, "cinnamon twig") referred to all forms of cinnamon with the the bark that is recommended in small doses today being the likely part

[1] He (2013) identifies 1 *liang* as being 13.875 g in the Eastern Han dynasty and the classic formula "Cinnamon Twig Decoction" written during this period recommends using 3 *liang* of cinnamon twigs (Zhang & Liu, 2016).

used (Liu, J. et al., 2020). This can be explained by hormesis. *C. cassia* contains high levels of coumarins which are produced by plants for many purposes, one of which is to protect the plant from infections (Lončar et al., 2020) and mitochondria are theorised to have evolved from a bacterium that entered into a symbiotic relationship with a eukaryotic cell (Roger, Muñoz-Gómez & Kamikawa, 2017). Therefore, compounds metabolised by a plant to have a disruptive effect on infectious bacteria may also have a disruptive effect on mitochondria. This is seen in the literature with several components of cinnamon having antibacterial effects via multiple mechanisms, some of which would affect mitochondria such as the inhibition of ATPases (Vasconcelos, Croda & Simionatto, 2018). This can be seen in the warming effect of *C. cassia* deriving from an uncoupling effect (Li X. et al., 2021). This is when protons which generate the $\Delta\Psi m$ are disassociated from ATP synthesis, causing the excess energy from the dissipation of the proton gradient to be dispersed as heat, leading to thermogenesis (Demine, Renard & Arnould, 2019). This stimulates a hormetic response with mitochondrial biogenesis (Gannon et al., 2015), especially in adipocytes leading to browning of adipose tissue (Kwan et al., 2017) and alleviating insulin resistance (Couturier et al., 2016) when taken in doses generally considered safe (2 g / day) by the Food Standards Agency (Apekey & Khokhar, 2009). When taken in higher doses, this disruptive effect might be enough to inhibit bacteria and potentially mitochondria too, but the mitochondria have the added protection of being inside a cell so will only be exposed to the level in the cytoplasm, whereas free floating bacteria will have a far higher exposure. In addition the mitochondria may already have developed compensatory mechanisms through chronic exposure to low doses of cinnamon.

3.2.5.1. Antioxidants

Many herbs are marketed for their antioxidant potential despite there being little evidence that antioxidants can help *in vivo* (Berger et al., 2012). This situation came about when ROS were discovered to be responsible for atherosclerosis, cancer, vision loss and a number of other chronic degenerative diseases which caused an immediate marketing drive among the media, food and supplement industries who were keen to promote products rich in antioxidants (Harvard School of Public Health, 2021). Subsequent research failed to meet the anticipated expectations and may even be detrimental by preventing the ROS mediated signalling behind hormetic responses to exercise (Pingitore et al., 2015; Merry & Ristow, 2016b; Higgins, Izadi & Kaviani, 2020), although it may be useful in certain populations where exercise causes oxidative stress (Wang, H. et al., 2023). Concerns have even been raised that antioxidants could facilitate cancer progression through inhibition of ROS mediated apoptosis (Harris & DeNicola, 2020).

Antioxidants have a controversial history with chemotherapy. Many chemotherapeutic agents rely on the induction of oxidative stress, initiating apoptosis in cancer cells, presenting the theoretical possibility that antioxidants may reduce the effectiveness of chemotherapy in tumour cells (Lawenda et al., 2008) or encourage tumour growth (Harris & DeNicola, 2020), but several papers present the opposite to be true (Poljsak & Milisav, 2019; Athreya & Xavier, 2017). Publication bias may prevent more studies from demonstrating an interfering effect of antioxidants on chemotherapy (DeVito & Goldacre, 2019) which makes many clinicians err on the side of caution. However, since ROS is necessary to stimulate cancer cell proliferation, invasion and metastasis, antioxidants could slow progression (George & Abrahamse, 2020). Alternatively, some authors suggest that antioxidants may have a biphasic dose-response leading to them being both

pro- and anti-oxidant at different doses, with them potentially interacting differently with the metabolisms of cancerous and non-cancerous cells (Shin et al., 2020; Mahanta & Challa, 2022; Zhang & Zhang, 2014; Fernando, Rupasinghe & Hoskin, 2019). This model proposes a different hormetic redox curve for different cell types with the greater reliance of normal cells on OXPHOS making them capable of tolerating higher doses before having a pro-oxidant effect (figure 33).

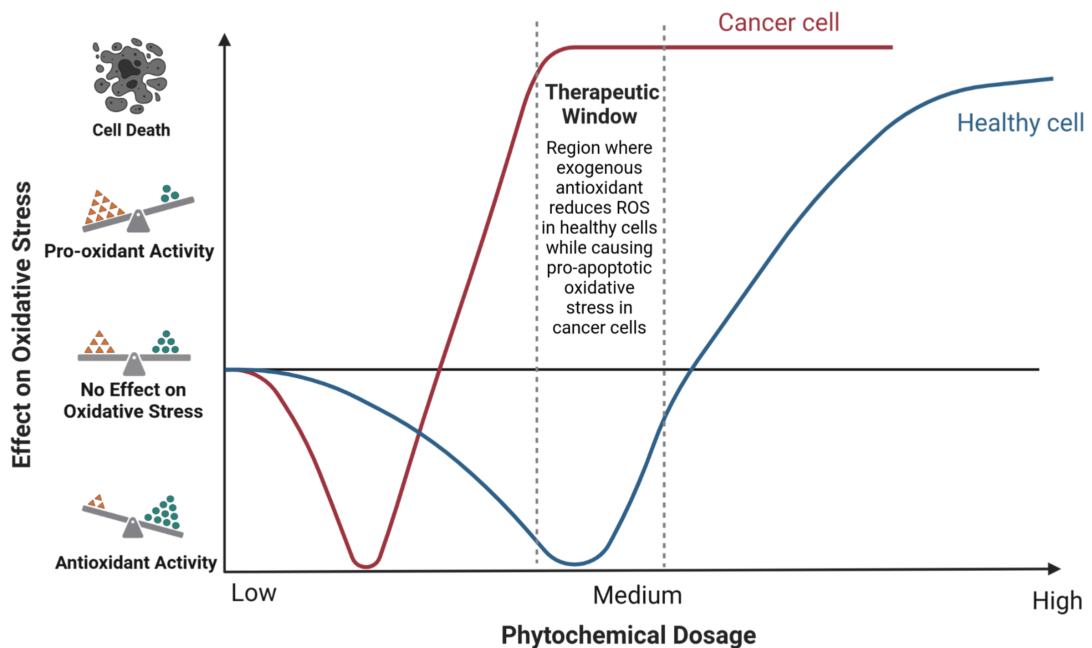


Figure 33: Theoretical Model of the Simultaneous Pro- and Anti- Oxidant Effects of Phytochemicals on Cancerous and Normal Cells (created with BioRender.com)

Quercetin is one example of a phytochemical that has this dual effect on redox states, simultaneously being reported to induce apoptosis in cancer cells through induction of oxidative stress, and providing neuroprotection through a reduction in ROS. It is theorised that it may achieve this effect through the common target of modulating the mitochondrial ETC (Carrillo-Garmendia, Madrigal-Perez & Regalado-Gonzalez, 2023). In cancer cells this disrupts the ETC which is

still necessary for the production of ATP, resulting in ΔΨm disturbance and ATP deficiency with cytotoxic effects. In neurodegenerative disorders, this same effect is able to restore the impaired ETC complexes, resulting in a pro-survival effect.

It is therefore possible for the right dose to exert an anti-oxidant effect on normal cells, protecting them from oxidative stress of chemotherapy, while having a pro-oxidant and pro-apoptotic effect on tumour cells (Prasad, Gupta & Tyagi, 2017). Using phytochemicals to prevent the side effects of chemotherapy has long been investigated, but the notion that they may enhance the efficacy of chemotherapeutic drugs is relatively new and has become known as "priming" the cells for destruction (Mould, 2019). This mechanism has been demonstrated with herbal extracts including cannabidiol (Henley, 2015) and ashwagandha (*Withania somnifera* (L.) Dunal*, Solanaceae*; Henley et al., 2017).

3.2.5.2. Adaptogens

One of the most popular classes of herbs are adaptogens which aim to enhance our non-specific responses to stress. The term adaptogen was defined by Dardymov & Brekhman (1967) as:

> "*non-specific remedies against a very broad spectrum of harmful factors ("stressors") of different physical, chemical and biological natures, and must be innocuous to have a broad range of therapeutic effects without causing any disturbance (other than very marginally) to the normal functioning of the organism*" (cited in Tian et al., 2022).

Many medicinal herbs and phytochemicals have been investigated for their adaptogenic properties, often through their antioxidant potential, assistance with improving energy levels, boosting the immune system and enhancing

mental functioning (Panossian & Wikman, 2010) but their multi-target effects have made it difficult to determine any singular mechanisms by which they can be measured (Gerontakos et al., 2020).

Early models suggested they may work by suppressing the General Adaptation Syndrome of Selye (1936) and an antioxidant capacity to reduce the damage caused by ROS generated by the stressor (Passovian, Wikman & Hagner, 1999). More recently, hormesis has been presented as a potential mechanism for the effect of adaptogens (Lamming, Wood & Sinclair, 2004). This suggests that foreign molecules produced by plants under stressful conditions can mimic the stress response in animals that feed on them and generate adaptive responses that then increase their tolerance when actual stressors are experienced.

This method of inducing hormesis with adaptogenic plants has been coined xenohormesis (Baur & Sinclair, 2008). One example of a xenohormesis inducing compound is resveratrol, which is generated by grapes in response to harsh environments and activates Silent Information Regulator (SIRT)1 in humans, associated with the health enhancing effects of calorie restriction, making resveratrol a compound of interest in metabolic hormesis (de Ligt, Timmers & Schrauwen, 2015). Another example are ß-glucans present in medicinal mushrooms that activate microbial Pattern Recognition Receptors, training the immune system for when an actual pathogen invades (van Steenwijk, Bast & de Boer, 2021). This implies that different strategies may be appropriate before and after the onset of disease, similar to the CHM concept of “tonics” that help to prevent disease but are of little use and even contraindicated once a pathogen has taken hold (Tian et al., 2022; Bensky et al., 2004, p.721). Given the role of mitochondria in many of the functions adaptogens claim to improve, it seems possible that mitochondria are the main target of adaptogenic herbs.

As well as adding to the general knowledge on these herbs, potentially providing new avenues for treatments to be developed, mitochondria may provide a means of evaluating the quality of a herbal blend, by observing their activity on these organelles and comparing future batches to see if they have the same effect. A system such as this can profile a single herb with complex ingredients such as adaptogens, or a blend of herbs which may also have multiple targets and mechanisms since the only important measure is the end result. Initial research will require some pilot work to find the ideal cell lines and specific assays to use for any particular herb or blend, but once profiled and recorded, future batches can be tested against the same standard and observed to see if they have the desired effect. Potency can also be measured by comparing the degree to which the herb or blend creates the known effect as the reference standard. The advantage of using biological assays instead of chemical testing is that knowledge of the active ingredients or their proportion, which may be numerous, have complex interactions with each other, and vary according to batch or harvest, is not necessary. Instead the effect of the herbal tea on its target tissues is measured directly and a statement of quality pronounced based on its final effects rather than the current methods of measuring quantities of a specific compound. The first herb to be selected was liquorice due to its ubiquity in TCM, and commercial supplements and herbal teas blends sold in the UK based on Chinese and Ayurvedic medicine, including Pukka (Rolfe, 2023) and Yogi Tea (2023), and Aura Nutrition (2023a), making it an important herb to test to ascertain its effects in any subsequent blends that contain liquorice.

Chapter IV

4. Liquorice

Figure 34: Liquorice (*G. uralensis*); Raw, Dried and Sliced Roots (Photo by Hazeena Azeez, 2023)

Liquorice (*G. glabra* L., *G. uralensis* Fisch. ex DC. & occasionally *G. inflata* Batalin) was chosen as the first herb to test for its capacity to modulate mitochondrial activity. It has a long history of medicinal use in every documented herbal medical tradition in the world and is still one of the most commonly consumed herbal medicines today (Brinckmann, 2020). It has a range of pharmacological properties and industrial uses, is considered an adaptogen, and present in many commercially available medicinal herbal teas in the UK (Rolfe, 2023; Yogi Tea, 2023; Aura Nutrition, 2023a). This means that as well as being

highly likely to have observable mitochondrial effects, these effects need to be documented before any future studies of more complex blends is undertaken in case the modulations detected in future blends can be attributed to liquorice alone.

The name liquorice derives from its Greek genus name *Glycyrrhiza* meaning “sweet root” and is renowned for its sweet taste, reputedly 50 times sweeter than refined sugar (Isbrucker & Burdock, 2006). Its name in other languages has similar translations: in Ayurveda it is called *Yashtimadhu*, यष्टिमधु, meaning “sweet stick” (Pole, 2012, p.220), and in Chinese it is called *Gan Cao*, 甘草, meaning “Sweet Herb” (Wilms, 2017, p.40).

As well as being one of the most widely consumed medicinal herbs today (Brinckmann, 2020), liquorice also has many industrial uses. Its sweet taste makes it valuable as a sweetener in the food industry, and it has been manufactured into confectionery since the 19th century. It is used in pharmaceuticals where an extract of glycyrrhizin is used as a hepatoprotective agent in much of asia (Hayashi & Sudo, 2009) and the surfactant properties of its saponin content facilitate the absorption of some drugs such as anthraquinone glycosides (Öztürk et al., 2017). It is used in cosmetics for its anti-inflammatory and skin whitening properties, and more recently as an emulsifier (Ralla et al., 2020) and shampoo (Azadbakht et al., 2018) for its effect on steroid dehydrogenases, potentially stimulating hair growth in androgenetic alopecia (Dhariwala & Ravikumar, 2019), while also suppressing body odour causing *Staphylococcus* bacteria (Hara, Matsui & Shimizu, 2014). The tobacco industry is a major consumer of liquorice which is used to sweeten, enhance and balance the smoke flavour, improve absorption of other flavouring agents, reduce roughness that causes dryness in mouth and throat, and prevent desiccation to improve shelf life (Carmines, Lemus & Gaworski, 2005). Recently it has also attracted attention as a potential alternative for antibacterial agents and artificial growth stimulants in animal feeds and aquaculture (Ding et al., 2022). In 2007, its global market value was US$42 million which increased to US$848.9 million in 2022 with an estimated growth of 5.3% predicted to reach US$1, 418. 4 million in 2032 (Market Future Insights, 2022).

4.1. History

The earliest evidence of liquorice being used by humans dates the ancient Assyrian and Egyptian empires (Ding et al., 2022). It is mentioned in the ancient Assyrian stele, the code of Hammurabi, and clay tablets excavated from the library of Assyrian King Ashurbanipal. Large amounts were also found preserved in the tomb of Egyptian pharaoh Tutankhamun, sealed c. 1330 B.C.E. and there is mention of liquorice in Egyptian papyri (American Botanical Council, 2022). In India it is first described in Ayurvedic sources (सुश्रुतसंहिता, *Sushrata Samhita*, Sushruta's Compendium and चरक संहिता, *Charaka Samhita*, Charaka's Compendium, both 1st Millenium BC), and then in Europe by Theophrastus in the 3rd - 4th century B.C.E., who said the Greeks learned of its therapeutic uses from the Scythians (Fiore et al., 2005). In China, it is mentioned in the earliest known medical writings, "Recipes for Fifty-two Ailments" (*Wushier Bingfang*, 五十二病方) found in a tomb sealed in 168 B.C.E. (Harper, 1998, p.221-304) and also in the earliest pharmacopoeia of Chinese medicine, The Divine Farmer's Materia Medica Classic (*Shen Nong Ben Cao Jing*, 神農本草經, 2nd Century C.E.) and takes a prominent role in in the earliest canonical work of Chinese herbal medicine, the Discussion of Cold Damage and Miscellaneous Diseases (*Shang Han Za Bing Lun*, 傷寒雜病論) compiled by Zhang Zhongjing (張仲景), c. 220 C.E., where it is used in almost half of the formulas presented (Jiang et al., 2020).

Liquorice has remained a popular herb through most of history. Its popularity appears to have been limited in Anglo-Saxon England where the only surviving herbarium from this period, *The Old English Herbarium Manuscript V*, includes a single line entry on liquorice, referring to it as "herb glycyrida" with no attempt to provide a common name suggesting it was peripheral in this herbal tradition (Pollington, 2008, p.135) and notably omitting its main contemporary indication for coughs (ibid., p.269). However, soldiers returning from the crusades brought some specimens back to England where it was cultivated in Pontefract, before demand for confectionery made its import a more economical option (Butler, 2022, p.709-710). Its therapeutic uses were also imported from the Islamic world with the indications for respiratory, gastrointestinal, cardiac and genitourinary

disorders mentioned by Avicenna in the 10th century (Fiore et al., 2005) matching those given by Culpeper (1653) who recommends it for “dry cough or hoarseness, wheezing or shortness of breath, and for all the griefs of the breast and lungs, phthisic or consumptions … in all pains of the reins [kidneys], the stranguary, and heat of urine.” By the time of Grieve’s *Modern Herbal* (1931) it was no longer used in urinary disorders or chest pain but was “an ingredient in almost all popular cough medicines on account of its valuable soothing properties" and was recognised that its sweetness is safe in diabetic patients for the alleviation of thirst.

4.2. Main Constituents

The non-active constituents of liquorice are reported to be 20% moisture, 3–16% sugars, 30% starch, and 6% cinder (Esmaili & Karami, 2022). The active constituents of liquorice have been well characterised (Pastorino et al., 2018; El-Saber Batiha et al., 2020) and include saponins, flavonoids and phenolic compounds. The most notable of these are:

4.2.1. **Glycyrrhizin**, also known as **glycyrrhizic acid**

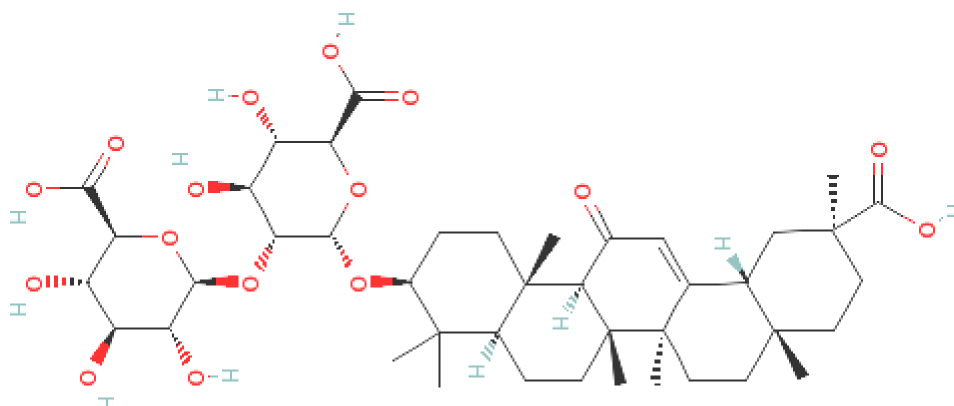


Figure 35: Glycyrrhizin (National Center for Biotechnology Information [NCBI], 2023a)

Glycyrrhizin (figure 35) is a pentacyclic triterpenoid saponin compound that is reputed to be 50x sweeter than sucrose (Isbrucker &

Burdock, 2006). All species should contain over 2% glycyrrhizin (Hsueh et al., 2022, p.193) with the dried root usually containing between 5-10% (Rizzato et al., 2017). It is also the compound responsible for the antiinflammatory therapeutic actions of liquorice through its non-selective inhibition of 11β-hydroxysteroid dehydrogenase, which converts active cortisol into inactive cortisone, resulting in accumulation of glucocorticoids with anti-inflammatory and mineralocorticoid effects (Lim, 2016). This same action is responsible for the toxic effects of overconsumption of liquorice since both cortisol and aldosterone bind to the same receptors, resulting in mineralocorticoid-related hypokalemia and hypertension (Nazari, Rameshrad & Hosseinzadeh, 2017).

Recently glycyrrhizin has gained attention for its antiviral properties. Its ability to bind to ACE2 receptors, which also have an important role in SARS-CoV2 infection, have led to its potential role as a competitive inhibitor to initial infection (Chrzanowski, Chrzanowska & Graboń, 2021). It has also been researched for its ability to inhibit replication in a large number of viruses including hepatitis B, C and E, herpes, Epstin-Barr, Acquired Immuno-Deficiency Syndrome, influenza and others (Zuo et al., 2023) but the large dose required for an independent antiviral effect have led to it being disregarded as a primary therapy and limited to use as an adjuvant, assisting in the antiviral activity of other drugs while reducing comorbidities through its anti-inflammatory and steroid enhancing effects (Sun et al., 2021).

Glycyrrhizin has also attracted interest in its potential as a drug delivery system (Butler, 2022). This is because the molecule is amphiphilic, with its sugar end being hydrophilic and the steroid end, hydrophobic. At sufficient concentration in an aqueous solution, this causes it to aggregate in a ball shape called a micelle, with the sugar end pointing outwards, bonding to the water, and the hydrophobic end in the centre. This can be exploited by incorporating some of the molecules of a lipophilic drug into the micelle, enhancing its solubility and bioavailability (figure 36). Glycyrrhizin also appears to influence the permeability and elasticity of cell membranes,

further enhancing the ability of glycyrrhizin to deliver a drug to its intended target (Selyutina et al., 2016; Selyutina & Polyakov 2019), showing promise in cancer therapy (Su et al., 2017) and enhancing the delivery of Atorvastatin (Kong et al., 2018).

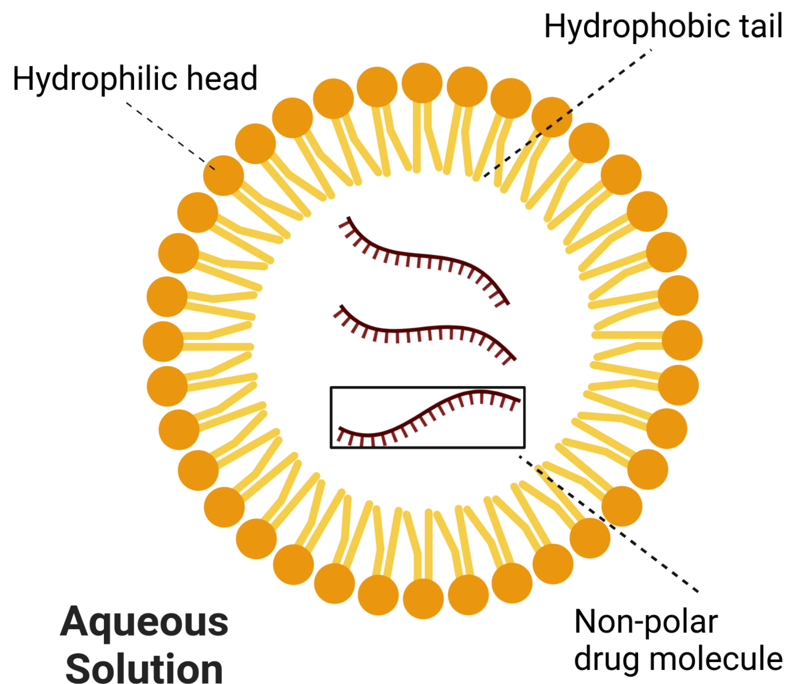


Figure 36: Diagram of a Micelle and its use as a Drug Delivery System (created with BioRender.com)

Besides medical usages and food usages, glycyrrhizin is also used in the cosmetic industry as a flavouring and an emulsifier. It has been demonstrated to form nano-sized emulsion droplets at neutral pH which were stable across a broad range of pH values, ionic strengths and temperature ranges (Ralla et al., 2020).

4.2.2. **Glycyrrhetinic acid**, also known as **enoxolone**

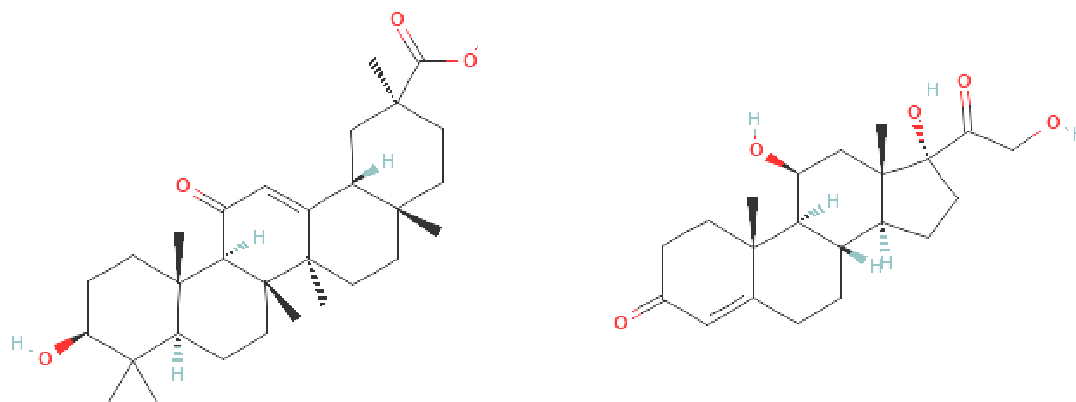


Figure 37: Glycyrrhetinic acid (left) and its similarity to Hydrocortisone (right) (NBCI, 2023b; NCBI 2023c)

Glycyrrhetinic acid (figure 37) is the aglycone metabolite of glycyrrhizin and found within the extract of the plant itself (Graebin, 2018) but also obtained by enzymatic hydrolysis by the intestinal flora after consumption (Hattori et al., 1983). It is considered to be the active part of glycyrrhizin, able to act on cortisone due to its structural similarity to hydrocortisone (Aronson, 2018). It can have two stereoisomers, 18α and 18**β**-glycyrrhetinic acid with its 18**β** form being the most often studied. 18**β**-glycyrrhetinic acid is of interest in dermatology for its anti-inflammatory, antioxidant and antimicrobial properties combined with its low toxicity to normal cell lines (Kowalska & Kalinowska-Lis, 2019). Like glycyrrhizin, it has also received interest as a drug delivery system but with a particular focus on cancer, especially liver cancer due to its ability to bind with receptors on the surface of hepatocytes, leading to the scaffold being used to create semisynthetic compounds aimed at targeting cancers (Hussain et al., 2021; Wu et al., 2018; Speciale et al., 2022). However, the removal of the glycone component also makes glycyrrhetinic acid lose the amphiphilic properties of glycyrrhizin and become almost completely insoluble in aqueous solutions (Cayman Chemical, 2022).

4.2.3. **Glabridin** and **Glabrene**

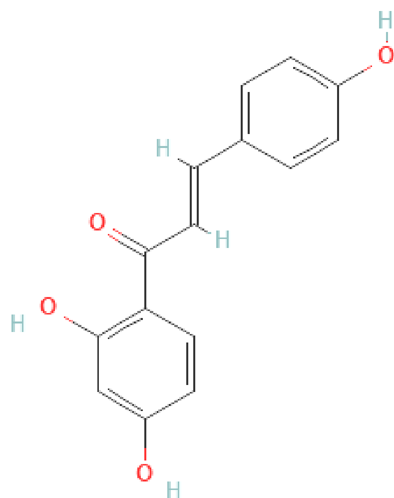


Figure 38: Glabridin (NCBI, 2023h)

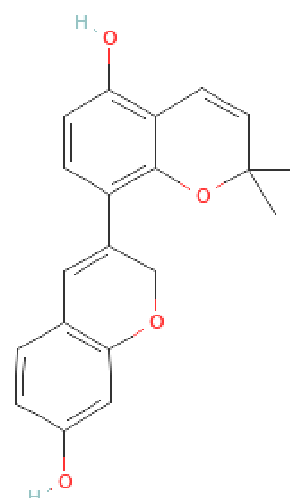


Figure 39: Glabrene (NCBI, 2023i)

Glabridin (figure 38) and glabrene (figure 39) are prenylated isoflavonoids which have demonstrated both agonist and antagonistic activities with different oestrogen receptors (Simons et al., 2011). Glabridin is present almost exclusively in *G. glabra*, comprising between 0.08 and 0.35% of the root's dried weight (Hayashi et al., 2003), and can be used as a marker compound to differentiate species (Rizzato et al., 2017; Avula et al., 2022). It has been primarily studied in relation to its anti-inflammatory, cardioprotective, oestrogenic and metabolic effects, but has also been reported to have antioxidant, neuroprotective, anti-osteoporotic and skin-whitening effects (Simmler, Pauli & Chen, 2013). However, it has poor water solubility (Tian, Yan & Row, 2008), so is unlikely to be extracted in teas.

Glabrene has been primarily researched for its potential oestrogenic activity (Tamir et al., 2001), protective effects against osteoporosis (Liu, J. et al., 2021; Somjen, Katzburg et al., 2004) and cardioprotective effects (Somjen, Knoll et al., 2004) but also acts as an tyrosinase inhibitor making it of potential interest to cosmetics as a skin whitening agent (Cerulli et al., 2022).

4.2.4. **Liquiritin**, its precursors, isomers and apiosides

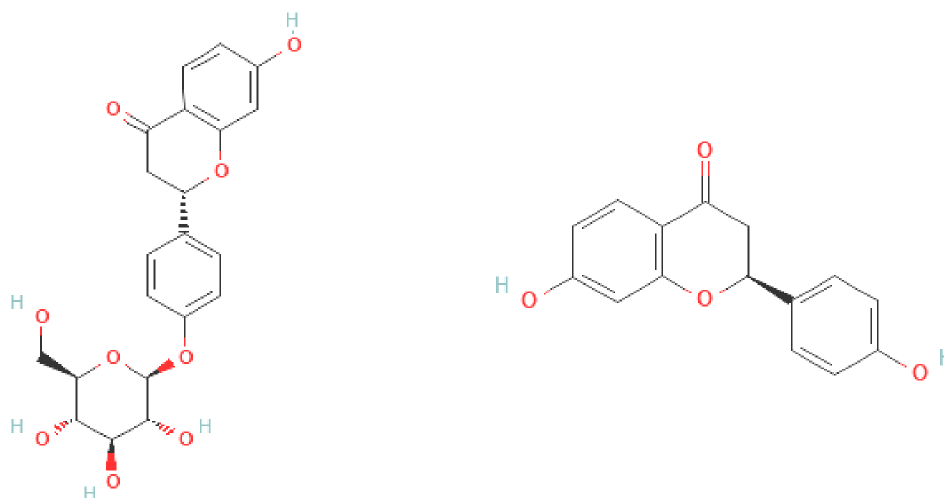


Figure 40: Liquiritin (NCBI, 2023d) **Figure 41:** Liquiritigenin (NCBI, 2023e)

Liquiritin (figure 40) is a flavonoid glycoside with numerous pharmacological effects including anti-Alzheimer's disease, antidepressant, antitumor, anti-inflammatory, cardiovascular protective, antitussive, hepatoprotective and skin protective effects (Qin et al., 2022). Its aglycone component, liquiritigenin (figure 41), also has reported neuroprotective, antioxidant, anti-inflammatory, antibacterial, anti-asthmatic, anti-diabetic, anti-oesteoposis, anti-neoplastic and oestrogen receptor signalling activities (Ramalingam et al., 2018). Isoliquiritigenin (figure 42), the precursor and isomer chalcone to liquiritigenin with a similar pharmacological profile, has recently come to the attention of the media for a potential role in prevention of pancreatic cancer (Tetzlaff-Deas, 2023; Zhang, Yung & Ko, 2022; Zhang, Z. et al., 2022) and has previously been identified as a tyrosinase inhibitor with potential use as a skin whitening agent in cosmetics (Cerulli et al., 2022). Liquiritin, liquiritigenin and their isomers isoliquiritin and isoliquiritigenin, are found predominantly in *G. uralensis* although liquiritin apioside (figure 43) and isoliquiritin apioside are found equally distributed among species (Kondo et al., 2007) or predominantly in *G. glabra* (Avula et al., 2022). Together these comprise approximately 1% of liquorice aqueous extracts making the most abundant phenolics (Rizatto et al., 2017).

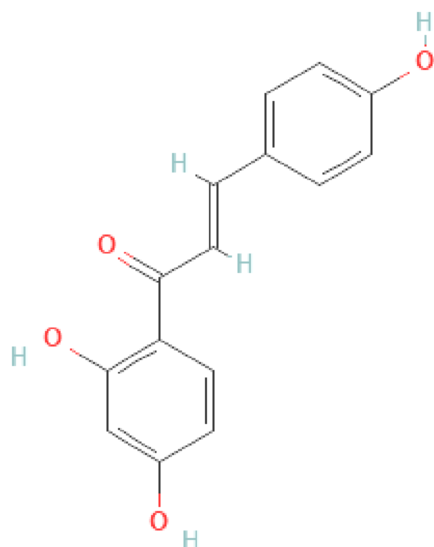


Figure 42: Isoliquiritigenin (NCBI, 2023f)

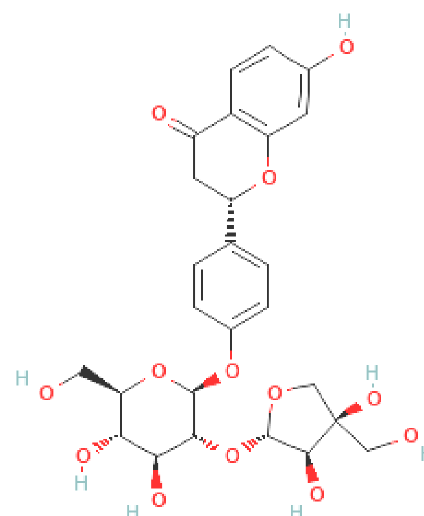


Figure 43: Liquiritin apioside (NCBI, 2023g)

4.2.5. Glycycoumarin

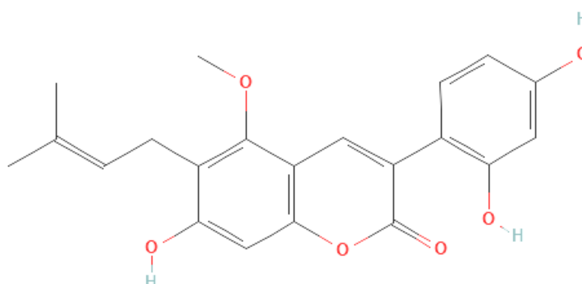


Figure 44: Glycycoumarin (NCBI, 2023j)

Glycycoumarin (figure 44) is a coumarin compound which has demonstrated anti-spasmodic, antimicrobial, antitumor and hepatoprotective activities (Tang et al., 2022). It is found exclusively in *G. uralensis* where it can be used as a marker compound (Avula et al., 2022) although different coumarins exist in other species.

4.2.6. Licochalcones

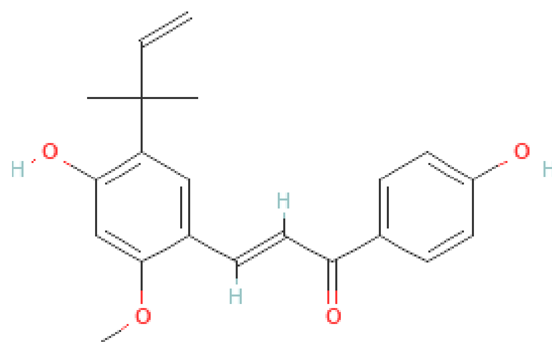


Figure 45: Licochalcone A (NCBI, 2023k)

Licochalcones are a chalcone group found in substantial quantities only in *G. inflata* so can be used as marker compounds for this species (Kondo et al., 2007; Rizzato et al., 2017). Smaller quantities of licochalcones A and B have also been reported in *G. glabra* and either absent or in trace amounts in *G. uralensis* (Avula et al., 2022; Fu et al., 2013; Cerulli et al., 2022). Licochalcone A (figure 45) has attracted particular attention for reported anticancer, anti-inflammatory, antioxidant, antibacterial, anti-parasitic, bone protective, blood glucose and lipid regulatory, neuroprotective and skin protective effects (Li, M-T. et al., 2022). Its anticancer effects are suspected to be due to activation of the mitochondrial apoptosis pathway.

Minor compounds include licocoumarin A, licoarylcoumarin, licopyranocoumarin, prenyllicoflavone A, glisoflavone, semilicoisoflavone B, licoriphenone, kanzonol R, shinpterocarpin, 1-methoxyficifolinol, furfuraldehyde and tetramethyl pyrazine (El-Saber Batiha et al., 2020).

4.3. Use in Western Herbal Medicine

In WHM, liquorice is primarily used for digestive discomfort, acid reflux and coughs (European Medicines Agency (EMA), 2012). Its main actions are described as expectorant, demulcent, anti-inflammatory, anti-hepatotoxic,

antispasmodic and mild laxative (Hoffman, 2003, pp.554-5). Less common actions include anti-ulcer, immunomodulatory, anti-allergy, chemopreventive, antitumor, hepatoprotective, antimicrobial, neuroprotective, antidepressant, cognitive enhancing, able to help with management of dyslipidemia and insulin resistance, and in the management of polycystic ovarian syndrome (Bone & Mills, 2013, p.726-30). Its interaction with steroid hormones has also given it indications in the treatment of Addisons's disease and steroid withdrawal (Brown, 2014). Its actions on coughs, viruses and steroid metabolism has also encouraged its use in the treatment of mild to moderate cases of Covid-19 (Silveira et al., 2020; Bailly & Vergoten, 2020; Diomede et al., 2021) making it the most commonly prescribed herbal medicine for this condition in a survey of herbal medicine practitioners in the UK (Frost et al., 2021).

4.4. Use in Traditional Ayurvedic and Unani Medicine

In Ayurvedic medicine, liquorice (*G. glabra*) is used as a tonic, able to balance all three *doshas* (the three primary energies), strengthen the kidneys and calm the nervous system, in addition to the common WHM indications of cough and digestive disorders (Pole, 2012, p.220). It is also suggested as an aphrodisiac in the *Kama Sutra* and its sequel, the *Ananga Ranga* (Aronson, 2018). In the Unani tradition, liquorice was recommended by Rhazes in the 9-10th century for cough and by Avicenna in the 10th century for making the voice clear, reducing hoarseness of the throat, disorders of the kidneys and bladder, palpitations, skin ulcers and as a remedy for gastrointestinal disorders (Fiores et al., 2005) but most of these were later dropped for many centuries and it retained only expectorant, demulcent and antiinflammatory actions until modern research suggested it be used for peptic ulcers (Saad & Said, 2011, p.264).

4.5. Use in Chinese Herbal Medicine

In CHM, liquorice (usually *G. uralensis* but *G. glabra* and sometimes *G. inflata* are considered viable species) is the most commonly used herb, present in between a quarter to half of all Chinese medical formulas (Wang et al., 2013; Guo

et al., 2014) and is said that "nine out of ten formulas contain liquorice" (*Shifang Jiucao*, 十方九草) (Jiang et al., 2020). It is one of the only herbs prescribed as a single herb without any additional supporting herbs in the classic Han dynasty formulary text, *Discussion of Cold Damage* (*Shang Han Lun*, 傷寒論, c. 220 C.E.), where a decoction of liquorice (*Gan Cao Tang*, 甘草湯) is indicated for sore throat that has persisted for 2-3 days (Liu, 2016, p.739). Although it retains the WHM indications for coughs and digestive discomfort, its primary indication is as a *Qi* tonic and it contains a few additional indications unique to CHM such as resolving toxicity, harmonising the effects of other herbs and acting as an envoy, enabling other herbs to effect all twelve traditional organ systems (Bensky et al., 2004, pp.732-4; Chen & Chen, 2000, pp.866-71). This has earned it the title of "Nations Elder" (*Guo Lao*, 國老), a term used to refer to the Emperor's teachers, the only people in the traditional hierarchy that were masters to the Emperor (Li, 2022, p.36).

In traditional theory, sweet taste and yellow colour are associated with the neutral, central element of Earth (Marshall, 2020) making liquorice "the king of herbs with regard to the neutral earth element" (Liu, 2020, p.487) and providing it with the power to treat both "Hot" and "Cold" disorders, and resolve toxicity since the earliest Chinese medical writings (Wilms, 2017, p.40), by bringing either extreme back to the centre. Its effects on the cardiovascular system have also been recognised since the Han dynasty with a large dose being used as the chief herb to treat irregular pulse and palpitations in the formula "Prepared Liquorice Decoction" (*Zhi Gan Cao Tang*, 炙甘草湯) that remains one of the most popular formulas in Taiwan today for improving myocardial contractility, alleviating oxidative stress and reducing inflammation in ischaemic heart disease (Wang, L. et al., 2022). This prepared form involves stir-frying with honey to increase its ability to augment the TCM organs of Heart and Spleen, strengthen the digestion (中焦 *Zhong Jiao*) and harmonise the properties of other herbs (Bensky et al., 2004, p.734). Liquorice is also often combined with fresh ginger (*Zingiber officinale*, Roscoe, *Zingiberaceae*) and jujube berries (*Ziziphus jujuba*, Mill., *Rhamnaceae*), which also have actions of settling the stomach, improving digestion by enhancing "Spleen *Qi*" and moderating the harshness of other herbs (Bensky et al., 2004, p.30 & 730), working in a triad as assistants and envoys to the main herbs in the

formula, to smooth the diverse actions of the other ingredients into a harmonious whole that is appended to many herbal remedies (Fruehauf, 1995; Scheid et al., 2009, p.272).

4.6. Risks Associated with Liquorice

Liquorice has a well documented history of toxicity when consumed in large amounts over a long term causing a loss of potassium and an elevation in blood sodium levels leading to mineralocorticoid excess with potentially fatal consequences due to hypertension (Corsi et al., 1983; Omar et al., 2012; Deutch et al., 2019). This may be exacerbated in cases of anorexia nervosa where even ordinarily safe levels can cause hypokalemia and low plasma aldosterone (Fetissov & Crook, 2011). Some incidents have made international news (Associated Press, 2020) and prompted warnings from the NHS (NHS, 2022) making it even more imperative to understand the mechanisms of this herb in as much detail as possible. This is mainly due to the action of glycyrrhizin which has resulted in the development and sale of deglycyrrhizinated liquorice as a safer alternative to liquorice in the treatment of peptic ulcer and intestinal spasms (Tewari & Trembalowicz, 1968).

Liquorice also has the potential to interact with several drugs. Corticosteroids can be potentiated by liquorice, and interactions with the oral contraceptive pill where the pill may increase sensitivity to glycyrrhizin and increase the risks of hypokalemia and hypertension due to the oestrogen modulating activity of liquorice have also been reported (Fugh-Berman, 2000). Interactions with CYP450 enzymes and P-gp mediated transport have also been suggested, which could increase efficacy of chemotherapy but also increase toxicity to normal cells, decrease bioavailability of some drugs, enhance blood–brain barrier permeability and neurotoxicity, and decrease renal elimination (Nazari, Rameshrad & Hosseinzadeh, 2017).

4.7. Quality Evaluation of Liquorice

Liquorice quality is traditionally evaluated through macroscopic inspection and the three species can be differentiated through their gross morphological characteristics (figure 46; Zhao & Chen, 2014, pp.107-8). *G. uralensis* has a reddish-brown cork that is unevenly tight with obvious lenticels with a firm, slightly fibrous but powdery texture. *G. inflata* has a rough, greyish-brown or tan skin with a hard, woody and fibrous texture. *G. glabra* has a smooth, greyish-brown exterior with thin lenticels that may not be obvious and a relatively firm texture. The centre of all species should contain a radial lines called a "chrysanthemum heart" (*Ju Hua Xin*, 菊花心) but this ring is not unique to liquorice though and is found in the centre of other roots such as astragalus (*Astragalus membranaceus*, Fisch. ex Bunge, *Fabaceae*) (ibid., p.129). Inferior quality specimens usually have rough, greyish-brown surface, loose texture with a dark yellow cross-section (Bensky et al., 2004, p.734).

Figure 46: Macroscopic Characteristics of Liquorice.
(a) and (b) are roots and rhizomes of *G. glabra*, (c) and (d) are *G. uralensis* (Raman et al., 2022; reproduced with permission)

Cross sectional microscopic analysis can also be used for identification (Hong Kong Baptist University, 2023; Shah, 2020). Liquorice contains an outer cork layer with many rows of yellow-brown cells, a narrow secondary cortex, 1-3 cells wide, and a thick, curved phloem with cracks. Xylem rays are 3~5 rows wide in bundles and the root and rhizome centre has no pith. Fibres are often grouped and parenchyma cells often contain starch and calcium oxalate crystals. Differences between species can be observed including variations in cork thickness, medullary ray width, vessel element size and arrangement, and pore frequency (Raman et al., 2022) (figure 47).

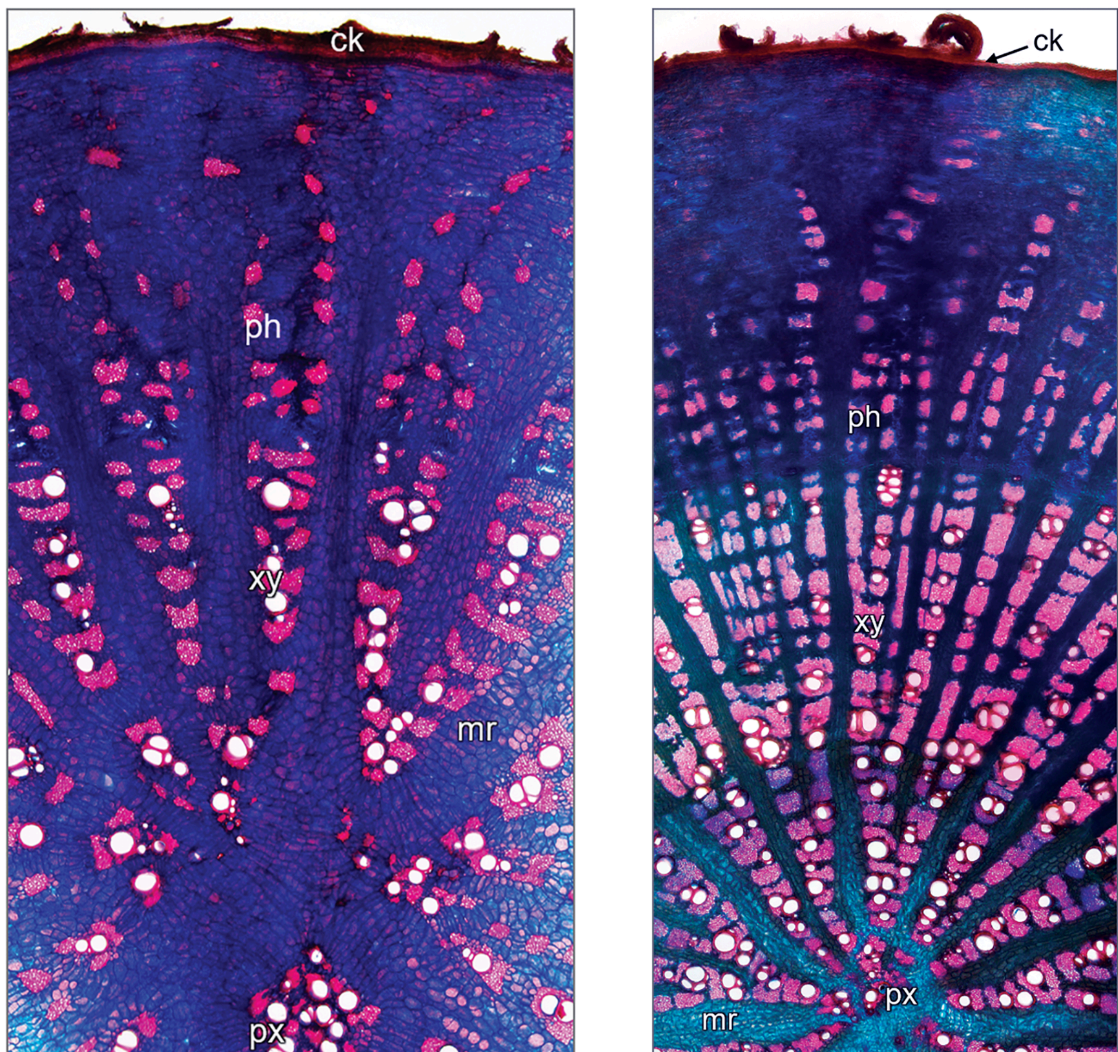


Figure 47: Microscopic Characteristics of *G. glabra* (left) and *G. uralensis* (right). Abbreviations: ck: cork; ph: phloem; xy: xylem; px: primary xylem; mr: medullary rays (Raman et al., 2022; reproduced with permission).

Chemometric analysis can also differentiate between species. Some compounds are unique to each species and others which are present in several have differing levels which can be used for identification (Avula et al., 2022). These include glabridin which is unique to *G. glabra* and glycycoumarin which is unique to *G. uralensis* while *G. inflata* is the only species to contain a significant amount of licochalcones. In addition, different forms of liquiritin are abundant in different species with *G. glabra* containing the most liquiritin apioside and isoliquiritin apioside, while *G. uralensis* contains the most liquiritin, neoliquiritin and isoliquiritin. These differences can be seen using HPTLC which reveals some unique quenching zones for *G. uralensis* compared to *G. glabra* (figure 48).

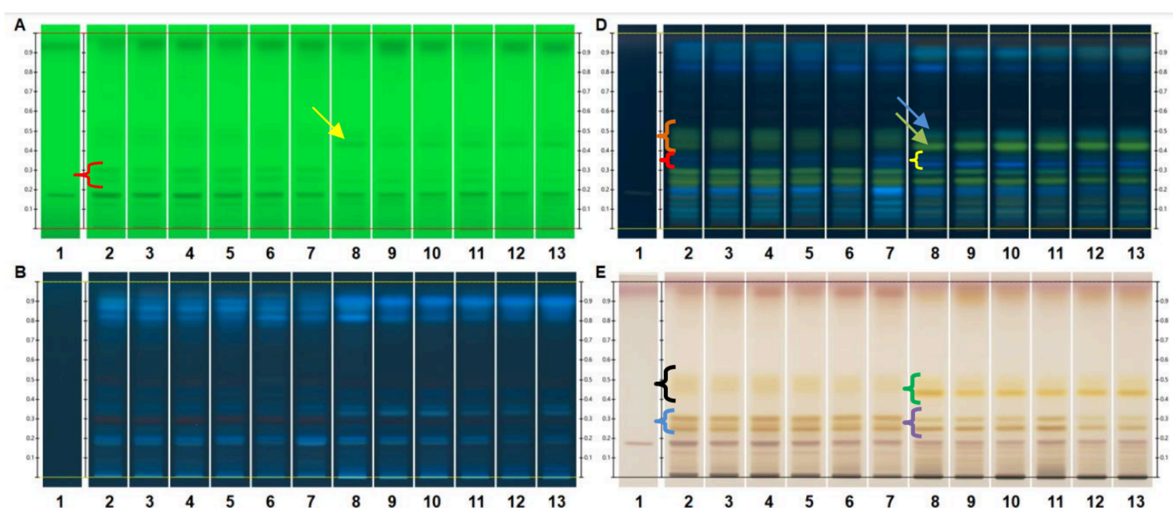


Figure 48: HPTLC Profiles of *G. glabra* and *G. uralensis* (HPTLC Association, 2019; reproduced with permission).

Left shows underivatized plates under UV 254 nm (top), and UV 366 nm (bottom). Right shows derivatized plates under UV 366 nm and visible light. Track 1 shows glycyrrhizic acid (0.5 mg/mL). Tracks 2-7 show *G. glabra*, tracks 8-15 show *G. uralensis* with additional quenching zone.

4.8. Adulteration of Liquorice

With its high global market value increasing rapidly due to its expanding uses, the chances of liquorice becoming adulterated for economic gain are also likely to increase. Before the advent of chemometric testing, adulteration of the

powdered herbs with sucrose has been reported since it can imitate the sweet taste of liquorice while increasing its bulk to command a higher price by weight (Lee, 2018). This is still common today with the majority of “liquorice” confectionery on the market being primarily sugar, molasses, cane syrup, treacle or low sugar sweeteners combined with anise (*P. anisum*) oil, due to anise having a similar smell and taste to liquorice (National Center for Complementary and Integrative Health [NCCIH], 2020; O’Kelly, 2021). These could all be detected quite easily through chemometric or spectroscopic means (Process NMR Associates, LLC, 2007).

Liquorice confectionery has been the main source of adulteration throughout most of history because it has already been extracted and boiled down, giving the possibility of adding other ingredients during this process. This was first described and published in the 1820s when Frederick Accum recorded the practice of mixing gum arabic, used for shoe polish, with a small amount of genuine liquorice juice and reconstituting the mixture into cylinders to be sold as liquorice confectionery (Accum, 1822, p.332). Other common adulterants in liquorice confectionery from the 19th century came from the copper in the cooking pots being scraped off and ending up in the mixture (Hassall, 1876, pp.607-8). These could also be easily detected today with chemometric analysis.

In the raw herb trade, stones have been reported inside bales of liquorice root and powdered olive stones mixed with powdered herbs (Jiwaji University, n.d.). The former may simply increase the weight for the unsuspecting buyer but can also contain toxic mineral compounds such as lead which may be preferred as they are heavier but will also negatively affect the safety of the herb for the end user. The toxicity of heavy metals involves disruption of mitochondrial function (Sun et al., 2022) and so could be a potential target for adulteration detection by mitochondrial analysis, but spectroscopic analysis would be more sensitive and could detect the presence of heavy metals at doses that would not produce a marked biological effect. The use of olive stones in powdered herbs will reduce the efficacy of the herbal powder and could potentially be a candidate for functional evaluation but may also be detected with the presence of foreign marker compounds.

A common source of adulteration with raw herbs is incorrect species being gathered, either through incorrect identification during foraging or harvesting, or purchased incorrectly at markets. Russian, Iranian and Manchurian variants (*G. glabra var. glandulifera*, *G. glabra var. Violacea* and *G. uralensis*) have been listed as adulterants for *G. glabra* (Pharmacognosy, 2022) but these species also have similar constituents and are listed as accepted variants for medicinal use in other sources (EMA, 2013; Shah, 2020). *G. glabra* and *G. uralansis* are often considered interchangeable in many herbal sources of both WHM and CHM traditions (American Botanical Council, 2000; EMA, 2012; Bensky et al., 2004, p.732). *Taverniera cuneifolia* (Roth) Arn., *Fabaceae* can also sometimes be misidentified but has a very similar phytochemical profile with an appreciable glycyrrhizin content (Zore et al., 2008; Nagar, Rane & Dwivedi, 2022) and considered by some herbalists to be an acceptable substitute (Bone & Mills, 2012, p.720). It would be easier to detect the presence of this different species with genetic analysis on whole plant samples, or chemometric analysis which does show a clear interspecies fingerprint despite common zones but mitochondrial analysis could potentially be used to detect mixed samples.

Liquorice is usually wild harvested due to the cultivated roots lacking the same levels of constituents (Brinckmann, 2020; Wang, H. et al., 2021). The herbs used in this study are also wild harvested from Georgia, Kazakhstan and Spain (Rolfe, 2023). The superiority of wild liquorice compared to cultivated varieties also introduces the prospect of deliberate adulteration of the wild harvested crop with a cultivated one. As demand grows and the natural habitat of wild liquorice declines (Khaitov et al., 2022), the likelihood of over-foraging increases and suppliers may attempt to adulterate wild harvested crops with cultivated ones. This would represent exactly the form of functional adulteration described by Tao Hongjing in the 5th Century China (Liu, 2021). Therefore, a practical functional adulteration test could involve examining the difference in mitochondrial modulating activity between wild and cultivated liquorice and whether a mixture of the two can be detected. This would be difficult to detect by genetic analysis and simple chemometric analysis but could establish whether the adulterated crop is capable of producing the same biological effect as genuine wild-harvested liquorice.

Finally, there is the possibility of exhausted herbs being re-marketed for the herbal industry. Exhausted liquorice has been used historically in China for external use by reboiling it into a coarse cloth to make a medicinal towel but not for internal consumption (Ding et al., 2022). However, approximately 6-7 million tonnes of liquorice compost is produced every year in China alone, for which no industrialised applications have been successfully applied (Santulli et al., 2020). Even if a potential use for this waste is found, it is unlikely to be as profitable as resale for medicinal use, and if mixed with authentic herbal material, or used in extract form to increase the volume of the extracted material, it would be almost undetectable but may affect the potency, making this another form of functional adulteration that mitochondrial analysis may be able to detect.

4.9. Current Mitochondrial Research into Liquorice

Being such an essential herb in CHM, WHM, confectionery, food and cosmetics, liquorice has been very well researched. A search on Pubmed, conducted on 18th August 2023 reveals almost 6,000 papers returned when using the term "liquorice" alone. 102 are returned for "liquorice AND mitochondria," with the earliest result dating from 1973, seeing a steady increase from 2000 onwards suggesting this is an area of growing interest (figure 49).

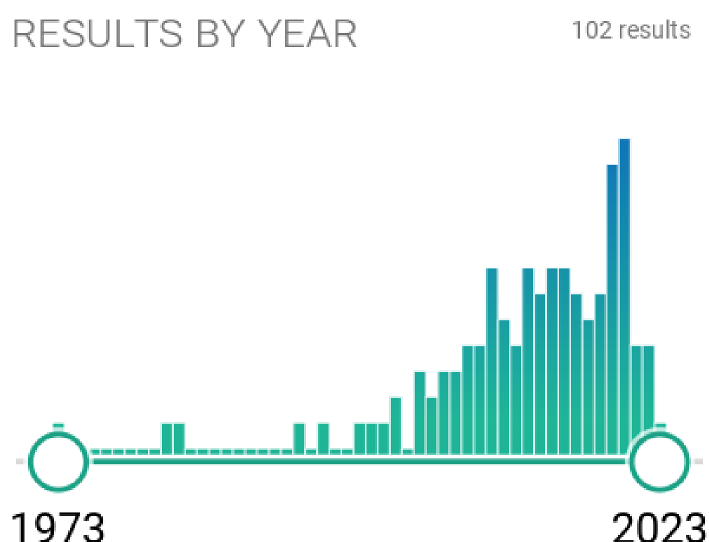


Figure 49: PubMed search results by year for "Licorice AND mitochondria" Search performed on 18th August 2023

The majority of these focus on isolated extracts rather than the entirety of the herb. When “Seahorse Flux Analyzer” is added to the search then no studies are found suggesting that despite many of the active ingredients working via mitochondrial modulation, no complete profile of whole liquorice root extraction on mitochondrial activity has been undertaken.

A brief summary of these results reveals a few core themes. Almost half (40) of these are looking at cancer, either by inducing apoptosis via the mitochondrial pathway, especially using licochalcones (Yuan et al. 2013; Kang et al., 2017; Wang et al., 2019; Hong et al., 2019; Oh et al., 2018; Lin et al., 2019; Kim et al., 2015), glycyrrhizin (Lin et al., 2018; Chueh et al., 2012), glycyrrhetinic acid (Luo et al., 2021; Wang, D., Wong et al., 2014; Yang, J. et al., 2012; Lee et al., 2010; Sharma et al., 2012) and isoliquirtigenin (Hirchaud et al., 2013; Ma et al., 2001; Jung et al., 2006; Chen et al., 2019; Hsu et al., 2009), although the recent interest in glycyrretinic acid as a scaffold with which to target cancers has also led to the possibility it may be able to direct delivery of other compounds to mitochondria (Jin et al., 2022; Zhang et al., 2018). Others looked at the attenuation of toxic side effects from chemotherapy and other xenobiotics which were discussed in Section 4.6. Another important category is those investigating neuroprotective effects against apoptosis initiated by endogenous neurotoxic factors such as glutamate (Wang, D., Guo et al., 2014; Lee et al., 2018; Yang, E. et al., 2012), corticosterone (Zhou et al., 2017; Li, X. et al., 2020) and 7-ketocholesterol (Kim et al., 2009). There were also studies into protection from mitochondrial dysfunction caused by metabolic disorders in renal cells (Yokozawa et al., 2005; Cheng, Qiu & Wang, 2020), liver damage in rats (Sil, Ray & Chakraborti, 2015; Sil & Chakraborti, 2016), peripheral neuropathy (Ciarlo et al., 2021) and pancreatic islets (Rahimifard et al., 2014), as well as a mitohormesis effect of isoliquiritigenin which inhibited ATP production in mice with Type 2 diabetes mellitus that triggered mitochondrial biogenesis to restore balance (Yang, L. et al., 2022). Another looked at reduction of mitochondrial ROS mediated inflammatory damage in ulcerative colitis (Kong et al., 2023). The common theme in almost all these studies is the amelioration of ROS generated through mitochondrial dysfunction leading to apoptosis.

Chapter V

5. Research Aim, Objectives, Questions and Hypothesis

5.1. Research Aim

The research aim of this project is:

> To profile the ways in which a medicinal herbal tea (liquorice) modulates mitochondrial activity and determine whether this can be used to detect a functionally adulterated extract

5.2. Research Objectives

To achieve this aim, the research objectives are:

- To develop a body of research data into the mitochondrial effects of liquorice tea (*G. glabra* L.)
- To discover unique characteristics which can be used to develop reference standards and validate the testing protocol
- To refine the methodology so that it is rapid and efficient
- To develop a pipeline methodology that can discover the mitochondrial fingerprint of novel herbs and apply this to functional quality assessment

5.3. Research Questions

To develop these objectives, some specific questions to be answered are:

- What unique effects does liquorice have on mitochondria that can be used as a reference standard to enable identification and measure potency?
- What kind of adulteration can be used that would be challenging to detect via chemical or genetic testing but detectable with mitochondrial analysis?
- Can a standard set of tests be developed for a pipeline that will generate a profile?

5.4. Hypothesis

The null hypothesis (H0) is:

> A medicinal herbal tea will not induce specific mitochondrial modulations that enable a measurement of functional potency and identification of adulteration.

And the alternative hypothesis (H1) is:

> A medicinal herbal tea will induce specific mitochondrial modulations that enable a measurement of functional potency and identification of adulteration.

Materials and Methods

Chapter VI

6. Cells and Culture

6.1. Cell Lines

Michigan Cancer Foundation 7 (MCF7) human breast cancer cell lines were grown in modified Eagle's medium (MEM) (Sigma, UK), supplemented with 10% filter sterilised Foetal Bovine Serum (FBS), 1% L-glutamine and 1% Penicillin/Streptomycin. Michigan Cancer Foundation 10A (MCF10A) non-cancerous human breast cell line were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK), supplemented with 5% filter sterilised Horse Serum, 1% Penicillin/Streptomycin, 20 ng/mL epidermal growth factor (EGF) (Sigma, UK), 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma, UK) and 10 µg / mL insulin (Sigma, UK). All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

MCF7 and MCF10A cell lines were chosen as well characterised cancerous and non-cancerous analogues of breast cells with different respiratory mechanisms and sensitivities that may respond differently to herbal teas. Cancer cells metabolise differently, favouring glycolysis even in the presence of oxygen, discussed in section 3.1.6.3, and MCF7 cells are oestrogen-sensitive (Comşa, Cîmpean & Raica, 2015) which may mean that they will respond to phytoestrogens found in medicinal herbs, of which liquorice (*G. glabra*) has some of the highest of any known herbs (Jungbauer & Medjakovic, 2014). Both of these cell lines were also available to the lab despite the constraints Covid-19 exerted on supply chains. If a herb targets specific organs, systems, or tissues then cell lines from these origins would be preferred. Fortunately liquorice (*G. glabra*), has a wide

range of therapeutic uses, from bacterial and viral infections, cough, sore throat, digestive problems, menopausal symptoms and topical application to the skin, with indications in traditional medical systems for lung, liver, circulatory, and kidney diseases (NCCIH, 2020) and said in Chinese medicine to be one of the only herbs to enter all 12 systems (Bensky et al., 2004: 732). This means we may expect to see a response from a wide range of cells.

6.2. Cell Culture

All cell culture work was performed using aseptic technique in a laminar flow hood. Cells were grown in sterile T75 flasks and passaged based on visual inspection. For passage, media was removed from cells, washed with sterile phosphate-buffered saline (PBS) and then detached from the surface of the flask using 1 mL TrypLE for 7 minutes (MCF7) or 20 minutes (MCF10A). Following detachment, the cells were resuspended in fresh media and diluted into new flasks. Only passages between 5 and 25 were used for experimentation.

For seeding cells for experimentation, cells were detached as described above, centrifuged at 1000 rpm for 5 minutes, the media and TrypLE removed, then resuspended in fresh media and counted using a Countess automated cell counter (Thermofisher, UK). The resulting suspension was then diluted to the appropriate seeding density for the experiment and added to the wells with the appropriate volume of media for the plate being used (2.5×10^4 for 96-well plates and 3×10^4 for SeaHorse assay). The seeding for the original liquorice tea extract was performed by the researcher, and for the exhausted liquorice tea extract it was performed by the research associate.

Chapter VII

7. Herbal Tea

7.1. Original Liquorice Tea Extract Preparation

The Original Liquorice Tea Extract (OLTE) was made from “Three Licorice“ tea (Pukka, UK) which consists of three liquorice (*G. glabra*) cultivars that are wild harvested from organic soils in Kazakhstan, Georgia and Egypt or Spain (Pukka Herbs, 2020a; Rolfe, 2023). As an industry product, this was considered an ideal sample for testing the potential industrial applications of this method of quality and potency evaluation. The reasons for selecting liquorice as the initial herb to test are described in section 4.

In order to maintain a consistency as close to that of a cup of tea made by a typical consumer, the herbs were prepared following Pukka’s Standard Operating Procedure for organoleptic testing of herbal teas (Zoani, 2019; personal communication by email from Marion Mackonochie, 1st October 2020). This suggests using 1 tea bag in 250 mL of boiled water, left to steep for 15 minutes and being agitated after 2 minutes. For consistency across experiments the amount was scaled up to result in the following preparation method:

- 5 teabags were added to 1.25 L of boiling tap water. Tap water was chosen to make the result as close to an actual cup of tea as possible.
- The tea bags were left to steep for 15 minutes and agitated after 2 minutes. The tea bags were then removed and the liquid left to cool.
- Once cool, 25 mL aliquots were measured into 50 mL Falcon tubes and frozen at -20°C in preparation for freeze drying.
- A desired quantity was then freeze dried and kept at -20°C for future use.

The 5 bags of liquorice tea in a single batch produced 2.83 g of freeze dried powder. This was calculated to be 566 mg per bag. Each bag contained 1.5 g of raw material, equating to 377.33 mg / g of raw material.

When ready to use:

- The dried material was weighed and 566 mg (1 bag equivalent) of freeze dried material was measured
- This was rehydrated using 10 mL filter sterilised distilled water to create a solution 56.6 mg / mL solution
- This stock solution was then frozen at -20°C in 2 mL aliquots for future use.

On the day of the experiment:

- The stock solution was thawed and 35.4 µL was added to 1mL of media to make a solution with 2000 µg / mL freeze dried liquorice.
- Further serial dilutions were made from this initial dilution to obtain lower concentrations. The most common were:
    - 250 µL of 2000 µg / mL solution in 1 mL media to obtain 500 µg / mL
    - 20 µL of 500 µg / mL solution in 1 mL media to obtain 10 µg / mL
- Remaining stock was then refrozen at -20°C and stored in aliquots for future use.

7.2. Pre-extracted Liquorice Tea Extract Preparation

The supplier of the original liquorice tea (Pukka Herbs, UK) was contacted to find any samples which had failed their inspection process and a PhD student at another university (UCL), also investigating quality control methods using handheld Raman spectroscopy devices, was contacted to find samples that had failed quality tests but neither had any samples available. Instead it was decided to adulterate our own sample with a method that would be undetectable to genetic analysis and difficult to detect by chemical analysis using pre-extracted herbs to make an Pre-Extracted Liquorice Tea Extract (XLTE).

Pre-extraction has already been identified as a potential problem in herbal medicine and a promising test for functional adulteration. This has been fully detailed in sections 2.6.1 and 4.8 but to summarise: pre-extracted resold herbs are a recognised form of adulteration and reported in several instances of medicinal and culinary herbs and spices (Ilyas, 1980; Al-Khafaji, 2016; Centre for the Promotion of Imports from Developing Countries, 2018; Etheridge, 2023). Liquorice is one of the most widely consumed medicinal herbs around the world (Brinckmann, 2020) with up to 7 million tonnes of liquorice waste being produced each year in China alone, which lack alternative industrial applications (Santulli et al., 2020). Combined with a dwindling natural habitat (Khaitov et al., 2022) and the superiority of the wild harvested crop over cultivated ones (Wang, H. et al., 2021), there is a considerable economic motive to exploit this waste for fraudulent financial gain. In addition, these samples will share the identical genes and similar HPTLC profiles (Gafner et al., 2023), enabling them to evade the two most common tests currently performed in herbal quality evaluation today.

The method used to achieve this was to perform the extraction procedure outlined in section 7.1 once, and then leave the tea bags dry over 72 hours before performing the same extraction procedure again. This second batch was then frozen, freeze dried and stored in aliquots using the same method as before.

Due to problems with the freeze drying process which caused some of the extract to boil over and be lost, a precise total count of the exhausted extract was not possible. This was instead calculated from the yield produced by a single 20 mL tube which yielded 9.2 mg of freeze dried material. Based on a cup having 1.5 g of raw material in each bag extracted in 250 ml water, this meant that the adulterated tea yielded 115 mg per bag, or 76.6667 mg / g.

Compared to the original extraction, which yielded 377.33 mg / g, the total yield was 20.32% of the original, which itself could be used as a test for raw herbs, but it also suggests that companies selling extracts could use this procedure to increase their yields by 20% providing sufficient motivation to consider this a plausible form of adulteration.

7.3. Herbal Tea Treatment Protocols

Cells were treated for 24 hours with varying dosages of liquorice tea, diluted in media. Previous lab experience with herbal extracts and published literature showed 24 hours to be a reasonable starting point (Husni et al., 2015; Ling et al., 2016; Lombardi, Carrera & Cacabelos, 2017; Ziemlewska, Zagórska-Dziok & Nizioł-Łukaszewska, 2021). Once the results from 24 hour exposure were determined, the ROS and ΔΨm assays (described in sections 11.2 and 11.3 respectively) were repeated with a shorter (3 hour) and longer (72 hour) duration to determine the effects of acute and chronic exposure.

Initial dosages were decided by submitting a range of titrations of 1, 10, 100, 250, 500 and 1000 µg / mL to a cell viability assay (described in section 8.1) in order to find a toxic dose. Once no toxic effect was found at these doses an additional 2000 µg / mL was added. This also produced no toxic effects so low (10 µg / mL), medium (500 µg / mL) and high (2000 µg / mL) dosages were selected for subsequent assays. All treatments were performed by the researcher.

This experimental approach to finding an appropriate dosage was required because a review of the literature found no standardised dosage of herbs used when performing *in vitro* testing and the literature often appearing biassed towards obtaining the results desired. Chen et al. (2014) used doses of 0, 0.1, 0.3, 1, 3, and 10 µg / mL when testing the cytotoxicity of a Chinese medicine formula *Jia Wei Xiao Yao San* with Tamoxifen concluding that there is no interaction between the Chinese formula and the drug but there may potentially be interference at a molecular level. Conversely, Languon et al. (2018) used doses of 62.5 - 1000 µg / mL to assess efficacy and cytotoxicity of Ghanian herbal medicines against cancer cell lines. The low dose in the first study may be deliberately designed to not show interactions or toxicity of the most popular formula in Taiwan for the treatment of breast cancer (Lai, Wu & Wang, 2012), especially when the product was made by Sun Ten, a famous herbal granule company whose products are certified by the Taiwanese Department of Health and covered by National Health Insurance (Tso, 2009), and the funding for the study funding comes from several hospitals and a

government ministry. These suggest possible vested interests in demonstrating a popular brand of medicine as safe and effective which could have introduced bias. The Ghanian study may conversely be deliberately overdosing in order to demonstrate cytotoxicity on cancer cell lines to prove effectiveness, especially when they used no non-cancerous cell lines for comparison. This dosing bias is even more evident in Bailon-Moscoso et al. (2017) who used 100 µg / mL for their cell viability assays and 1000 µg / mL in their ROS assay in their investigations of "Horchata", a blend of Ecuadorian herbs. This variability between studies and the possibility of their concentrations being selected based on the desired outcome demanded that we develop our own titration system to find the optimal concentrations.

Chapter VIII

8. Mitochondrial Function Assays

8.1. Cell Viability

Cell viability assays are used to determine the effects of a substance on the proliferation or death of cells by measuring the number of viable cells remaining at the end of the experiment. Regardless of the experiment being carried out, the number of viable cells remaining at the end will affect the measurements so it is essential to determine the cytotoxic or proliferative effects of treatments first to know whether the changes are simply a change in cell number (Adan, Kiraz & Baran, 2016). The standard methodology behind most of these is to introduce a reagent to the cell culture whose substrate can be converted into a coloured or fluorescent product only by viable cells which can then be measured by a plate reader. The signal generated when examined by a plate reader is proportional to the number of living cells left in the assay.

There are several colorimetric assays that use tetrazolium dyes to assess cell viability. Gordon, Brown & Reynolds (2018) describe some of the main ones, with the full chemical name of the tetrazolium dye they use to be:

- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
- MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium)
- XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide)
- WST (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)

Each has their own advantages and disadvantages but the most common, and the one chosen for this study, was MTT. In the MTT assay, a yellow tetrazolium dye is introduced to a culture that penetrates living cells to be converted into purple formazan by reduced NAD(P)H-dependent oxidoreductase enzymes inside living, metabolically active cells (Riss, Moravec & Miles, 2013; figure 50). After incubating for 1-4 hours the resulting insoluble formazan which builds up inside the cells and on their surface is dissolved using a solvent and the colour transformation is evaluated by recording changes in light absorbance at 570 nm and subtracting this from a background absorbance reading at 630 nm using a plate reading spectrophotometer.

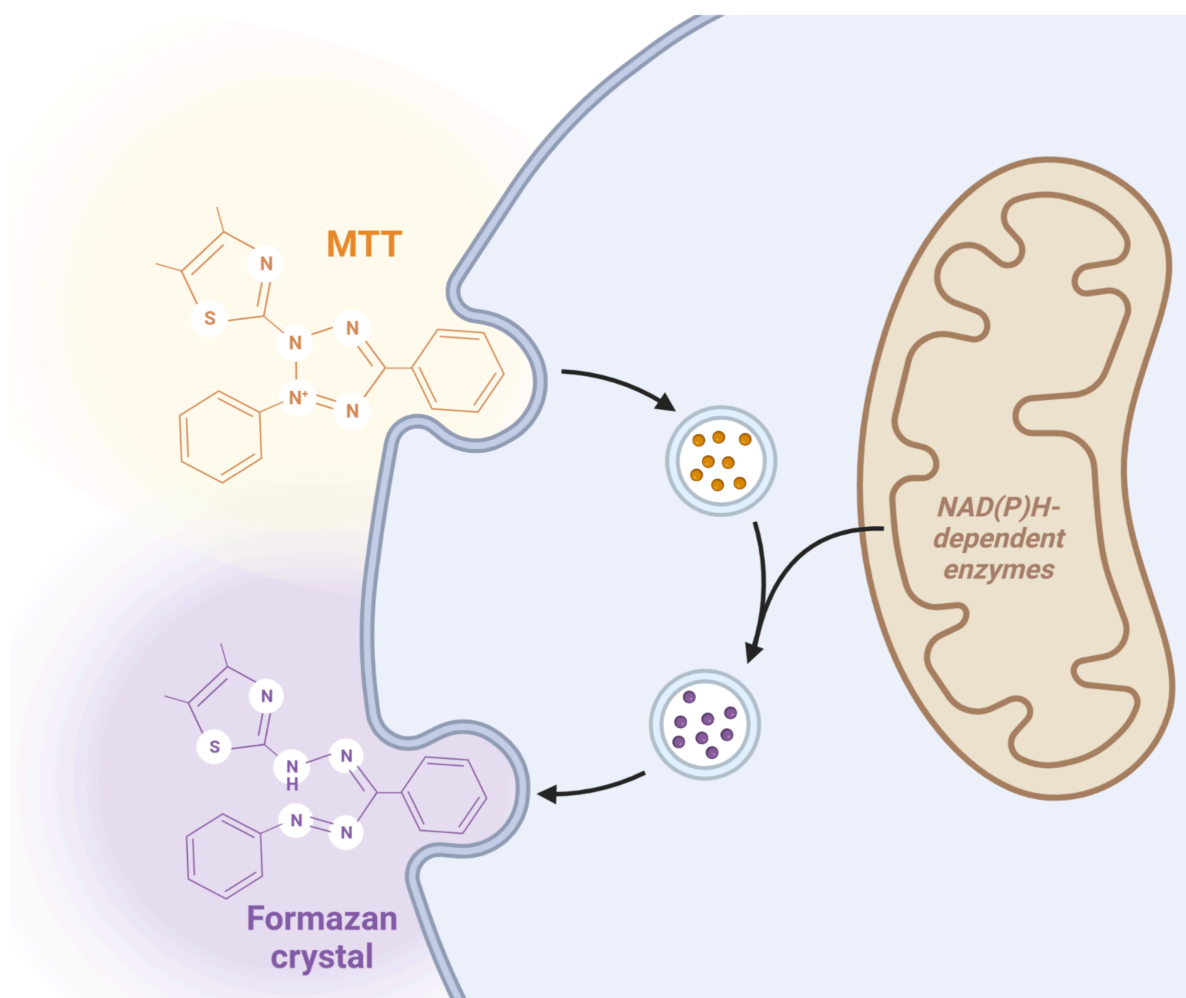


Figure 50: MTT Mechanism of Action (created with BioRender.com)

The advantage of the MTT assay is that it is an easy, cost-effective protocol with the potential for a high throughput (Gordon, Brown & Reynolds, 2018). This makes it ideal for getting an initial gauge of changes in viability prior to investigating with more specific methods that gather more detail.

The main limitation with the MTT assay is that it relies on a secondary measure of cell viability, the mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes, meaning that experiments which affect these enzymes or the metabolic activity of cells may distort the results (Zhang & Cox, 1996; Liu & Dalgleish, 2009). MTT assays are therefore usually the first test in a series in order to observe any obvious changes in viability, after which further tests can determine whether the result is due to a change in metabolic function, enzyme production, or genuine cell viability.

For this project, an MTT Assay kit (Sigma, UK) was used. Cells were seeded onto 96 well plates at a density of 2.5×10^4 and treated as described in section 7.3. Following treatment, cells were washed with PBS and the media replaced with phenol red-free media, then treated with 10 µL MTT solution (50 mg MTT in 10 ml PBS). After 3 hours the media was removed and the formazan crystals dissolved with 100 µL DMSO or 2-propanol and shaken for 5 minutes. Measurements were then taken using a microplate reader (SPECTROstar Nano, BMG Labtech, Germany) by subtracting absorbance at 570 nm with absorbance at 690 nm and presented as a percentage of the untreated control.

8.2. Detection and Quantification of Reactive Oxygen Species

2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) is one of the most widely used techniques for directly measuring the redox state of a cell (Eruslanov & Kusmartsev, 2010). It works through intracellular esterases which cleave the two ester bonds from the original molecule to produce H_2DCF that then accumulates intracellularly and oxidises to form highly fluorescent 2',7'–dichlorofluorescein (DCF). This can then be measured by detecting the increase in fluorescence at

535 nm when excited at 485 nm to provide a measure of generalised oxidative stress (figure 51). It is unable to provide a direct measure of any particular reactive species since neither H_2O_2 nor $O_2^{\bullet-}$ can oxidise H_2DCF directly but must be decomposed to radicals, while other substances in the cell may also produce the reaction including other radicals as well as cytochrome c, responsible for activating the caspase cascade that initiates apoptosis, making assessment of ROS during apoptosis using DCFDA especially problematic (Lawrence et al., 2003).

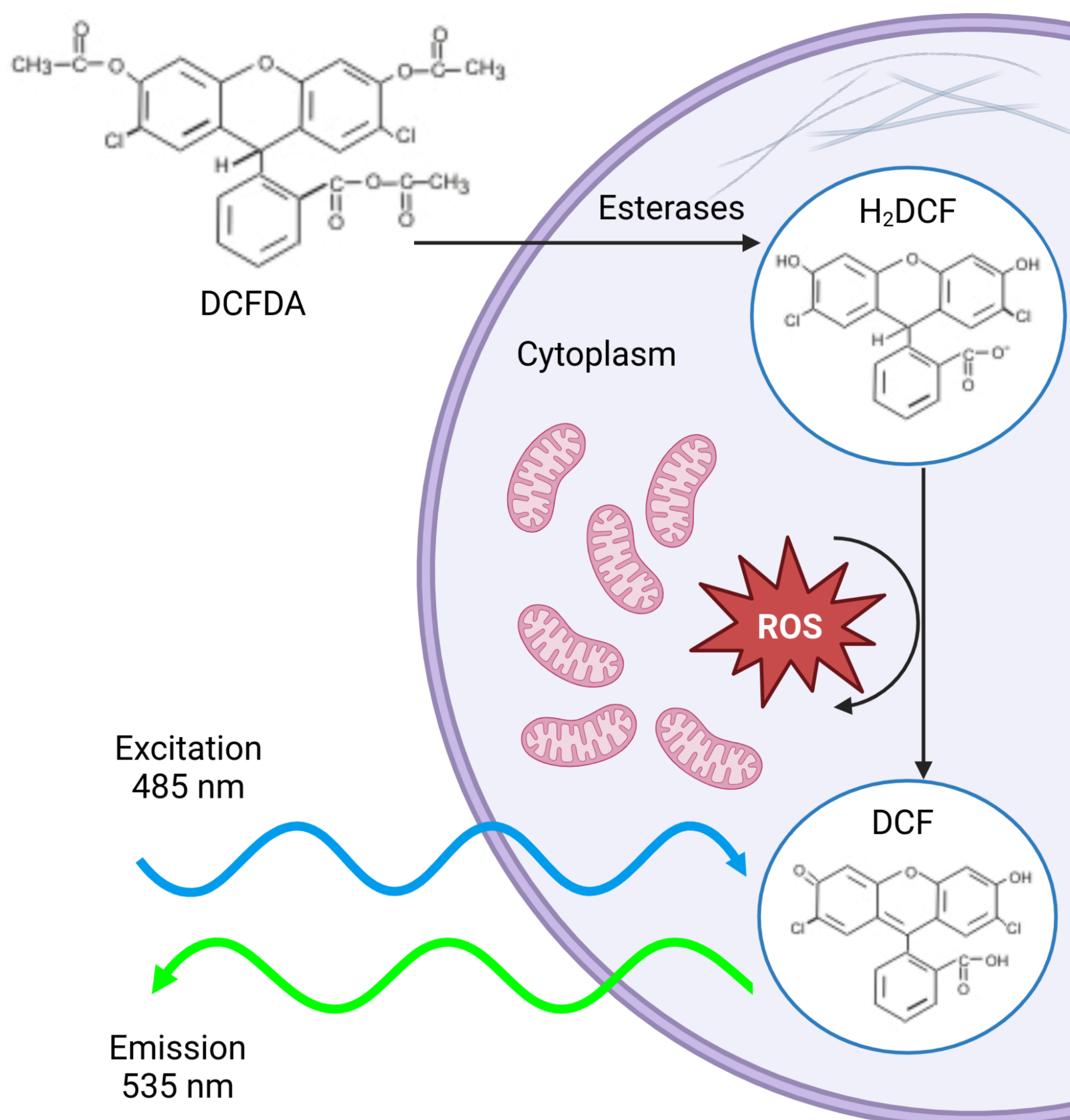


Figure 51: DCFDA Mechanism of Action (created with BioRender.com)

MitoSOX is another important assay used for detecting ROS, especially $O_2^{\bullet-}$, which contains a positively charged derivative of dihydroethidium (DHE) that rapidly accumulates in mitochondria where it is oxidised by $O_2^{\bullet-}$ to become 2-hydroxyethidium (2-OH-E^+), which then binds to DNA producing a red fluorescence (Kauffman et al., 2016). This is more specific than DCFDA but can still suffer from overlapping fluorescence from ethidium (E^+) which is not formed from $O_2^{\bullet-}$ and may be generated in larger quantities than the radicalised form (Nazarewicz, Bikineyeva & Dikalov, 2013; Zielonka & Kalyanaraman, 2010).

Although both assays have their drawbacks, a critical approach to measuring ROS combined with data from other tests and controls can give some valuable insights into the degree of oxidative stress that a cell culture is undergoing.

The DCFDA Cellular ROS assay kit (Abcam, UK) was chosen for this study because it is able to provide a generalised measure of oxidative stress. Following treatment, the cells were washed with 1x buffer solution and incubated with 20 μM DCFDA at 37°C 5% CO_2 in the dark for 45 minutes. Measurements were then taken using a fluorescence microplate reader (FLUOstar Optima, BMG Labtech, Germany) using excitation at 485 nm and emission at 535 nm.

8.3. Detection and Quantification of Mitochondrial Membrane Potential

Several fluorescent lipophilic cationic dyes dyes can be used to measure ΔΨm including:

- TMRM & TMRE (tetramethylrhodamine methyl and ethyl ester)
- Rhod123 (Rhodamine 123)
- DiOC6(3) (3,3′-dihexyloxacarbocyanine iodide)
- JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide)

The basic principle of these is all the same: they accumulate within the mitochondria in inverse proportion to Δψm making more polarised mitochondria accumulate more dye and depolarised mitochondria accumulate less (Perry et al., 2011) (figure 52). This can then be detected by measuring the fluorescence or imaged with a camera. Each dye has its particular advantages and disadvantages but the overall limitations of these methods are that changes in mitochondrial morphology, localization, or mass might also affect fluorescence measurements and so controls that assess whether these changes are also happening are advisable to conduct alongside the Δψm probes, although these tests are also not without their limitations and may affect Δψm and respiration themselves (Buckman et al., 2001).

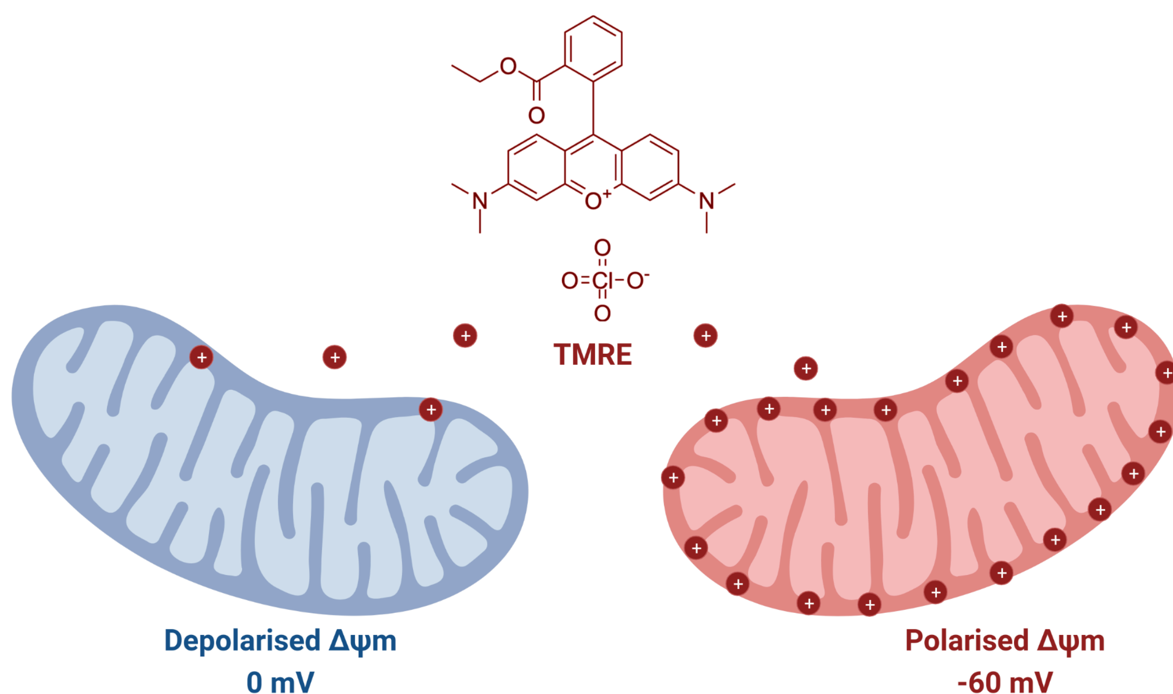


Figure 52: TMRE Mechanism of Action (created with BioRender.com)

TMRE Mitochondrial Membrane Potential assay kit (Abcam, UK) was selected for detecting and measuring modulations in ΔΨm. TMRE loads specifically into polarised mitochondria (Perry et al., 2011) and is especially

suitable for detecting changes in ΔΨm caused by oxidative stress in live cells (Tian et al., 2021). Following treatment, the media was removed and replaced with 20 nM TMRE in fresh media. The cells were then incubated at 37°C 5% CO_2 in the dark for 35 minutes before being washed with PBS and their media replaced with 100 µL PBS. Measurements were taken using a fluorescence microplate reader (FLUOstar Optima, BMG Labtech, Germany) using excitation at 549 nm and emission at 575 nm.

8.4. Detection and Quantification of Calcium Ions

Rhod-2 acetoxymethyl ester (Rhod-2) can be used to detect Ca^{2+} levels inside mitochondria by increases in fluorescence when it binds with Ca^{2+}, measured at excitation at 557 nm and emission at 581 nm (Cayman Chemical, 2021). It is not mitochondrially specific so another spectrally distinct dye such as MitoTracker Green should also be used to differentiate mitochondrial calcium from other sources on imaging (Maxwell et al., 2018). Combined with the previous assays, additional information can then be ascertained on the mechanism behind modulations in ROS, ΔΨm or apoptosis.

Rhod-2 dye (Thermofisher, UK) was used in this study. For MCF7 cells, following treatment, the media was removed and replaced with 1 µM Rhod-2 in fresh media. The cells were then incubated at 37°C 5% CO_2 in the dark for 30 minutes before being washed with PBS and their media replaced with 100 µL PBS. Measurements were taken using a fluorescence microplate reader (FLUOstar Optima, BMG Labtech, Germany) using excitation at 552 nm and emission at 581 nm. For MCF10A cells, this protocol had to be adjusted due to a failure to get any readings at this concentration. After experimentation with different concentrations, incubation periods and seeding densities, the results were obtained using a concentration of 4 µM Rhod-2 in fresh media and the seeding density reduced to 1 x 10^4 per well.

8.5. Observation of Mitochondrial Morphology via Confocal Microscopy

Cells were seeded and treated as per section 7.3, then washed with PBS and stained with 35 nM MitoTracker Red (Thermofisher, UK) and 4 drops of NucBlue (Thermofisher, UK) for 40 minutes at 37°C 5% CO_2. Images were taken using EVOS FL Auto 2 (Thermofisher, UK) using a 40X 0.65 NA plan fluorite objective.

8.6. Measurement of Mitochondrial Respiration

To measure mitochondrial respiration, oxygen consumption rate (OCR) via the SeaHorse XF_e24 Flux Analyzer (Agilent, USA) was chosen. This measures OCR in real time by isolating a monolayer of cells covered with an extremely small volume of media (about 2 µL) and placing a probe 200 microns above the monolayer (Agilent Technologies, 2020). The probe measures the concentrations of dissolved O_2 in the transient microchamber every 2-5 minutes and calculates the OCR. It then lifts, allowing the media to mix with the microchamber, restoring cell values to the baseline. This provides a direct measure of cellular respiration but other cellular processes also consume O_2 including NADPH-oxidase and oxidoreductase enzymes (Herst & Berridge, 2007) so these processes have to be controlled for. Up to four drugs can be injected during the assay, which can be used to inhibit particular respiratory processes, including the entire ETC, enabling this limitation to be controlled for and other parameters to be measured too.

The SeaHorse MitoStress Assay (Agilent Technologies, 2019) utilises four drugs with known effects on the ETC in order to measure how much O_2 is consumed by those processes through observing the change in OCR when these drugs are applied. These drugs are:

1) **Oligomycin**: inhibits ATP Synthase causing a drop in OCR which enables quantification of ATP linked respiration.

2) **Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP)**: an uncoupling agent that disrupts ΔΨm and collapses the proton gradient, disinhibiting electron flow through the ETC. O_2 consumption is then increased to offset the proton leak, drawing upon the spare capacity of the mitochondria to reach their maximal OCR (Marchetti, Fovez et al., 2020). By subtracting the basal rate from the maximal OCR after FCCP administration, the spare respiratory capacity can be calculated.

3) **Antimycin A** and **rotenone:** Antimycin A inhibits Complex III and rotenone inhibits Complex I of the ETC which causes mitochondrial respiration to shut down completely, revealing non-mitochondrial respiration. This can then be deducted from the other measurements to reveal mitochondrial OCR alone.

This results in a predictable wave (figure 53) whose modulations can be used to determine the mitochondrial effects of any prior treatments.

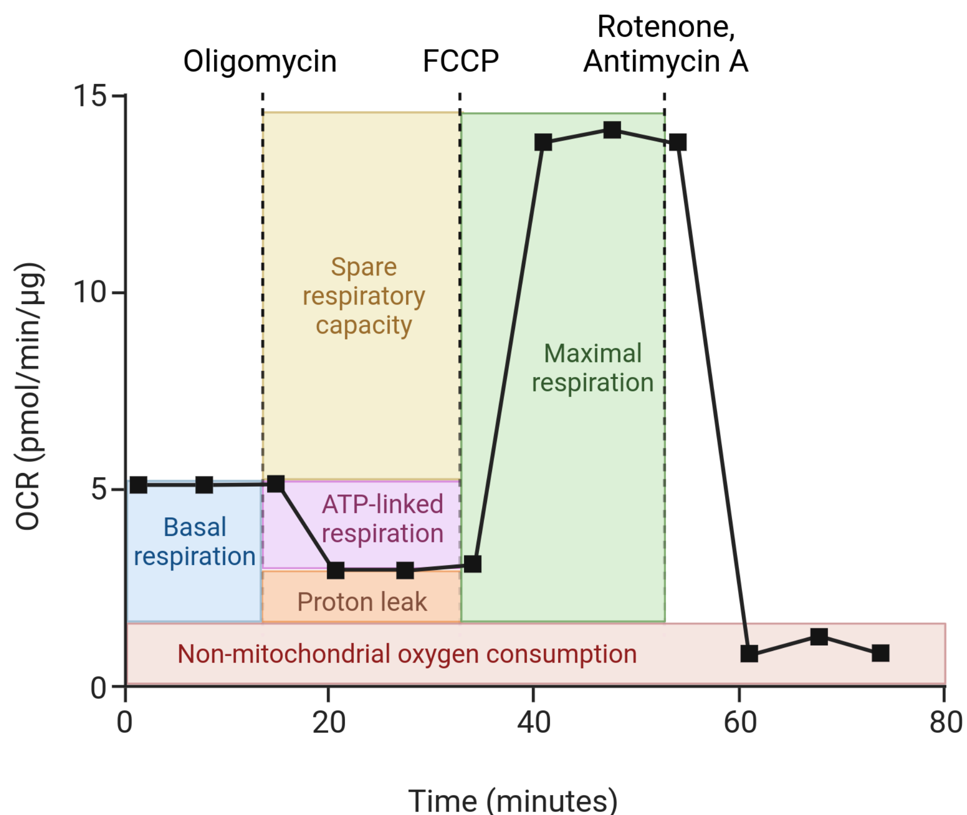


Figure 53: A Typical Seahorse MitoStress Assay Profile (created with BioRender.com, based on Agilent Technologies, 2022).

MCF7 and MCF10A cells were seeded onto SeaHorse plates at a density of 3 x 10^4 using 100 µL of media to ensure a monolayer, which was then topped up to 250 µL after adherence, 3 hours later. 24 hours after seeding they were treated as described in section 7.3 but using the maximum capacity of the well, 500 µL. For each assay, prepared Seahorse media was made appropriate to the cell line by using 50 mL of Seahorse XF Media (Agilent, USA) and adding 1% sodium pyruvate, 1% L-Glutamine and the amount of glucose required for the individual cell line (equivalent to 1 g / L for MCF7 and 3.152 g / L for MCF10A) and balancing the pH and filter sterilising, if required. The cells were then washed twice and replaced with 500 µL of the prepared Seahorse media and incubated for a further 45 minutes to 1 hour in a non-CO_2 incubator at 37°C while the drugs were prepared in the injection ports of the Seahorse Sensor Plate. The drugs were prepared in the following proportions, as outlined in the instructions (Agilent Technologies, 2019):

1) **Oligomycin** (Sigma, UK): 56 µL of 10 µg / mL solution in the prepared Seahorse media was added to injection port A

2) **FCCP** (Sigma, UK): 62 µL of 5 µM solution in the prepared Seahorse media was added to injection port B. This was later increased to 6 µM when the predicted response was present in the control group but seemed to have a weaker response than expected, suggesting the drug had lost some of its potency.

3) **Antimycin A** (Sigma, UK) and **rotenone** (Sigma, UK): 69 µL of a solution containing 10 µM of each drug in the prepared Seahorse media was added to injection port C.

The sensor plate was then calibrated using a utility plate filled with 1 mL calibrant fluid (Agilent, USA) per well which was entered into the Seahorse Flux Analyzer running Agilent Wave software. Once calibrated, the utility plate was replaced with the cell plate and left to run.

Once completed the OCR measurements were then corrected for total protein concentration using the Bradford or Pierce™ bicinchoninic (BCA) assays (see section 8.6.1). The protein estimations of each well were then entered into Agilent Wave software to normalise the recorded OCR pmol/min based on µg protein for each well.

8.6.1. Protein Assays for SeaHorse Normalisation

Following the SeaHorse Mito Stress Test, a protein assay was performed to quantify the total protein content and correct for variations in cell density. Initially a Bradford protein assay was conducted but after the first set of results this was changed to Pierce™ BCA assay due potential incompatibility of the Bradford assay with the surfactants in radioimmunoprecipitation assay (RIPA) buffer potentially introducing background noise, and a superior working range of the Pierce™ BCA assay (ThermoFisher Scientific, n.d.; CiteQ Biologics, n.d.).

For the Bradford assay, each well was washed twice with ice cold PBS before being lysed with 50 µL ice cold RIPA buffer. Each well was scraped and the contents transferred to individual ice cooled Eppendorf tubes before being centrifuged at 1600 G for 15 minutes at 4°C. During this time a series of protein standards were created using FBS against which a standard curve could be created to allow calculation of the unknown samples via linear regression (performed using Google Sheets). 45 µL of supernatant was then removed and placed into a new set of tubes, whereupon 5 µL was added to the wells of a 96-well plate in triplicate. 200 µL of Bradford Reagent was added to each well and the absorbance measured at 595 nm (SPECTROstar Nano, BMG Labtech, Germany).

For the Pierce™ BCA assay, each well was washed twice with ice cold PBS before being lysed with 30 µL ice cold RIPA buffer. Each well was scraped and the contents transferred to individual ice cooled Eppendorf tubes before being centrifuged at 1600 G for 20 minutes at 4°C. During this time a series of protein standards were created using Bovine Serum Albumin (BSA) against which a

standard curve could be created to allow calculation of the unknown samples via linear regression (performed using Google Sheets). 20 µL of water and 5 µL supernatant was added to the wells of a 96-well plate in triplicate. 200 µL of Working Reagent was then added to each well, the plate covered in foil and shaken for 30 seconds, incubated at 37°C with no CO_2 for 30 minutes and allowed to cool to room temperature. The absorbance was then measured at 562 nm (SPECTROstar Nano, BMG Labtech, Germany).

8.7. Statistical Analysis

Technical replicates were averaged to achieve a result for each biological replicate and compared as a percentage change from the control to eliminate interday variability. Statistical analysis was carried out using GraphPad Prism (version 9.5). A one-way analysis of variance (ANOVA) was performed with Dunnett's post-hoc analysis to compare all groups to a control, or a Tukey Test to compare treatment groups to each other (Frost, 2019). These were chosen because three or more groups were being compared, the data was not measuring points in the same population and so is unpaired, and can be assumed to have a normal distribution making it parametric. Significance was taken when $p < 0.05$.

Chapter IX

9. Drug Resistance Assays

9.1. Seahorse Drug Resistance Assay

The drug resistance assay was designed after observing the results obtained from the first cell line, MCF7, to be assayed by the SeaHorse Flux Analyzer, described in chapter 12. A review of the literature revealed the detoxification properties of liquorice in CHM (see section 4.5, and a full discussion in section 20.3). This included some research papers that suggested liquorice may confer resistance to the drugs used in the Seahorse assay. Glabridin was found to protect osteoblast cells from antimycin A induced cytotoxicity (Choi, 2011) and glycyrrhizic acid revealed protective effects against a rotenone induced model of Parkinson’s disease in an animal model (Ojha et al., 2016), which was subsequently investigated further with *in vitro* mechanistic studies (Karthikkeyan et al., 2020; Karthikkeyan, G., Prabhu et al., 2021; Karthikkeyan, G., Pervaje et al., 2021; Karthikkeyan et al., 2022). Although there was no literature on liquorice having any effect on oligomycin or FCCP, it prompted an *ad hoc* hypothesis that liquorice was responsible for the reduced effect of the SeaHorse drugs and may have a protective effect on a broad range of toxic substances that modulate mitochondrial activity.

This assay involved repeating the DCFDA assay outlined in section 8.2 but with the introduction of the SeaHorse drugs 10 minutes before the introduction of DCFDA. This timing is recommended in the TMRE instructions where FCCP can be used as a positive control for its effect on collapsing $\Delta\Psi m$ (Abcam, 2022) so was expected to have an effect. DCFDA was chosen over TMRE because changes in ROS measure oxidative stress caused by a number of mitochondrial dysfunctions and so was considered more likely to be measurable in response to the other drugs. The dosage for FCCP was chosen according to the Abcam

protocol with the remaining drug dosages determined based on their proportion to FCCP in the Seahorse assay. This resulted in:

1. Oligomycin: 40 μg / mL
2. FCCP: 20 μM
3. Antimycin A: 40 μM
4. Rotenone: 40 μM

9.2. Cisplatin Resistance Assay

The cisplatin resistance assay was designed as a modification of the initial Seahorse drug resistance assay.

Cisplatin (Sigma, UK) was dissolved in dimethylformahide (Sigma, UK) to achieve a stock concentration of 10 mM to be diluted to the appropriate amount on the day of experimentation in fresh media.

Cells were grown and seeded as per section 6.2 and treated as per section 7.3, except divided into groups treated with a high dose of liquorice (2000 μg / mL) and untreated groups for 24 hours. The cells were then washed with PBS and replaced with cisplatin in low (10 μM), medium (50 μM) and high (100 μM) concentrations for a further 24 hours, after which they were washed once again with PBS and their viability measured with MTT as per section 8.1. A reduction in cell viability of the high dosage cisplatin only treated group was used as a positive control to ensure the cisplatin was killing the cells as intended. Comparisons were drawn between the reduction in cell viability of the cells which were administered cisplatin only and the equivalent group which were administered liquorice for 24 hours prior, followed by the same dose of cisplatin.

9.3. Statistical Analysis

Statistical analysis was performed using the same method of averaging technical repeats, determining changes as percentage of the control and using GraphPad Prism (version 9.5) as outlined in section 8.7. The drug resistance assays differed in that comparisons were made between between a liquorice pretreated group and a group that received no pretreatment, before both were exposed to the same dosage of a specific drug. This made only two groups directly comparable so an unpaired t-test was used for statistical analysis, which is suitable to compare only two groups of unpaired parametric data (Mishra et al., 2019).

Chapter X

10. Adulteration Detection Assays

10.1. Adulteration Detection with Mitochondrial Functional Analysis

Assays using OLTE which scored a significant result ($p < 0.05$) in any arm were repeated using the adulterated liquorice tea to see if the same result would be obtained. Levels of significance were primarily used to determine if the same result was obtained with mean differences used to determine the degree of difference. This was considered preferable to directly comparing values between the original and adulterated tea samples to better reflect real-world cases where an unknown sample may be tested for its ability to produce a significant known effect and not always compared directly against a reference sample.

10.2. Visual Inspection

A simple visual inspection was undertaken to determine if XLTE could be easily differentiated from OLTE. Eppendorf tubes containing each sample were defrosted and placed in a well lit area of the laboratory and examined for differences in colour, transparency and sediment. A photograph was taken using a Samsung Galaxy A13 50.0 MP camera running software version 13.1.00.58.

10.3. High Performance Thin Layer Chromatography

To determine the likelihood of this form of adulteration being detected through other means, the original and adulterated liquorice samples were subjected to HPTLC analysis. HPTLC instruments from CAMAG (Muttenz, Switzerland) were used, including Automatic TLC Sampler (ATS 4), Automatic Development Chamber

(ADC 2) with humidity control, Plate Heater 3, TLC Visualizer 2, and Immersion Device 3.

The reference standards included the original dried herbal material from one of the “3 Licorice” tea bags (Pukka, UK), *G. glabra* from the University of Westminster Polyclinic, and a geno-verified botanical reference sample of *G. uralensis* (Brion Research Institute, Taiwan). The active samples included the OLTE and XLTE.

To prepare the samples, 0.5 g of finely powdered herbal material, or the equivalent of freeze-dried material from the samples, was extracted in 10 mL of ethanol, water 7:3 (v/v), sonicated for 10 minutes, centrifuged at 5000 rpm for 5 minutes, and the supernatant extracted. Since the sample material was limited, the amount of freeze dried material that would have been obtained from 0.125 g of dried material was used in 2.5 mL of solvent. Three sample groups were included:

1. OLTE, using 39.3 mg in 2.5 mL of alcohol, water 7:3 (v/v).
2. XLTE, using 39.3 mg in 2.5 mL of alcohol, water 7:3 (v/v), to see qualitative differences when used at the same concentration as the original extract, as was used in the biological assays.
3. XLTE, using 9.6 mg in 2.5 mL of alcohol, water 7:3 (v/v), to simulate the amount that would be extracted from the same quantity of dried material.

2 μL and 5 μL of each sample and reference standard solution were used to aid visual comparison. 2 μL could result in bands from low concentration components being too faded, while 5 μL could result in bands from high concentration components becoming blurred. Having two concentrations was chosen to increase the likelihood of capturing results in both situations.

For the stationary phase, a 20 x 20 cm aluminium HPTLC silica gel 60 F_{254} plate (Sigma, UK) was used. 2 μL and 5 μL of each of the reference and sample extractions were applied to the plate. The mobile phase used ethyl acetate, formic acid, glacial acetic acid, water 15:1:1:2 (v/v). The plate was developed in a saturated chamber with $MgCl_2$ at 33% humidity for 20 minutes up to 70 mm from the lower

edge. Plates were then read underivatized under white light and UV 254 nm and UV 366 nm.

For derivatization, a 10% sulphuric acid in methanol solution was used, dipped for 0 seconds at a speed setting of 5 and heated at 100℃ for 10 minutes, before being observed under white light and under UV 366 nm.

Besides the appearance or disappearance of particular bands, the overall strength of the columns was compared to determine if HPTLC would be able to conclusively determine if the adulterated tea could be easily recognised, or was within the parameters that might be considered normal variation between crops.

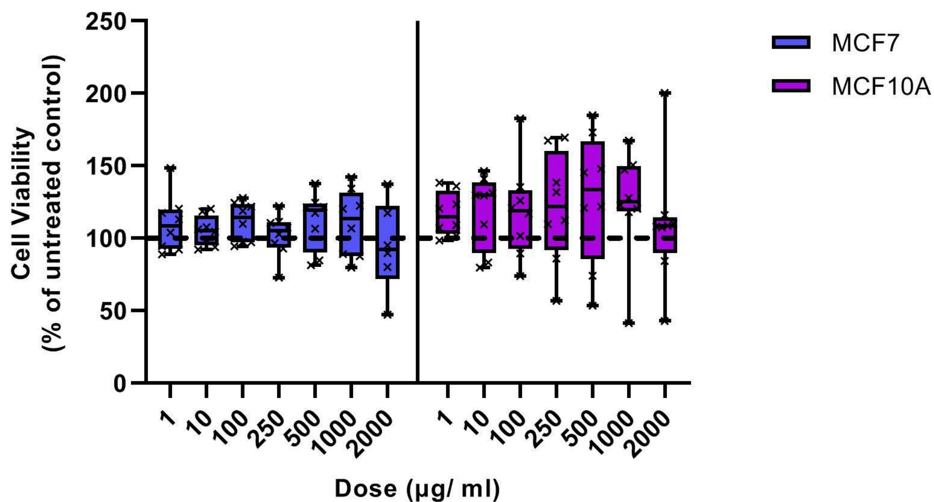
Results

Chapter XI

11. Results from Basic Mitochondrial Assays on Original Liquorice Tea Extract

11.1. Effects of Original Liquorice Tea Extract on Cell Viability

No significant change in cell viability was observed at any of the chosen doses after 24 hours treatment with OLTE (figure 54).



Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

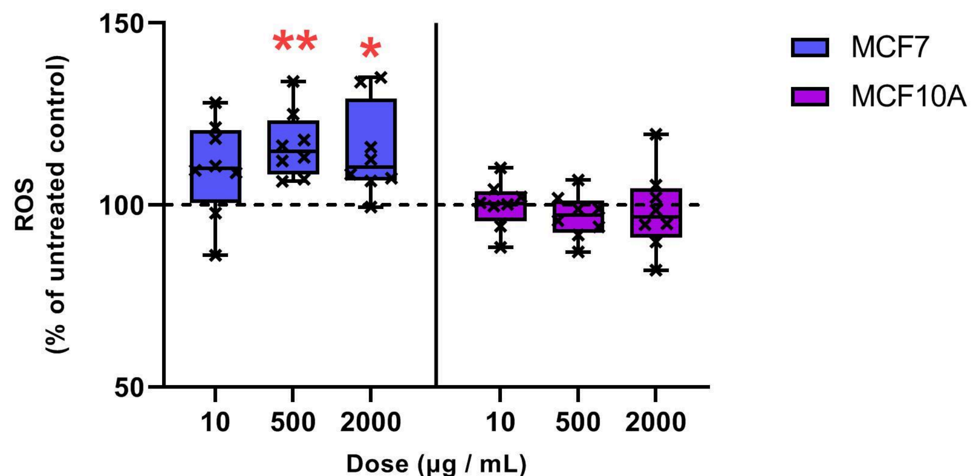
n=8, except MCF7 2000 µg/mL dose added later where n=6.

Figure 54: Results of MTT Assay Showing Changes in Cell Viability After Treatment with Original Liquorice Tea Extract

After finding no cytotoxicity at these doses, three were selected to represent the main dose ranges for the following assays: low (10 µg / mL), medium (500 µg / mL) and high (2000 µg / mL) dosage.

11.2. Effects of Original Liquorice Tea Extract on Reactive Oxygen Species

After 3 hours of treatment with OLTE, ROS increased 16.51% ($p < 0.01$) in the 500 µg / mL and 14.88% ($p < 0.05$) in the 2000 µg / mL dose groups in the MCF7 cell line (figure 55).



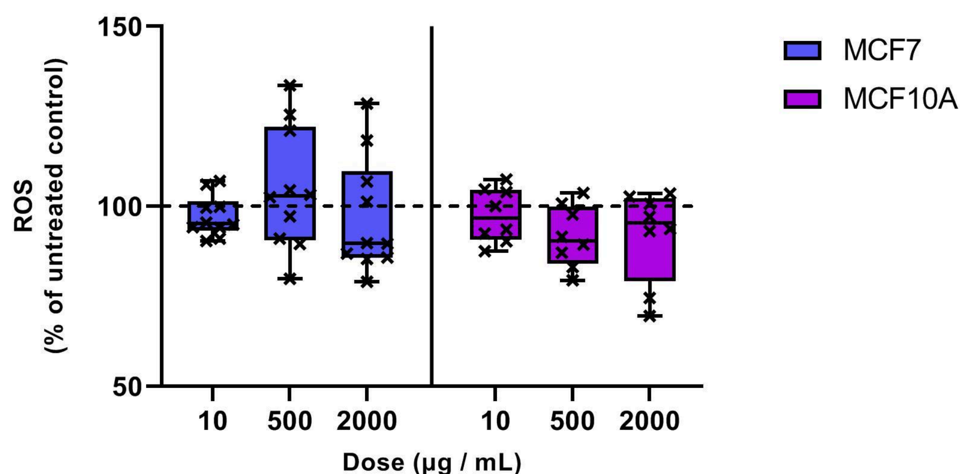
Reactive Oxygen Species (ROS) measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

n=8 for both groups.

* indicates significance ($p < 0.05$), ** indicates significance ($p < 0.01$), compared against control using one way ANOVA with Dunnett's multiple comparisons.

Figure 55: Results of DCFDA Assay Showing Changes in ROS 3 Hours After Treatment with Original Liquorice Tea Extract

After 24 hours treatment with OLTE, no change was observed in either line (figure 56).

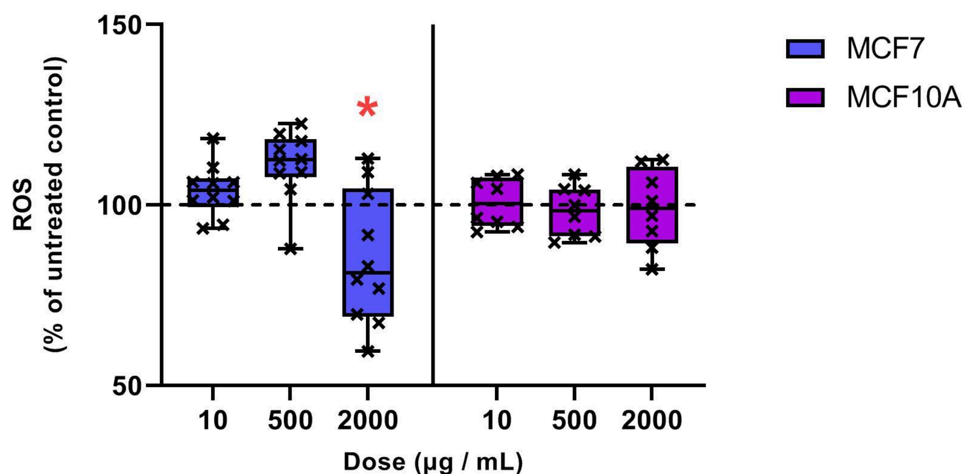


Reactive Oxygen Species (ROS) measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

MCF7 n=10, MCF10A n=8.

Figure 56: Results of DCFDA Assay Showing Changes in ROS 24 Hours After Treatment with Original Liquorice Tea Extract

After 72 hours of treatment with 2000 μg / mL OLTE, a 14.73% ($p < 0.05$) drop in ROS was observed in the MCF7 cells (figure 57).



Reactive Oxygen Species (ROS) measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

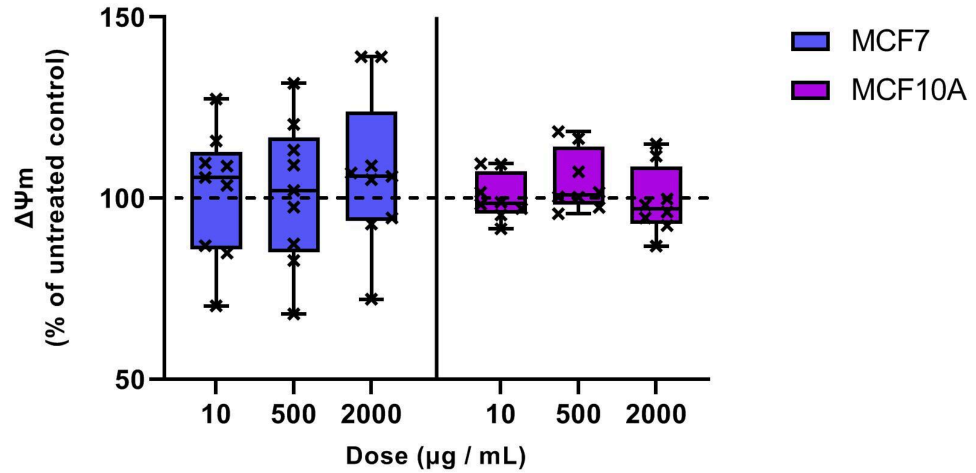
MCF7 n=10, MCF10A n=8.

* indicates significance ($p < 0.05$) compared against control using one way ANOVA with Dunnett's multiple comparisons.

Figure 57: Results of DCFDA Assay Showing Changes in ROS 72 Hours After Treatment with Original Liquorice Tea Extract

11.3. Effects of Original Liquorice Tea Extract on Mitochondrial Membrane Potential

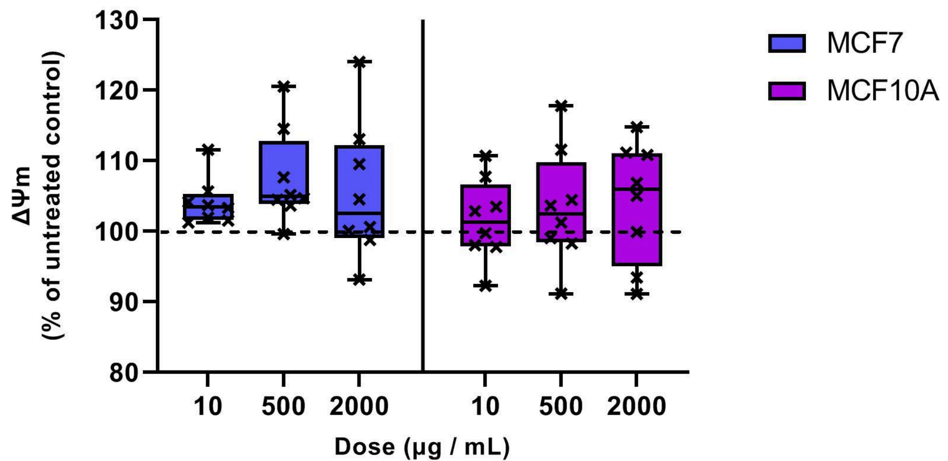
No significant change was seen in $\Delta\Psi m$ after treatment with OLTE at any dose, or any time point, in either cell line (figures 58 - 60).



Mitochondrial Membrane Potential (ΔΨm) measured with tetramethylrhodamine methyl ester (TMRE) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

MCF7 n=9, MCF10A n=8.

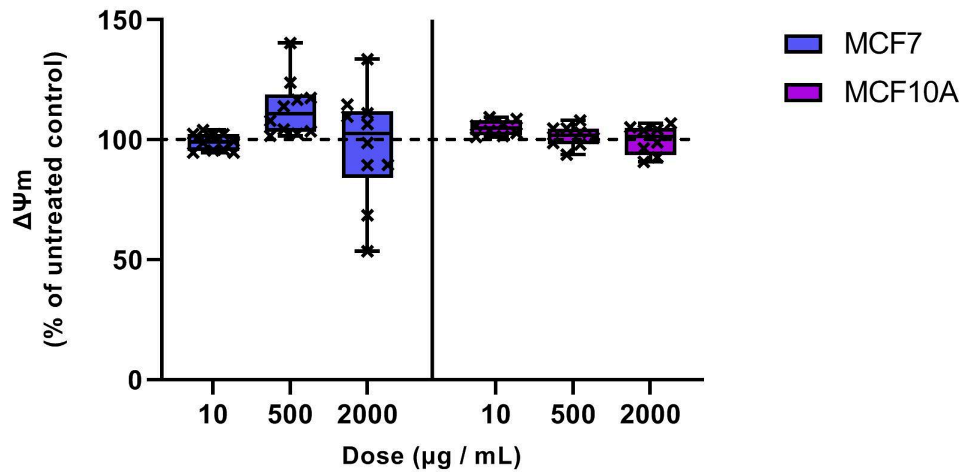
Figure 58: Results of TMRE Assay Showing Changes in ΔΨm 3 Hours After Treatment with Original Liquorice Tea Extract



Mitochondrial Membrane Potential (ΔΨm) measured with tetramethylrhodamine methyl ester (TMRE) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

n=8 for both cell lines.

Figure 59: Results of TMRE Assay Showing Changes in ΔΨm 24 Hours After Treatment with Original Liquorice Tea Extract



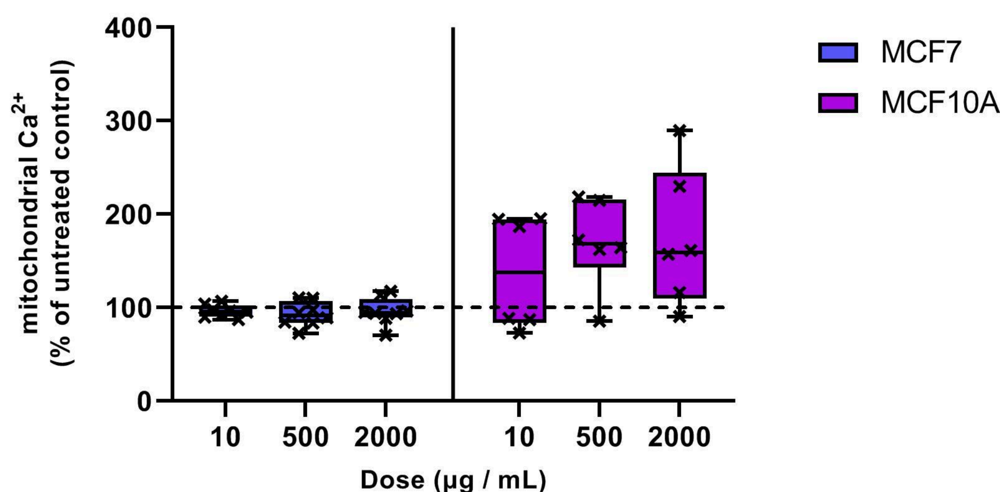
Mitochondrial Membrane Potential (ΔΨm) measured with tetramethylrhodamine methyl ester (TMRE) represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

MCF7 n=10, MCF10A n=8.

Figure 60: Results of TMRE Assay Showing Changes in ΔΨm 72 Hours After Treatment with Original Liquorice Tea Extract

11.4. Effects of Original Liquorice Tea Extract on Calcium Ions

No significant change in Ca^{2+} was observed after 24 hours exposure to OLTE in either cell line (figure 61). MCF10A cells proved to be highly resistant to the assay requiring 4 µM of Rhod-2 (compared to 1 µM in MCF7 cells) and modification of the seeding density time to produce any result (see section 8.4) and still had a much wider spread of data when a result was obtained. This may account for the large variation seen in the MCF10A data.



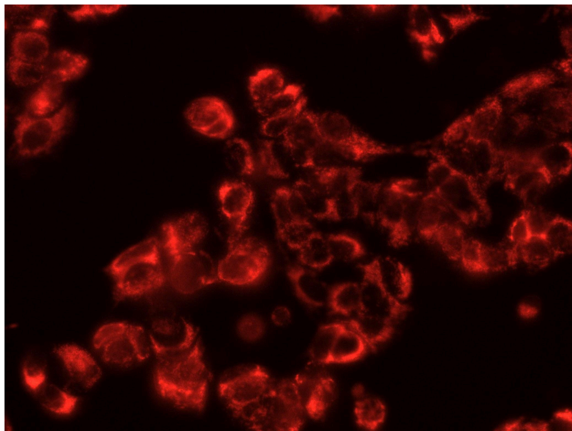
Mitochondrial Ca^{2+} measured with Rhod-2 represented as % change from untreated control drawn as dotted line at 100%. Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed.

MCF7 n = 8; MCF10A n = 6.

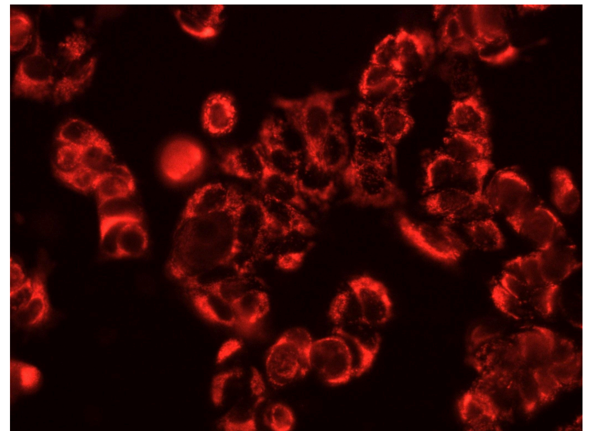
Figure 61: Results of Rhod-2 Assay Showing Changes in Ca^{2+} 24 Hours After Treatment with Original Liquorice Tea Extract

11.5. Effects of Original Liquorice Tea Extract on Mitochondrial Morphology

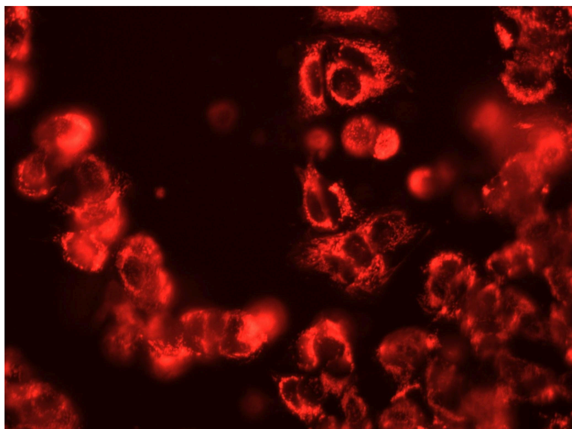
Preliminary confocal microscopy on MCF7 revealed no significant changes in mitochondrial morphology compared to untreated control (figure 62). In all images the mitochondria appear to be fused around the nucleus, apparent by the red stained mitochondria forming a circular network around the nucleus which was stained with NucBlue and so not visible under a red light filter. This is consistent with an absence of raised ROS or cytotoxicity.



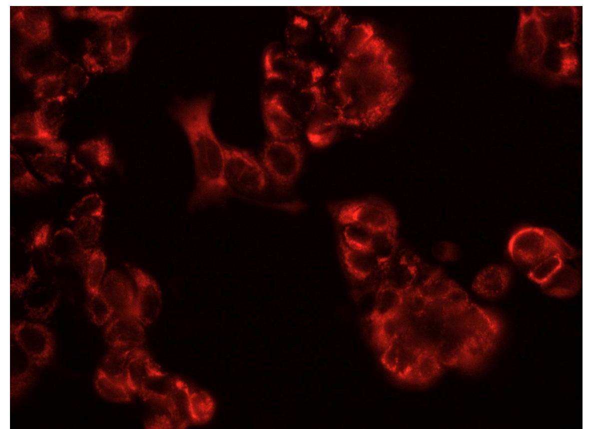
Control



24 hr treatment with 10 µg / mL original liquorice tea extract



24 hr treatment with 500 µg / mL original liquorice tea extract



24 hr treatment with 2000 µg / mL original liquorice tea extract

Figure 62: Mitotracker Red Stained Confocal Images of MCF7 Cells After Treatment with Original Liquorice Tea Extract

Since there was no visible evidence of mitochondrial dysfunction in the cell line which showed the greatest perturbations when exposed to OLTE (raised ROS at 3 hours, figure 55, and lowered ROS at 72 hours, figure 57) and no changes in cell viability were seen in either cell line at any dosage (figure 54), it was decided that visual inspection was highly unlikely to produce any useful data so no further microscopy was undertaken.

11.6. Summary of Results from Basic Mitochondrial Assays on Original Liquorice Tea

The results can be summarised as follows:

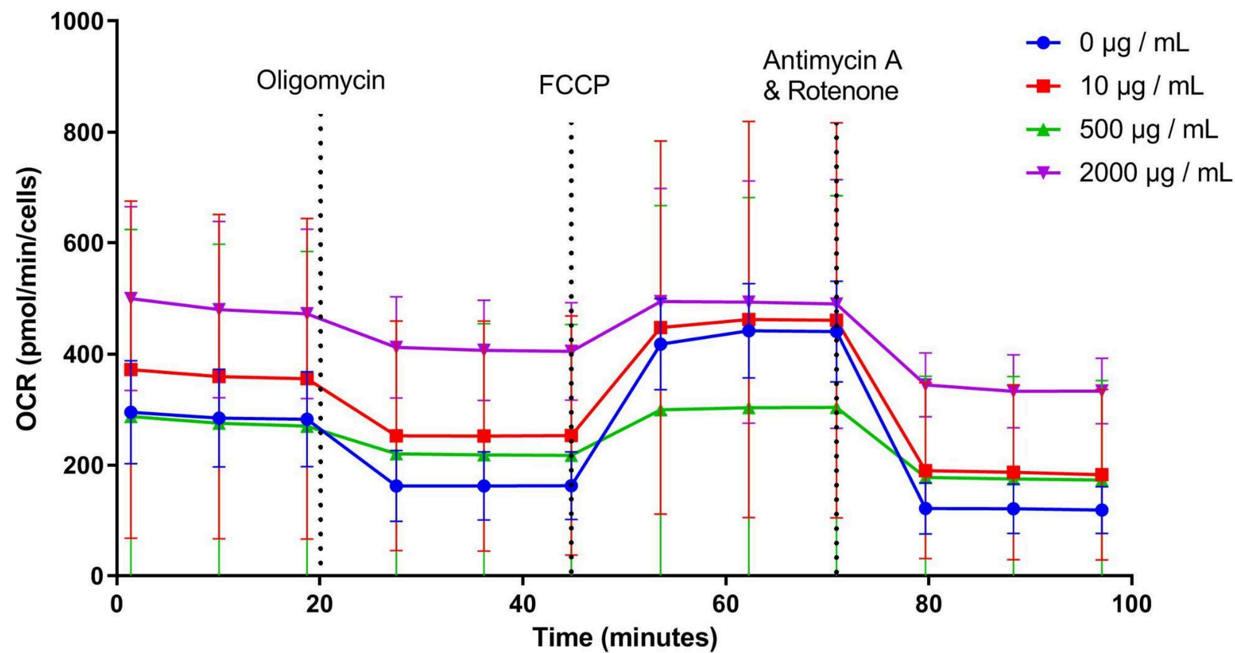
- 3 hours treatment with 500 µg / mL OLTE saw a 16.51% ($p < 0.01$) increase in ROS in MCF7 cells
- 3 hours treatment with 2000 µg / mL OLTE saw a 14.88% ($p < 0.05$) increase in ROS in MCF7 cells
- 72 hours treatment with 2000 µg / mL OLTE saw a 14.73% ($p < 0.05$) decrease in ROS in MCF7 cells
- No significant changes were observed on the MCF10A cells

Chapter XII

12. Results from Respiratory Measurements of Original Liquorice Tea Extract in MCF7 Cells

12.1. Effects of Original Liquorice Tea Extract on Mitochondrial Respiration and its Measurement in MCF7 Cells

After 24 hour treatment with OLTE the Seahorse MitoStress assay produced a surprisingly flat curve in response to the drug injections in MCF7 cells, which seemed to become flatter as the dosage increased (figure 63) in contrast to the expected response to the drug injections (figure 53, section 8.6)

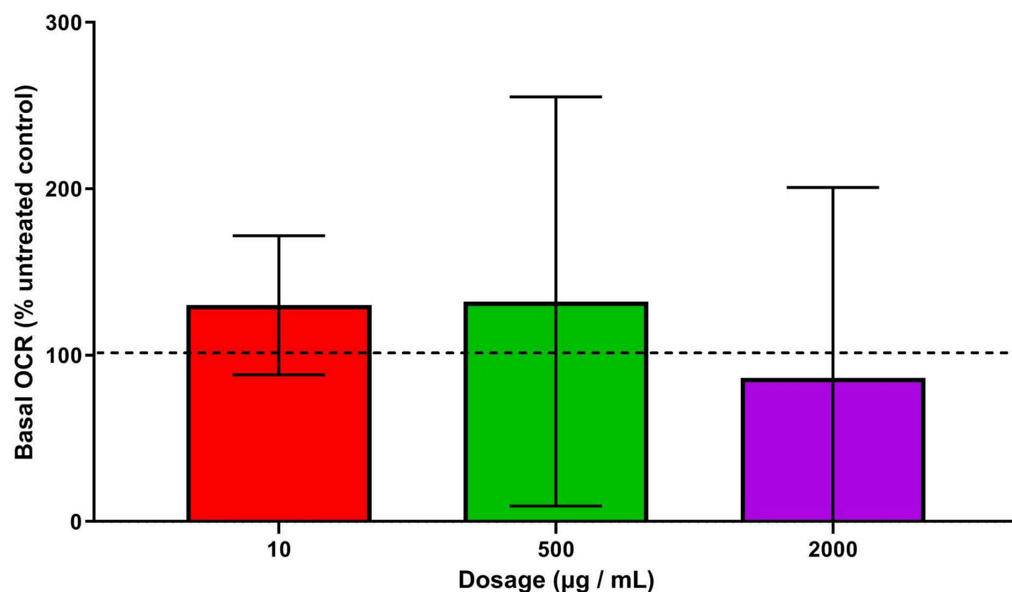


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

Figure 63: Results of SeaHorse MitoStress Assay Showing Changes in OCR of MCF7 Cells after Treatment with Original Liquorice Tea Extract

When comparing the basal rates of respiration as a percentage of the control, there was no significant difference in basal OCR (figure 64).

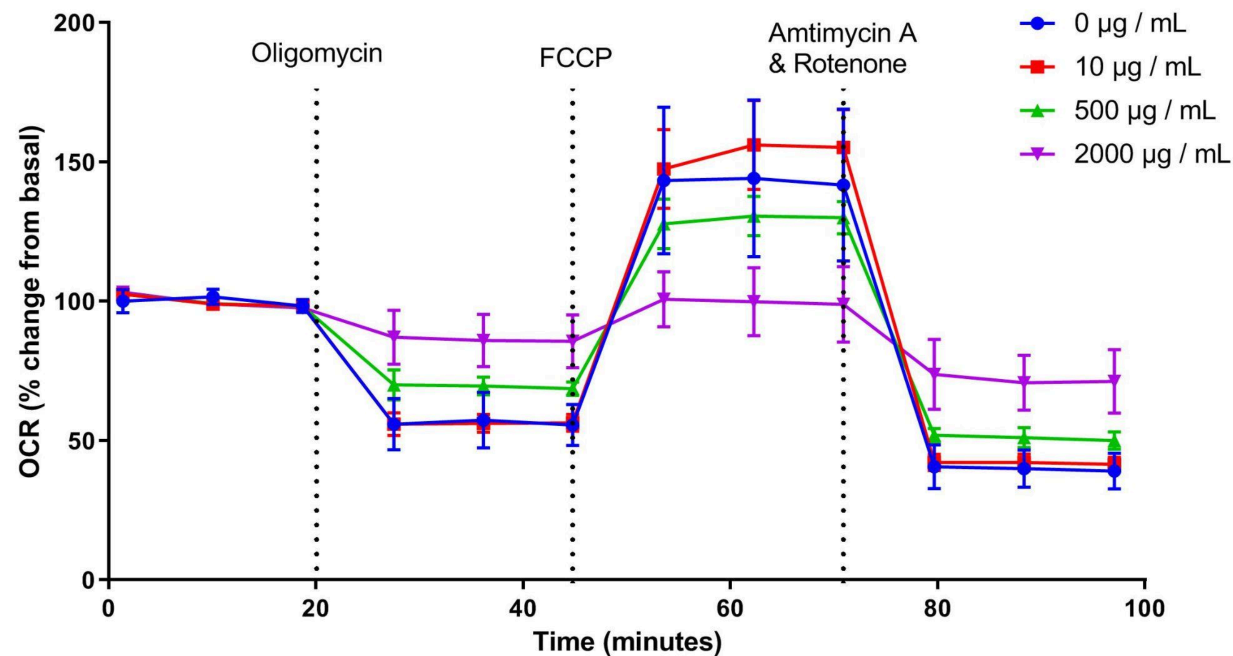


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content in mg / mL measured with Bradford and BCA assays to estimate cell count.

Basal OCR calculated by subtracting OCR after administration of antimycin and rotenone from initial OCR. Data shown as mean average +/- SD compared to untreated control shown as dotted line at 100%. n = 3.

Figure 64: Effect of Original Liquorice Tea Extract 24 Hour Treatment on Basal OCR of MCF7 Cells Shown as Percentage in Relation to Control

When plotted as a percentage change from the initial basal rate, the flattening of the expected curve is even more apparent (figure 65).

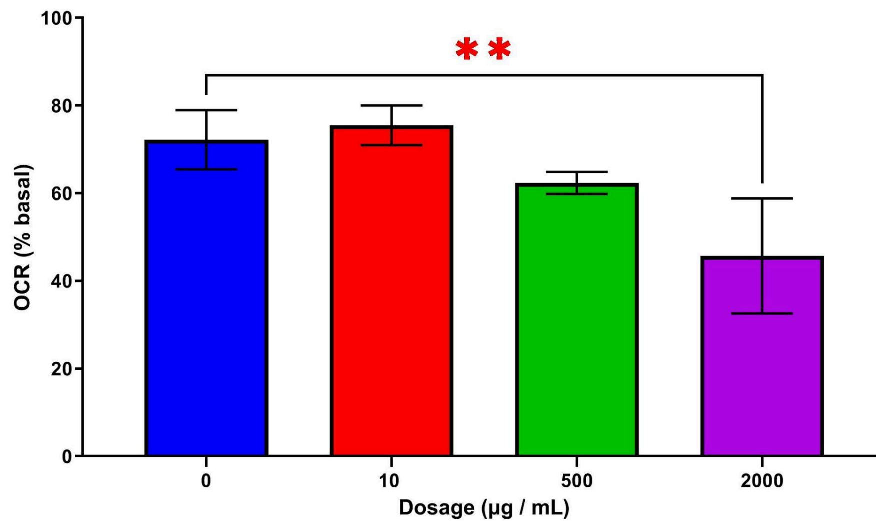


MitoStress assay performed with SeaHorse $XF_{e}24$ Flux Analyzer, normalised using protein content in mg / mL measured with Pearce BCA assays to estimate cell count shown as % of initial OCR of control. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

Figure 65: Results of SeaHorse MitoStress Assay Showing Changes in OCR of MCF7 Cells after Treatment with Original Liquorice Tea Extract as percentage of basal OCR

Calculated as a percentage change from the basal rate and compared to the control, 24 hour treatment with 2000 µg / mL OLTE saw a significant decrease in ATP linked respiration (45.7% compared to 72.24%; $p < 0.01$; figure 66) and proton leak (54.3% compared to 27.76%; $p < 0.01$; figure 67), a 26.54% difference.

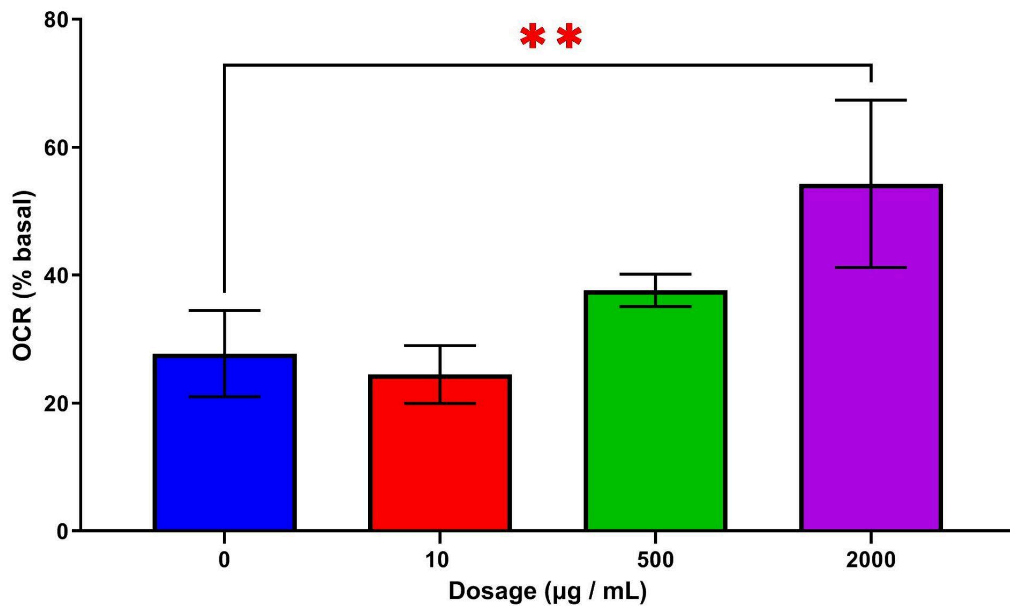


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content measured with Pearce BCA assays to estimate cell count.

ATP related respiration calculated by subtracting OCR after administration of oligomycin from initial OCR and shown as mean average +/- SD. n = 3.

** indicates significance ($p < 0.01$) compared against control analysed using one way ANOVA with Dunnett's multiple comparisons.

Figure 66: Effect of Original Liquorice Tea Extract 24 Hour Treatment on ATP linked OCR of MCF7 Cells



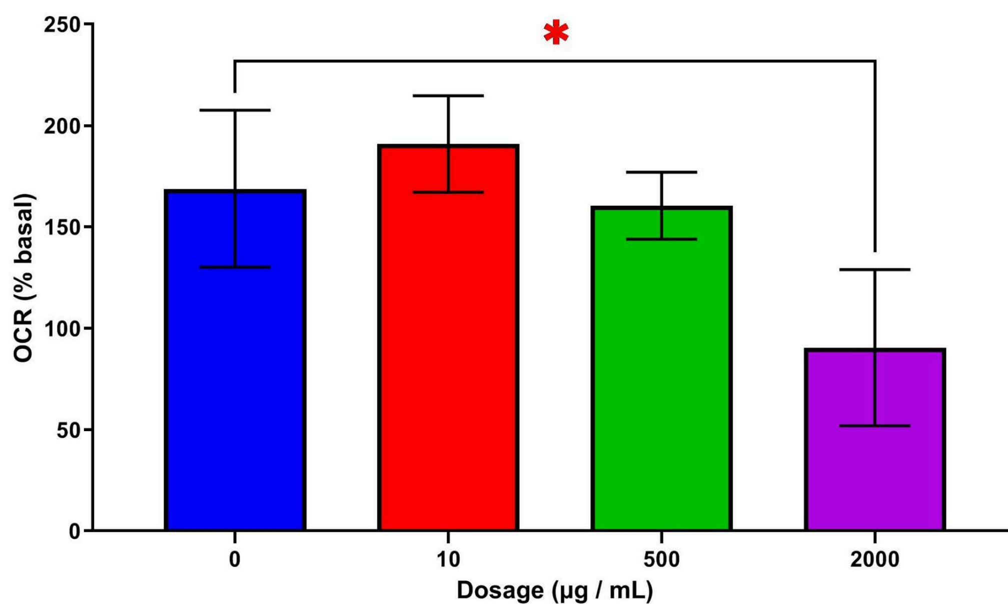
MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content with Pearce BCA assays to estimate cell count.

Proton leak calculated by subtracting OCR after administration of antimycin A & rotenone from OCR after administration of oligomycin and shown as mean average +/- SD. n = 3.

** indicates significance ($p < 0.01$) compared against control analysed using one-way ANOVA with Dunnett's multiple comparisons.

Figure 67: Effect of 24 hour Original Liquorice Tea Extract Treatment on Mitochondrial Proton Leak in MCF7 Cells

Calculated as a percentage change from the basal rate and compared to the control, 24 hour treatment with 2000 µg / mL OLTE showed a 78.42% reduction in maximal respiration compared to control, which as below the basal level (90.41% compared to 168.83%; $p < 0.05$; figure 68), resulting in a spare capacity in the minus figures (-9.59%; $p < 0.05$) (figure 69).

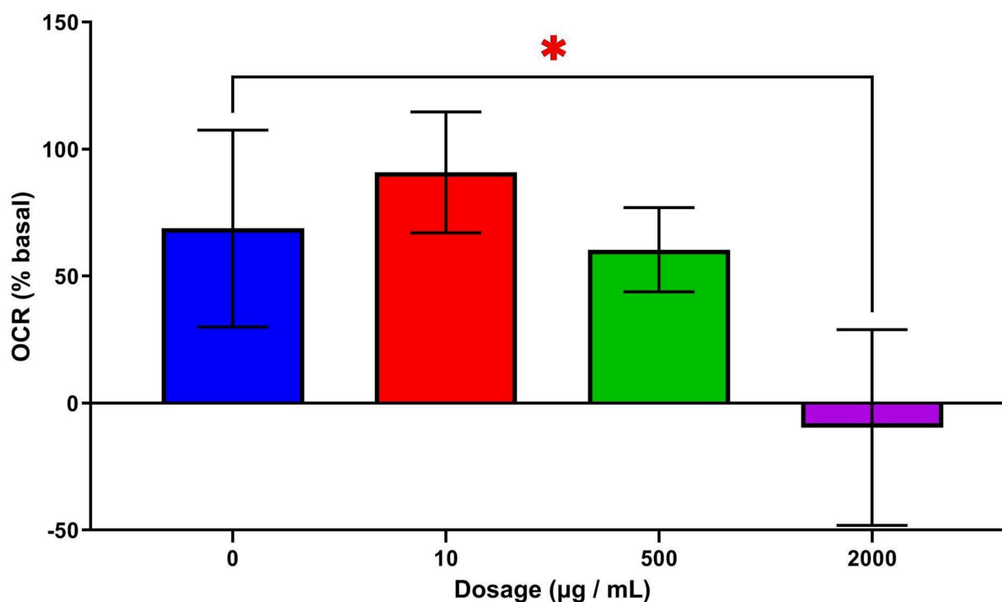


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

Maximal capacity calculated by subtracting OCR after administration of antimycin A and rotenone from OCR after administration of FCCP and shown as mean average +/- SD. n = 3.

* indicates significance ($p < 0.05$) compared against the untreated control analysed using one-way ANOVA with Dunnett's multiple comparisons.

Figure 68: Effect of Original Liquorice Tea Extract 24 hour Treatment on Mitochondrial Maximal Capacity in MCF7 Cells



MitoStress assay performed with SeaHorse XF $_e$ 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

Spare capacity calculated by subtracting initial OCR from OCR administration of FCCP and shown as mean average +/- SD. n = 3.

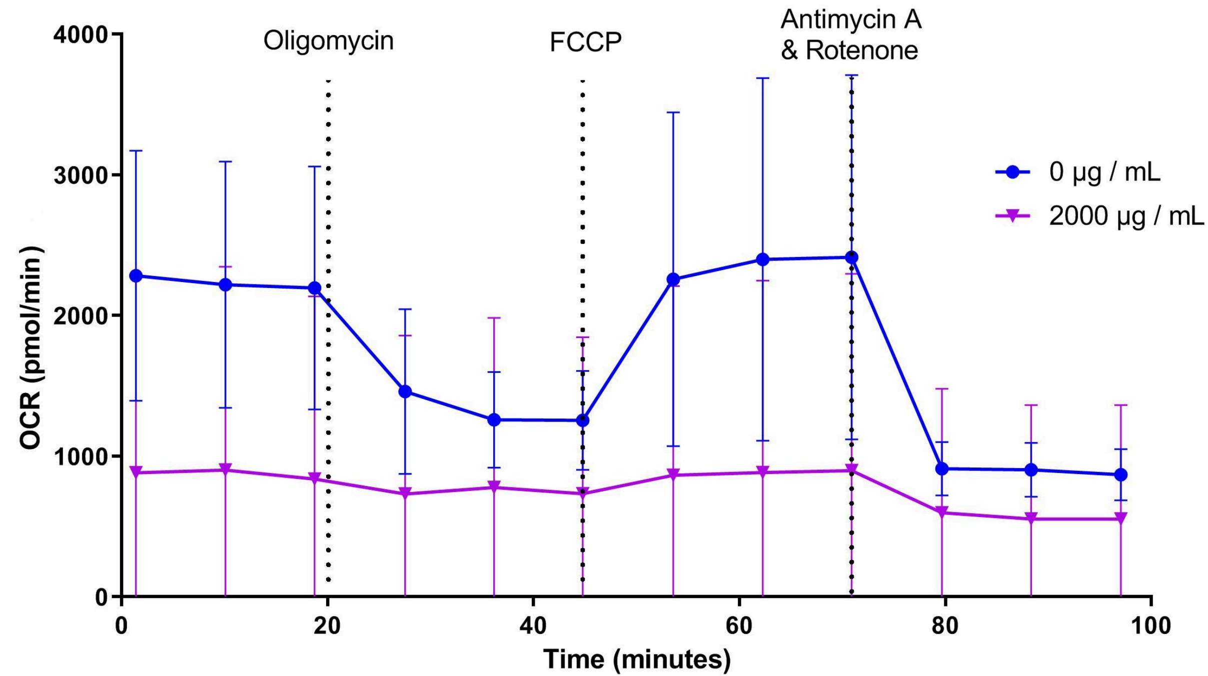
* indicates significance ($p < 0.05$) compared against control analysed using one-way ANOVA with Dunnett's multiple comparions.

Figure 69: Effect of Original Liquorice Tea Extract 24 hour Treatment on Mitochondrial Spare Capacity in MCF7 Cells

12.2. Error Checking the Results of Original Liquorice Tea Extract on Mitochondrial Respiration in MCF7 Cells

These unusual results from the MCF7 Seahorse assay (figures 65-69) prompted an investigation into whether there was a user error or equipment failure. The experiment was repeated using only the high dose and control group at different positions on the plate which confirmed that these findings were genuine and not due to technical error or equipment fault (figure 70).

Although the basal reading starts lower, it is clear that there is almost no effect from the injection of oligomycin and FCCP and only a small change after the injection of antimycin and rotenone. Statistical significance cannot be drawn due to there only being only a single repeat.



MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count as µg / L. n = 1, with 5 technical repeats for control and 13 technical repeats for 2000 µg / mL with positions on plate changed from previous assays.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal maximal and spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

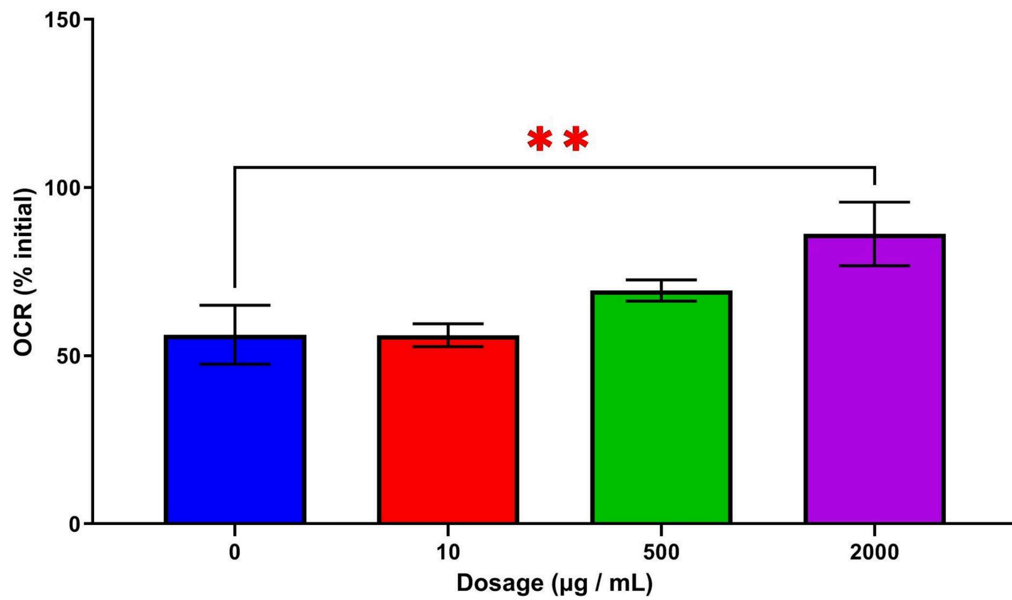
Figure 70: Results of SeaHorse MitoStress Assay Testing For Plate Positioning or Technical Error

12.3. Interpretation of the Results of Original Liquorice Tea Extract on Mitochondrial Respiration in MCF7 Cells as Protection Against Mitochondrial Modulating Drugs

Since there was no previous observations of cytotoxicity or respiratory inhibition at 24 hours with any dosage tested so far (see section 11), and the literature had reports of traditional use for detoxification with some modern mechanistic research supporting this use (see section 4.6), the results were interpreted around an *ad hoc* hypothesis that OLTE may be interfering with the actions of the drugs used in the Seahorse assay.

Reinterpreting the OCR readings as measurements of the effects that the drugs had on respiration, the following patterns were revealed:

Calculated as a percentage change from the basal rate and compared to the control, 24 hour treatment with 2000 µg / mL OLTE saw a 29.94% reduction in the effect of oligomycin to inhibit OCR (86.22% compared to 56.28%; $p < 0.01$; figure 71).



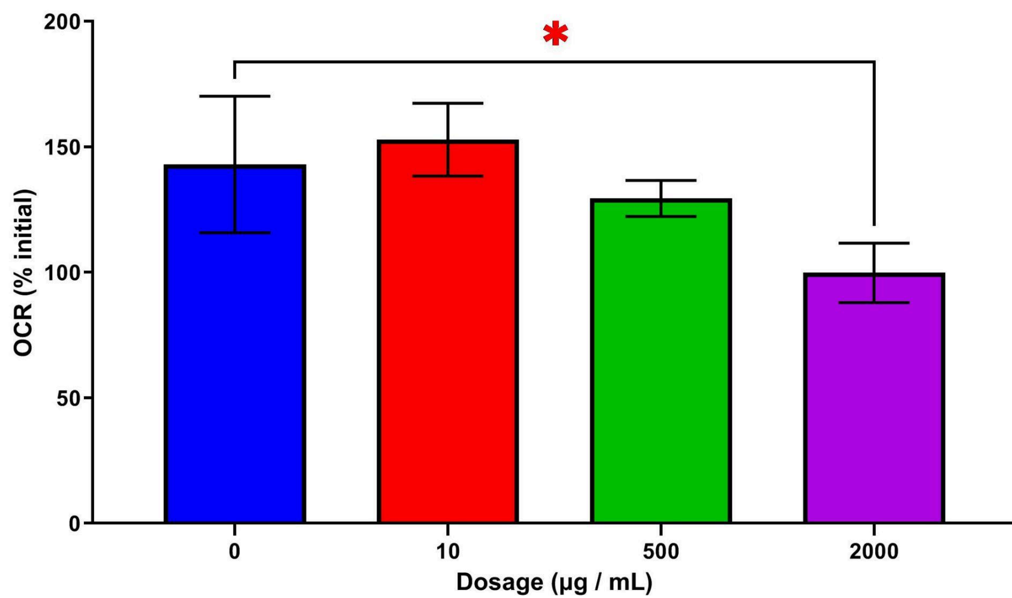
MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

** indicates significance ($p < 0.01$) compared against control analysed using one-way ANOVA with Dunnett's multiple comparisons.

Figure 71: Effect of Original Liquorice Tea Extract 24 Hour Treatment Followed by Oligomycin Administration on OCR of MCF7 Cell

Calculated as a percentage change from the basal rate and compared to the control, 24 hour treatment with 2000 µg / mL OLTE saw a 43.2% reduction in the effect of FCCP to increase OCR, remaining close to the initial basal reading (99.82% compared to 143.02%; $p < 0.01$; figure 72).



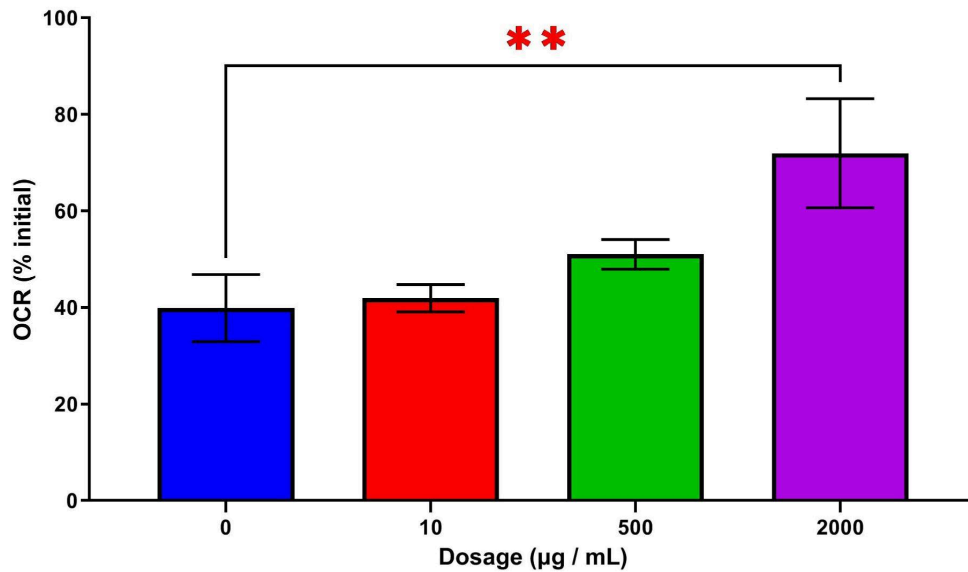
MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

** indicates significance ($p < 0.01$) compared against control analysed using one-way ANOVA with Dunnett's multiple comparisons.

Figure 72: Effect of Original Liquorice Tea Extract 24 Hour Treatment Followed by FCCP Administration on OCR of MCF7 Cells

Calculated as a percentage change from the basal rate and compared to the control, 24 hour treatment with 2000 µg / mL OLTE showed the effect of antimycin and rotenone to reduce OCR was reduced by 32.09% (71.95% compared to 39.86%; $p < 0.01$; figure 73).



MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

** indicates significance ($p < 0.01$) compared against control analysed using one-way ANOVA with Dunnett's multiple comparisons.

Figure 73: Effect of Original Liquorice Tea Extract 24 Hour Treatment Followed by Antimycin A and Rotenone Administration on OCR of MCF7 Cells

12.4. Summary of Results from Original Liquorice Tea on Respiration in MCF7 Cells

The results can be summarised as follows:

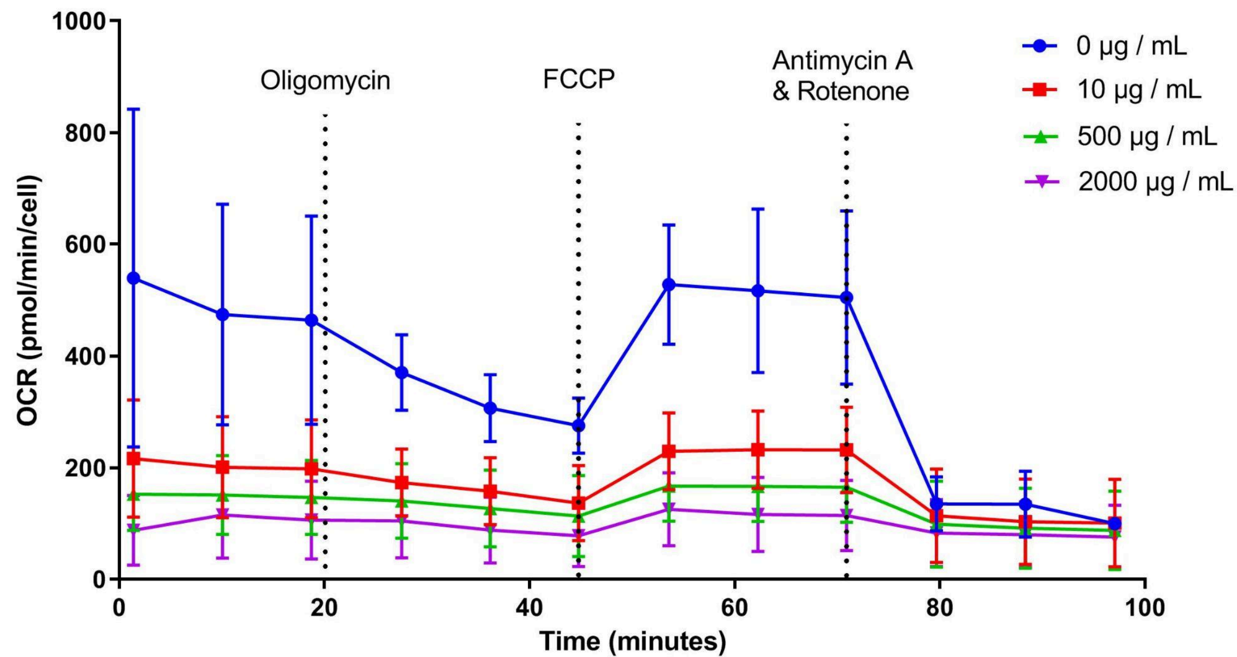
- There was a flattening of the predicted SeaHorse curve in response to all drugs including a minus value of spare capacity leading to a reinterpretation of results as the effect of OLTE on mitochondrial modulating drugs.
- 24 hours treatment with 2000 μg / mL OLTE saw a 29.94% ($p < 0.01$) decrease in the effect of oligomycin in MCF7 cells
- 24 hours treatment with 2000 μg / mL OLTE saw a 43.2% ($p < 0.01$) decrease in the effect of FCCP in MCF7 cells
- 24 hours treatment with 2000 μg / mL OLTE saw a 32.09% ($p < 0.01$) decrease in the effect of antimycin A and rotenone in MCF7 cells

Chapter XIII

13. Results from Respiratory Measurements of Original Liquorice Tea Extract in MCF10A Cells

13.1. Effects of Original Liquorice Tea Extract on Mitochondrial Respiration and its Measurement in MCF10A Cells

After 24 hours treatment with OLTE, there appeared to be a similar flattening of the expected curve in MCF10A cells (figure 74) to that seen in the MCF7 cells (figure 63, section 12.1), but the most noticeable difference was a decrease in the basal OCR.

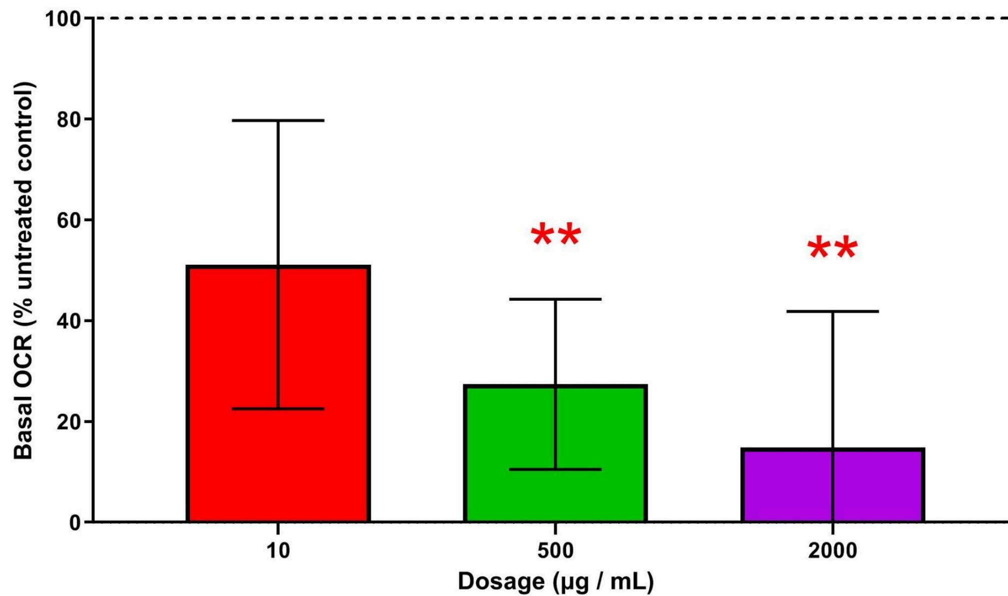


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient to reveal maximal & spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration by inhibiting complexes I & III revealing non-mitochondrial respiration.

Figure 74: Results of SeaHorse MitoStress Assay Showing Changes in OCR of MCF10A Cells After Treatment with Original Liquorice Tea Extract

When comparing the basal rates of respiration as a percentage of the control, 24 hours treatment with OLTE showed a significant reduction in basal OCR among the 500 μg / mL (72.6%; $p < 0.01$) and 2000 μg / mL (85.16%; $p < 0.01$) dose groups (figure 75).



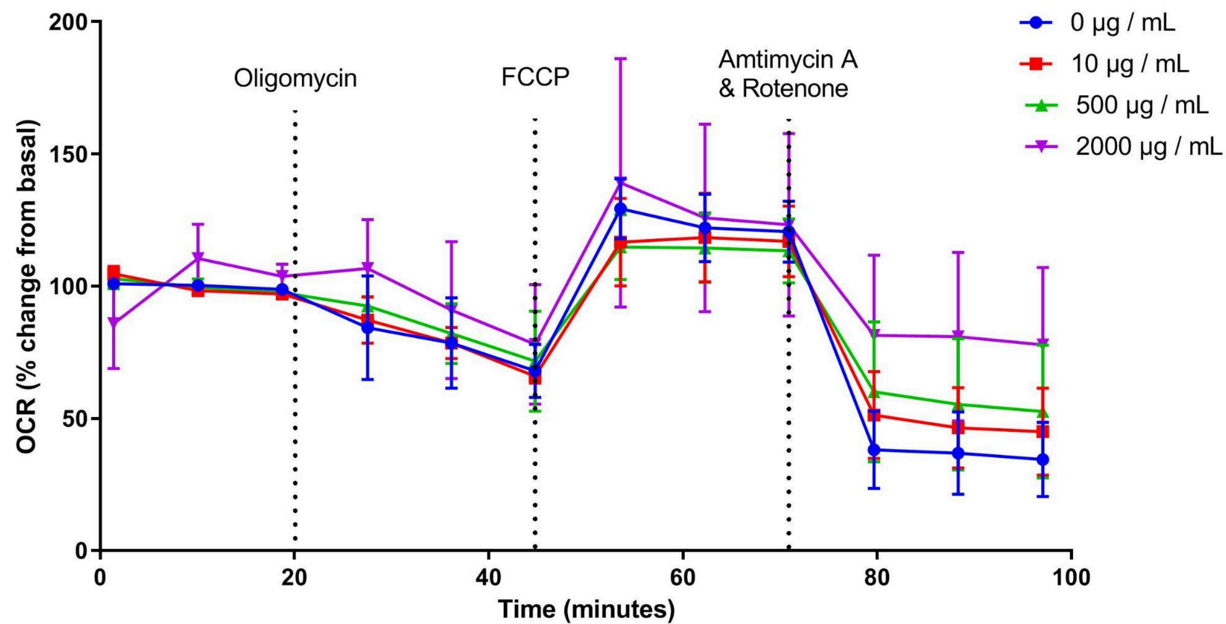
MitoStress assay performed with SeaHorse XF$_e$ 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

Basal OCR calculated by subtracting OCR after administration of antimycin and rotenone from initial OCR. Data shown as mean average +/- SD compared to untreated control shown as dotted line at 100%. n = 3.

** indicates significance ($p < 0.01$) compared against untreated control using one-way ANOVA with Dunnett's multiple comparions.

Figure 75: Effect of Original Liquorice Tea Extract 24 Hour Treatment on Basal OCR of MCF10A Cells Shown as Percentage in Relation to Control

When plotted as a percentage change from the basal OCR to see relative effects of the drugs, it was shown that the drop in basal OCR is the main factor and the relative effects of the drugs is the same (figure 76). It can also be seen that there is some possible degradation of the oligomycin which makes the initial drop in OCR after its administration a gradual decline in all groups, rather than the sharp decrease usually expected in a Seahorse MitoStress test (see section 8.6, figure 53)

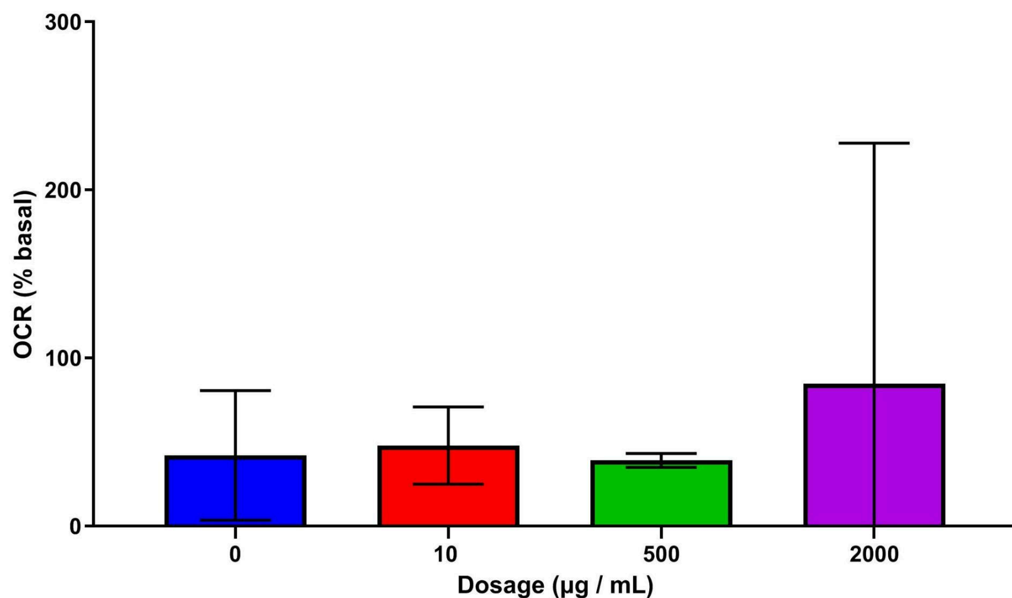


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content in mg / mL measured with Bradford and BCA assays to estimate cell count shown as % of initial OCR of control. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal spare capacity. Change at 70 minutes shows effect of administering Antimycin and Rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

Figure 76: Results of SeaHorse MitoStress Assay Showing Changes in OCR of MCF10A Cells After Treatment with Original Liquorice Tea Extract

This drop in the basal OCR meant that small differences in the modulations of the drugs produced large proportional differences, leading to a high standard deviation in the 2000 µg / mL group (142.98% in the ATP and proton leak calculations made from the administration of oligomycin and 650.74% in the maximal and spare capacity calculations made from the administration of FCCP) resulting a logarithmic scale being required to display the spare capacity and no significance being able to be drawn (figures 77-80).

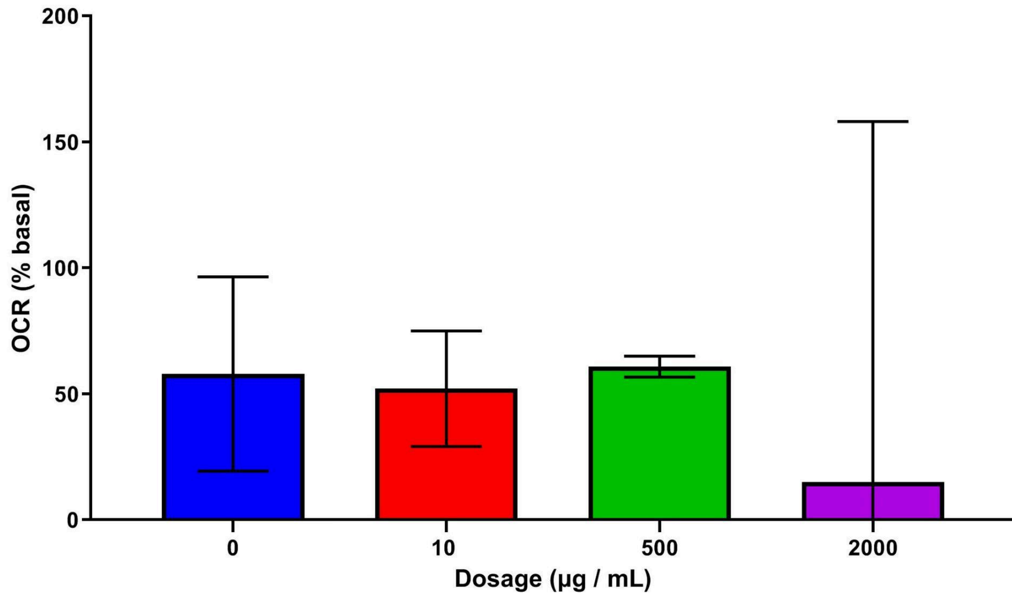


MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content measured with Pearce BCA assays to estimate cell count.

ATP related respiration calculated by subtracting OCR after administration of oligomycin from initial OCR and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 77: Effect of Original Liquorice Tea Extract 24 Hour Treatment on ATP linked OCR of MCF10A Cells

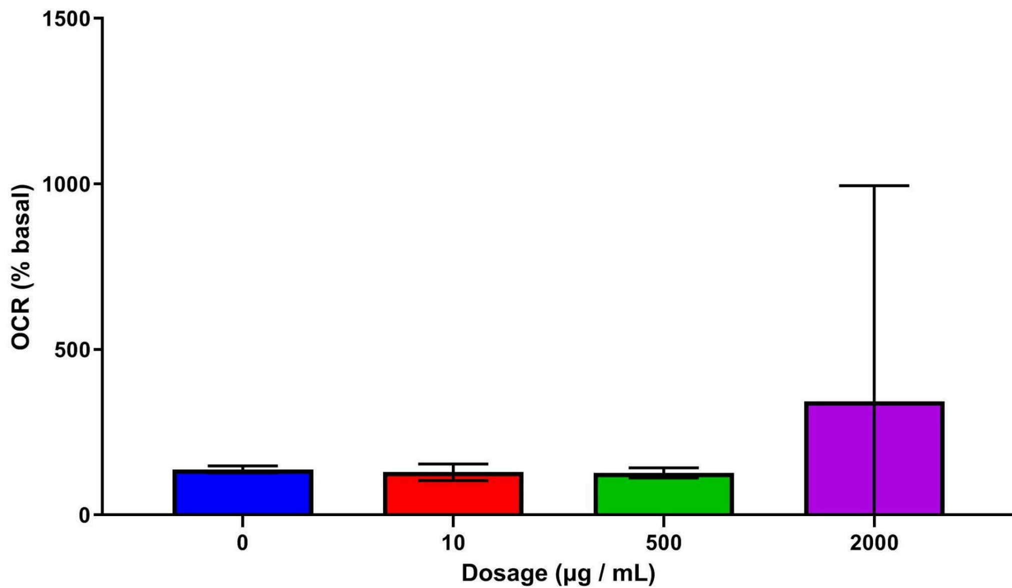


MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content with Pearce BCA assays to estimate cell count.

Proton leak calculated by subtracting OCR after administration of antimycin A & rotenone from OCR after administration of oligomycin and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

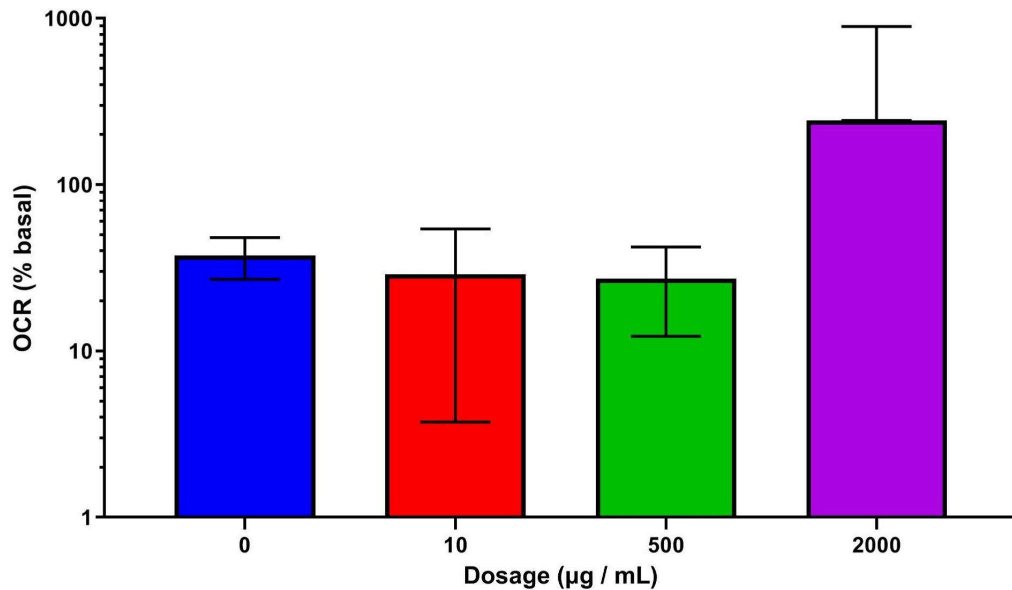
Figure 78: Effect of Original Liquorice Tea Extract 24 Hour Treatment on Mitochondrial Proton Leak in MCF10A Cells



MitoStress assay performed with SeaHorse XF $_e$ 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

Maximal capacity calculated by subtracting OCR after administration of antimycin A and rotenone from OCR after administration of FCCP and shown as mean average +/- SD. n = 3.

Figure 79: Effect of Original Liquorice Tea Extract 24 Hour Treatment on Mitochondrial Maximal Capacity in MCF10A Cells



MitoStress assay performed with SeaHorse XF $_e$ 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

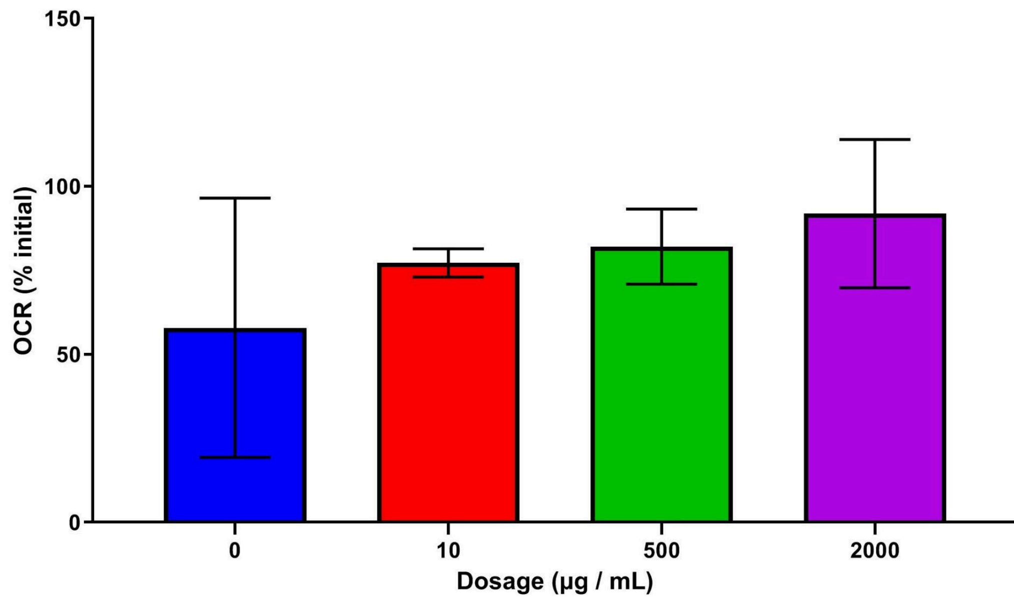
Spare capacity calculated by subtracting initial OCR from OCR administration of FCCP and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 80: Effect of Original Liquorice Tea Extract 24 Hour Treatment on Mitochondrial Spare Capacity in MCF10A Cells

13.2. Interpretation of the Results of Original Liquorice Tea Extract on Mitochondrial Respiration in MCF10A Cells as Protection Against Mitochondrial Modulating Drugs

When analysed as the effects of the Seahorse drugs on OCR, there was no significant difference of any group when treated for 24 hours with OLTE compared to control (figures 81-83).

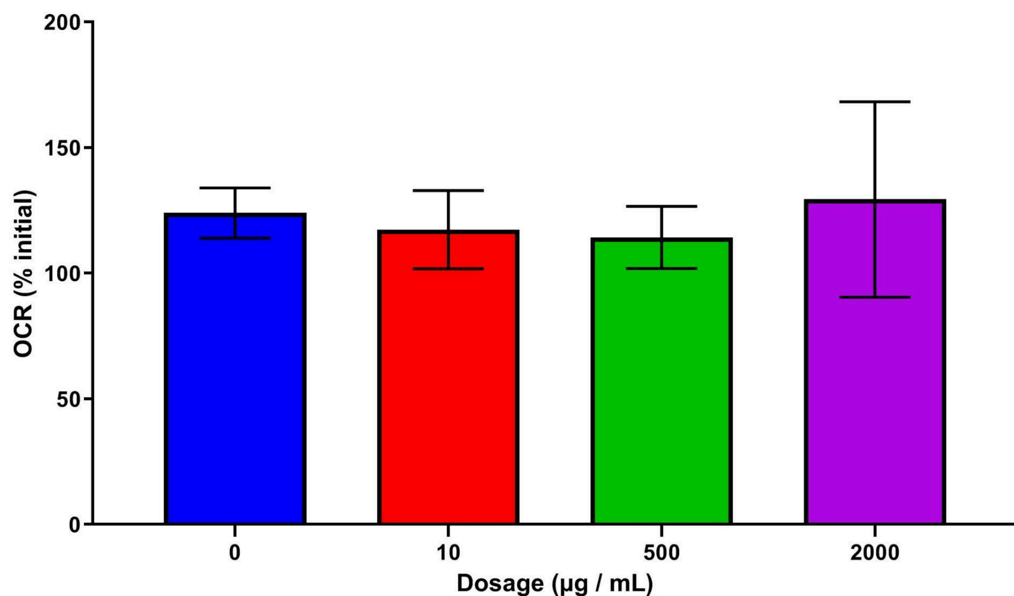


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 81: Effect of Original Liquorice Tea Extract 24 Hour Treatment Followed by Oligomycin on OCR of MCF10A Cells

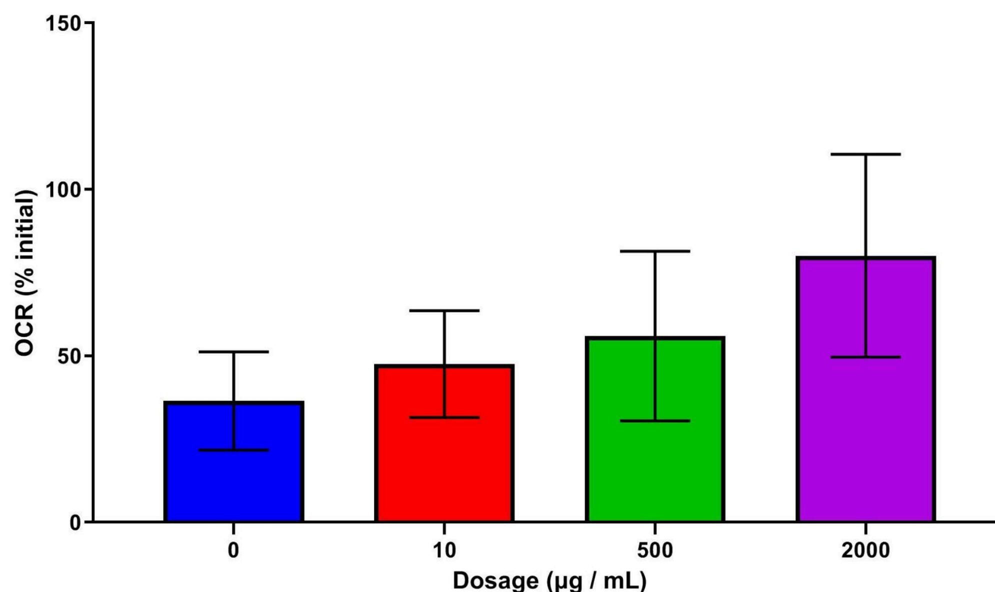


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 82: Effect of Original Liquorice Tea Extract 24 Hour Treatment Followed by FCCP on OCR of MCF10A Cells



MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 83: Effect of Original Liquorice Tea Extract 24 Hour Treatment Followed by Antimycin A and Rotenone on OCR of MCF10A Cells

13.3. Summary of Results from Original Liquorice Tea on Respiration in MCF10A Cells

The results can be summarised as follows:

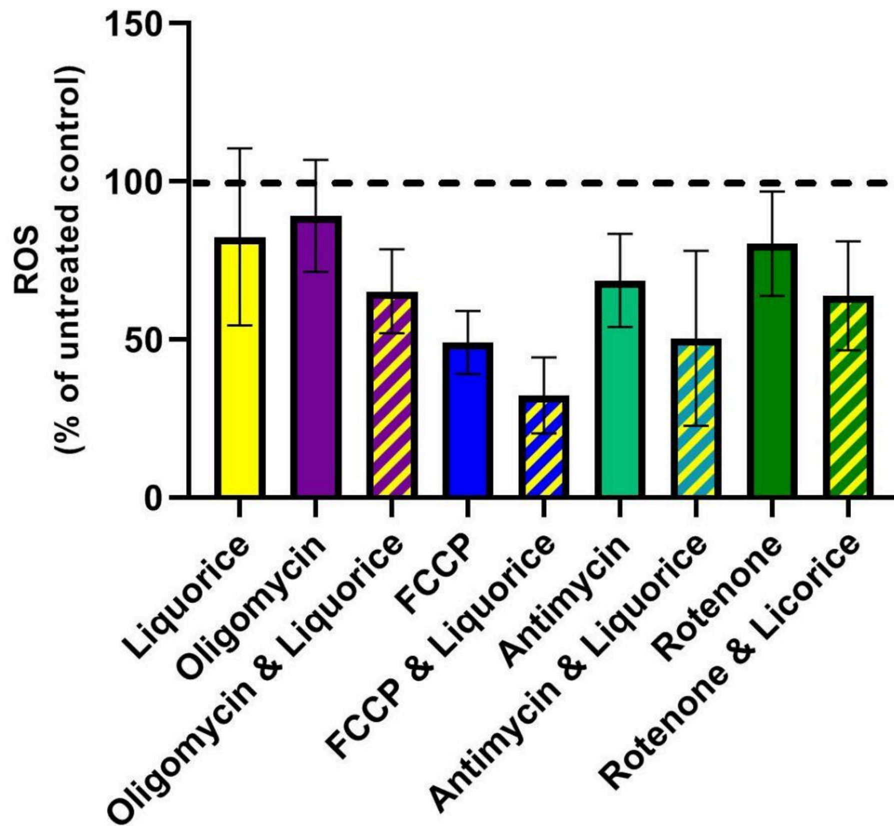
- 24 hours treatment with 2000 µg / mL OLTE saw an 85.16% ($p < 0.01$) decrease in the effect of basal OCR of MCF10A cells
- 24 hours treatment with 500 µg / mL OLTE saw an 72.6% ($p < 0.01$) decrease in the effect of basal OCR of MCF10A cells
- There were no other significant changes in respiration among the MCF10A cells.
- Some apparent differences were due to the decrease in basal respiration producing relatively large changes from the basal rate which led to high standard deviation

Chapter XIV

14. Results from Original Liquorice Tea Extract on Drug Resistance

14.1. Effects of Original Liquorice Tea Extract on Oligomycin, FCCP, Antimycin A and Rotenone Induced Perturbations in ROS in MCF7 Cells

24 hour pretreatment with OLTE followed by 10 minutes treatment with oligomycin, FCCP, antimycin A and rotenone saw no significant differences between the liquorice pretreated group and the group which received no pretreatment in MCF7 cells with any of the drugs (figure 84). This experiment was reasoned to not be a suitable test for drug resistance (see section 20.1.2) and subsequently abandoned in favour of the cisplatin resistance assays.



ROS production measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) represented as % change untreated control drawn as dotted line at 100%.

Liquorice treated cells were exposed to 2000 µg/mL of liquorice for 24 hours before being assayed. Mitochondrial drugs were applied 10 minutes before assay at dosages of:oligomycin 40 µg / mL, FCCP 20 µM, antimycin 40 µM, rotenone 40 µM.

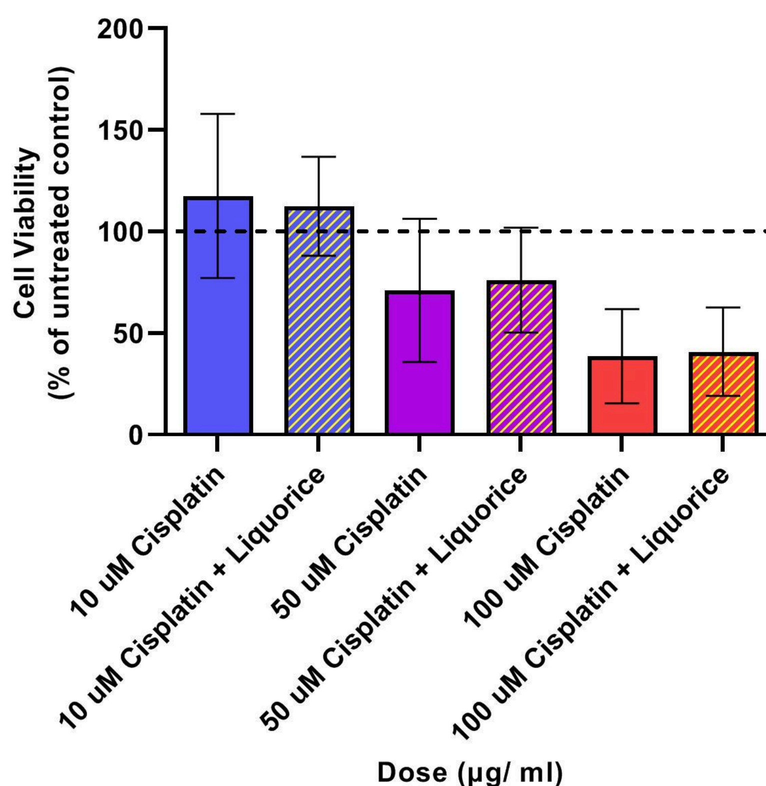
Data shown as mean +/- SD. n=5.

Significance assessed using unpaired t-tests to compare liquorice treated and non-liquorice treated groups for each drug. Additional liquorice only group with no drug included to assess the effect of liquorice alone on ROS.

Figure 84: Effect of Original Liquorice Tea Extract and Mitochondrial Modulating Drugs on ROS in MCF7 cells

14.2. Effects of Original Liquorice Tea Extract on Cisplatin Induced Reduction of Cell Viability in MCF7 Cells

24 hours pretreatment with 2000 µg / mL OLTE, followed by 24 hours treatment with cisplatin found no significant difference in cell viability between the liquorice pretreated and cisplatin only groups in MCF7 cells, both when compared as change against a group that received neither treatment (figure 85), or when comparing a liquorice pretreated group against the cisplatin only group (figure 86).

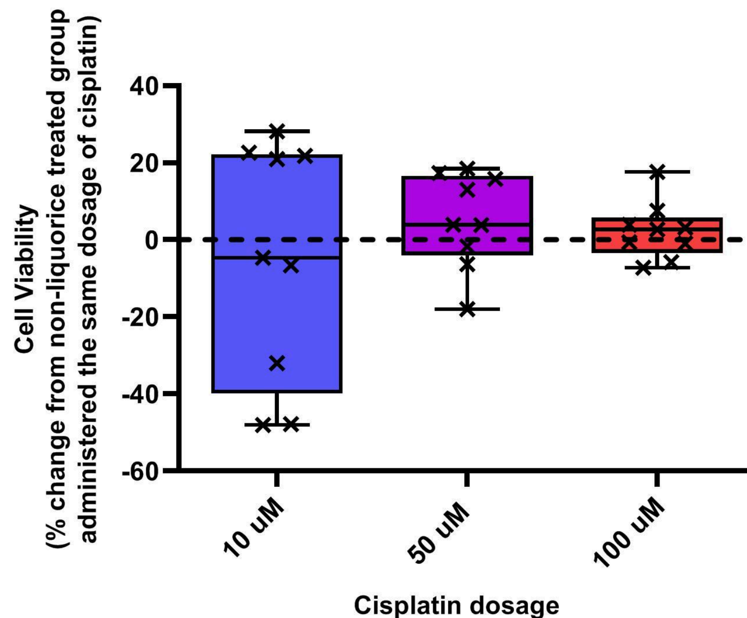


Liquorice groups pretreated with 2000 µg/ml freeze dried liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as the change in cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from the untreated control drawn as dotted line at 100%.

Data shown as shown as mean average +/- SD. n=9.

Significance assessed using unpaired t-test to compare each liquorice pretreated group to its untreated group given the same dosage of cisplatin.

Figure 85: Effect of Original Liquorice Tea Extract Pre-treatment on Cytotoxic Effects of Cisplatin in MCF7 Cells



Liquorice groups treated with 2000 µg/ml liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as difference between the cisplatin only group and the liquorice & cisplatin groups.

Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from group not treated with liquorice and given same dose of cisplatin.

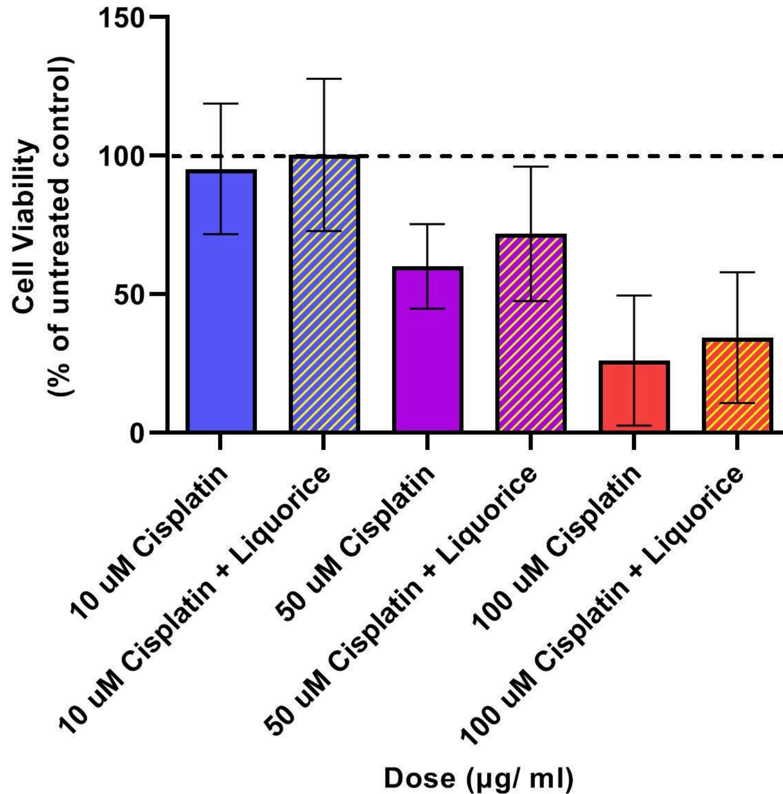
Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed. n=9.

Significance assessed using unpaired t-tests to compare liquorice treated and non-liquorice treated groups for each dosage of cisplatin.

Figure 86: Difference Between Original Liquorice Tea Extract Pre-treatment and No Pre-treatment on the Cytotoxic Effect of Cisplatin in MCF7 Cells

14.3. Effects of Original Liquorice Tea Extract on Cisplatin Induced Reduction of Cell Viability in MCF10 Cells

24 hours pretreatment with 2000 µg / mL OLTE, followed by 24 hours treatment with cisplatin found no significant difference when comparing the groups as changes from a control not treated with either liquorice or cisplatin (figure 87).



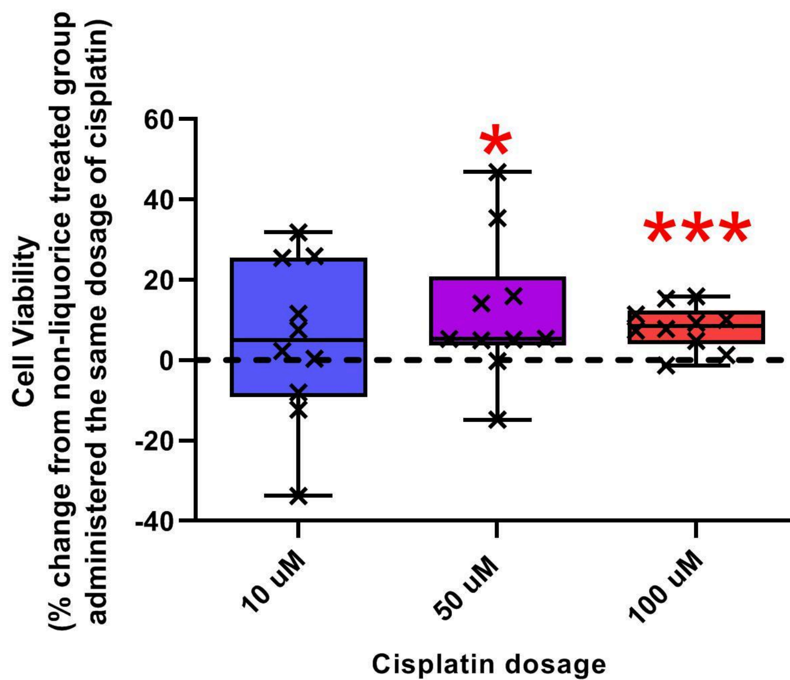
Liquorice groups pretreated with 2000 µg/ml freeze dried liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as the change in cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from the untreated control drawn as dotted line at 100%.

Data shown as shown as mean average +/- SD. n=10.

Significance assessed using unpaired t-test to compare each liquorice pretreated group to its untreated group given the same dosage of cisplatin.

Figure 87: Effect of Original Liquorice Tea Extract Pre-treatment on Cytotoxic Effects of Cisplatin in MCF-10 Cells

When the liquorice pretreated group was comprised directly against the group that received no pretreatment with liquorice, there was an 11.79% ($p < 0.05$) increase in cell viability among the MCF10A cells treated with 50 µM cisplatin, and a 8.186% ($p < 0.001$) increase in cell viability among the MCF10A cells treated with 100 µM cisplatin (figure 88). Comparing the 50 µM and 100 µM groups with an unpaired t-test reveals the effect is not significantly different between groups (3.6%; $p < 0.5465$).



Liquorice groups treated with 2000 µg/ml liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as difference between the cisplatin only group and the liquorice & cisplatin groups. n=9.

Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from group not treated with liquorice and given same dose of cisplatin.

Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed. n=10.

Significance assessed using unpaired t-tests to compare liquorice treated and non-liquorice treated groups for each dosage of cisplatin. * indicates significance ($p<0.05$) and *** indicates significance ($p<0.001$) compared to equivalent dose of cisplatin using an unpaired t-test.

Figure 88: Difference Between Original Liquorice Tea Extract Pre-treatment and No Pre-treatment on the Cytotoxic Effect of Cisplatin in MCF10A Cells

14.4. Summary of Results from Original Liquorice Tea on Drug Resistance

The results can be summarised as follows:

- MCF10A cells pretreated for 24 hours with 2000 μg / mL OLTE followed by 24 hour treatment with 50 μM cisplatin had a 11.79% ($p < 0.05$) increase in cell viability compared to those who did not receive liquorice pretreatment
- MCF10A cells pretreated for 24 hours with 2000 μg / mL OLTE followed by 24 hour treatment with 100 μM cisplatin had a 8.186% ($p < 0.001$) increase in cell viability compared to those who did not receive liquorice pretreatment

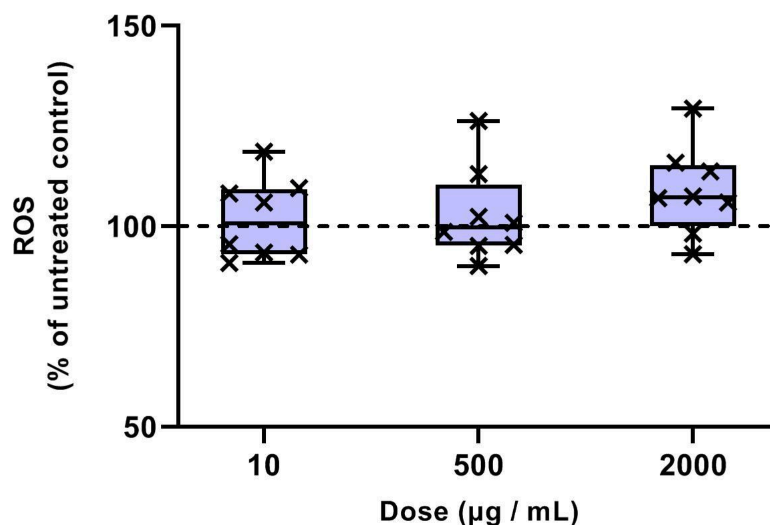
Chapter XV

15. Results from Basic Mitochondrial Assays on Pre-extracted Liquorice Tea Extract

Only the assays which produced significant results were replicated using XLTE. This included the ROS levels of the MCF7 cells after 3 and 72 hours exposure, the Seahorse assay in both cell lines, and the cytoprotective effect of liquorice pre-treatment against cisplatin induced cytotoxicity of MCF10A cells.

15.1. Effects of Pre-extracted Liquorice Tea Extract on ROS in MCF7 Cells

3 hours treatment with XLTE saw no significant difference in ROS in MCF7 cells (figure 89).



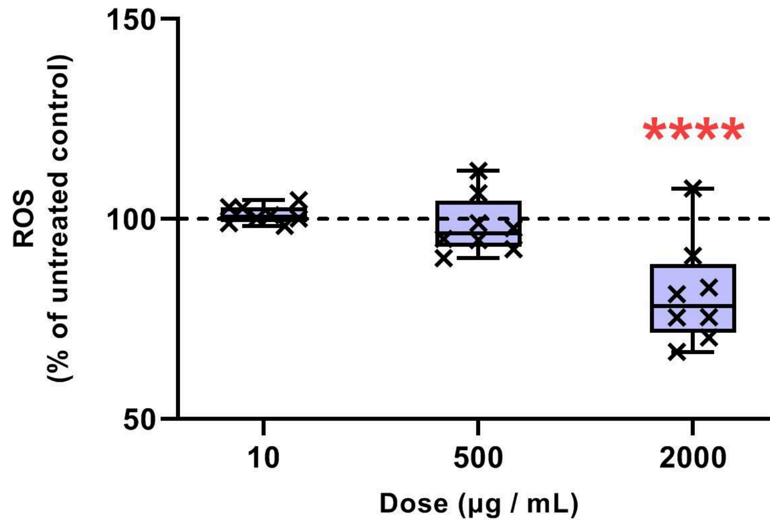
Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from untreated control drawn as dotted line at 100%.

Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed. n=8.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 89: Results of DCFDA Assay Showing Changes in ROS 3 Hours After Treatment with Pre-extracted Liquorice Tea Extract

72 hours pretreatment with XLTE saw an 18.7% ($p < 0.0001$) significant drop in ROS in MCF7 cells (figure 90).



Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from untreated control drawn as dotted line at 100%.

Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed. n=8.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons. **** indicates significance ($p < 0.0001$) compared to an untreated control.

Figure 90: Results of DCFDA Assay Showing Changes in ROS 72 Hours After Treatment with Pre-extracted Liquorice Tea Extract

15.2. Summary of Results from Basic Mitochondrial Assays of Pre-extracted Liquorice Tea

The results can be summarised as follows:

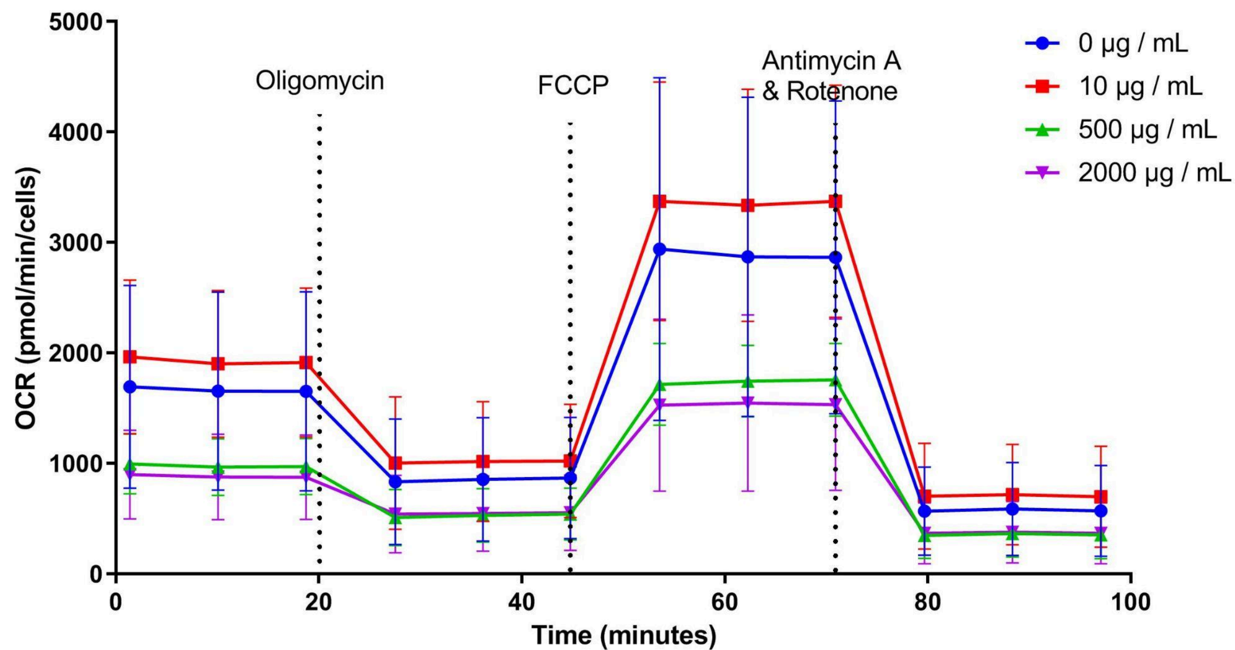
- 72 hours treatment with 2000 µg / mL XLTE saw an 18.7% ($p < 0.0001$) decrease in ROS in MCF7 cells

Chapter XVI

16. Results from Respiratory Measurements of Pre-extracted Liquorice Tea Extract in MCF7 Cells

16.1. Effects of Pre-extracted Liquorice Tea Extract on Mitochondrial Respiration and its Measurement in MCF7 Cells

24 hour treatment of MCF7 cells with XLTE showed no flattening of the expected curve of the Seahorse assay (figure 91).

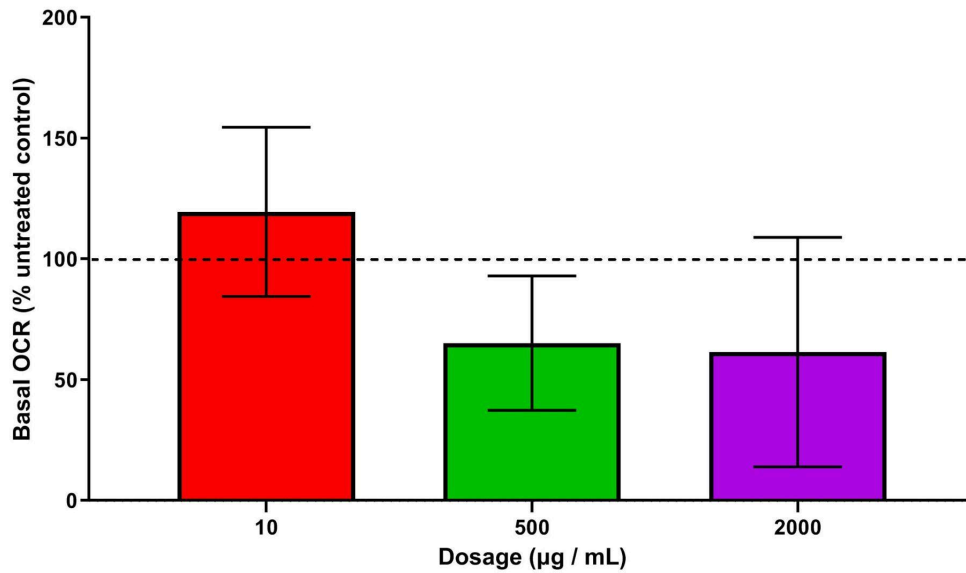


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

Figure 91: Results of SeaHorse MitoStress Assay Showing Changes in OCR of MCF7 Cells after treatment with Pre-extracted Liquorice Tea Extract

When comparing the basal rates of respiration as a percentage of the control, there was no significant difference in basal OCR (figure 92).



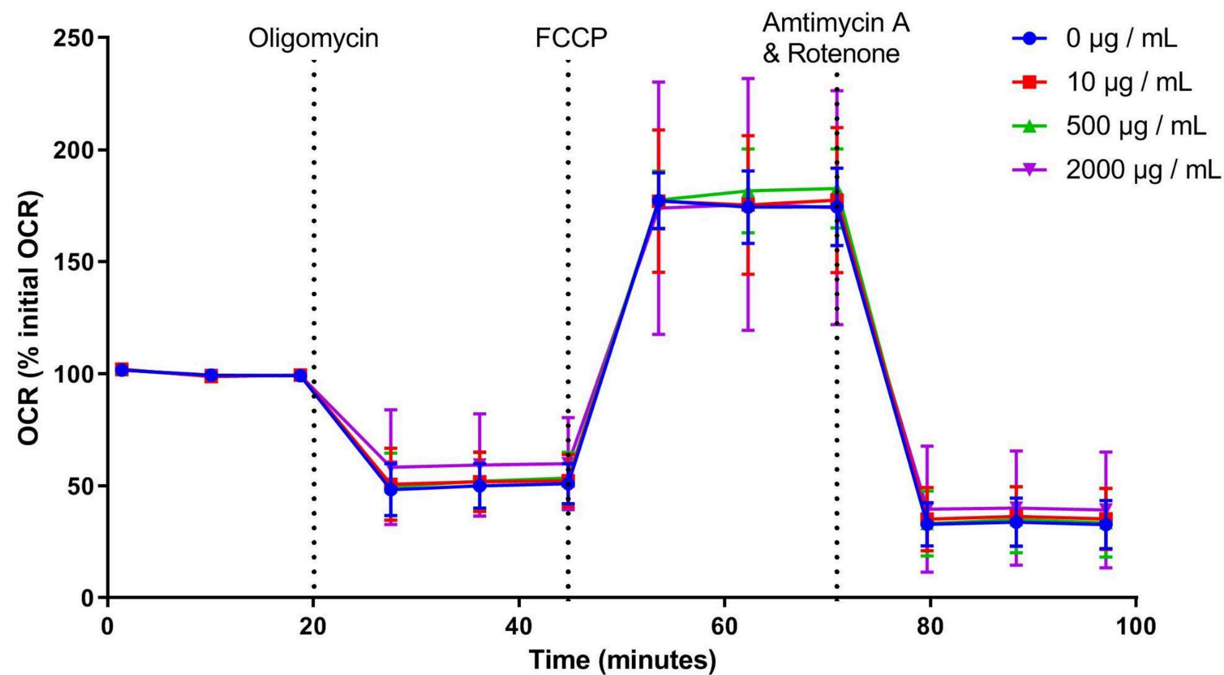
MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content in mg / mL measured with Bradford and BCA assays to estimate cell count.

Basal OCR calculated by subtracting OCR after administration of antimycin and rotenone from initial OCR. Data shown as mean average +/- SD compared to untreated control shown as dotted line at 100%. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 92: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment on Basal OCR of MCF7 Cells Shown as Percentage in Relation to Control

When plotted as a percentage change from the initial basal rate, it is apparent that there is no significant proportional difference between the groups treated for 24 hours with XLTE and the control (figure 93).

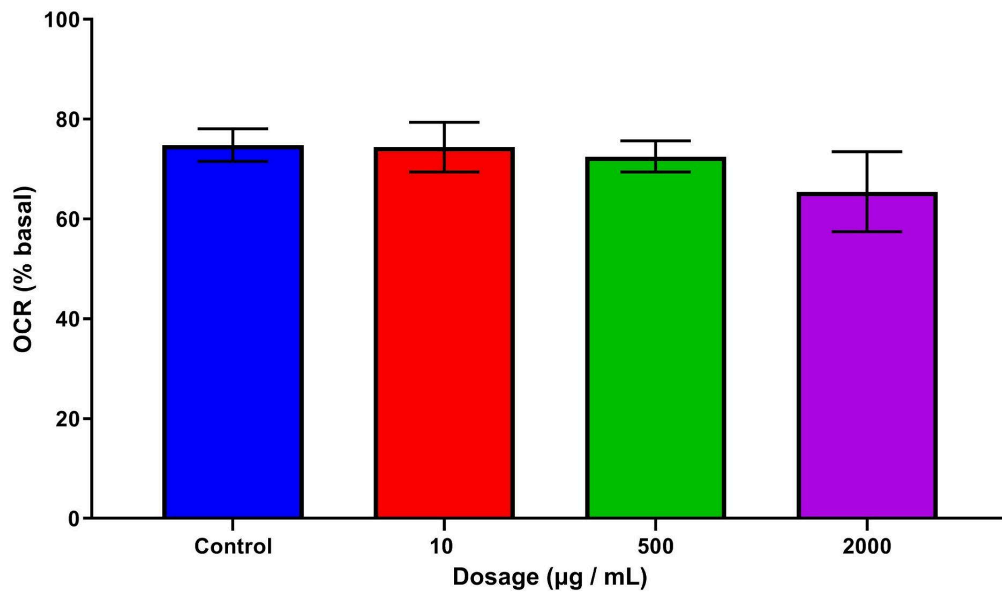


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content in mg / mL measured with Pearce BCA assays to estimate cell count shown as % of initial OCR of control. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

Figure 93: Results of SeaHorse MitoStress Assay Showing Changes in OCR of MCF7 Cells after treatment with Pre-extracted Liquorice Tea Extract as percentage of basal OCR

There were also no significant differences in any of the reported measures used for the Seahorse MitoStress assay (figures 94-97).

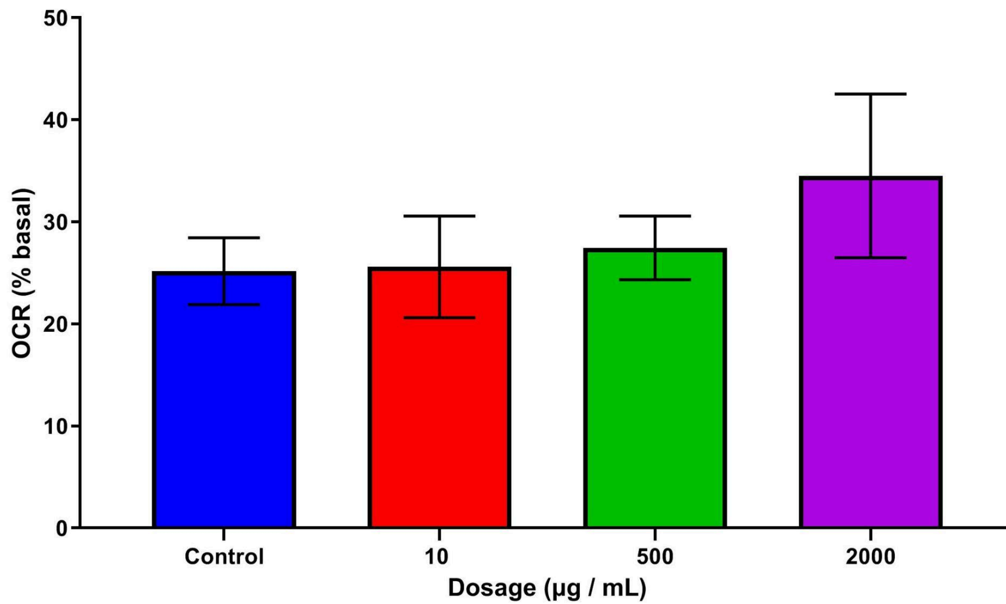


MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content measured with Pearce BCA assays to estimate cell count.

ATP related respiration calculated by subtracting OCR after administration of oligomycin from initial OCR and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 94: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment on ATP Linked OCR of MCF7 Cells

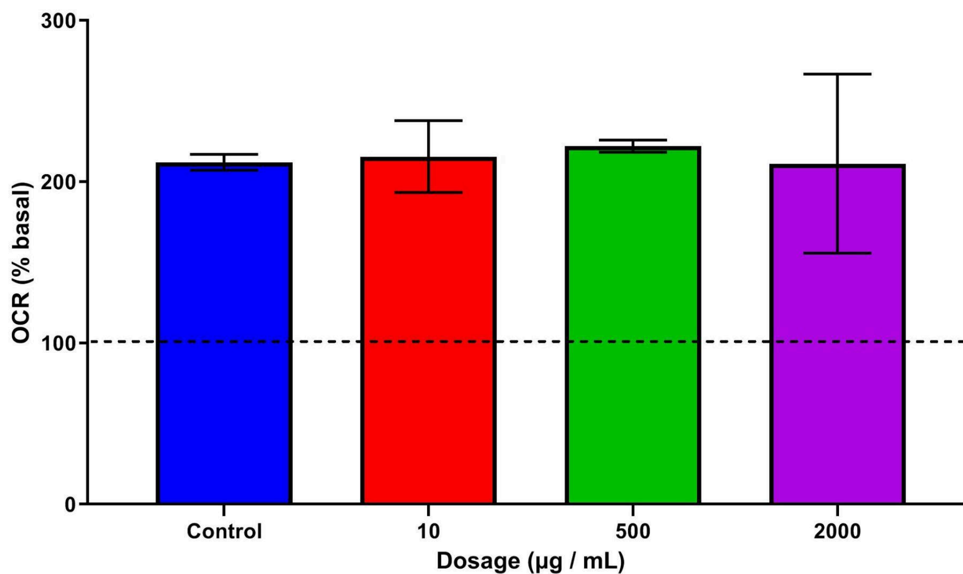


MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content with Pearce BCA assays to estimate cell count.

Proton leak calculated by subtracting OCR after administration of antimycin A & rotenone from OCR after administration of oligomycin and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 95: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment on Mitochondrial Proton Leak in MCF7 Cells

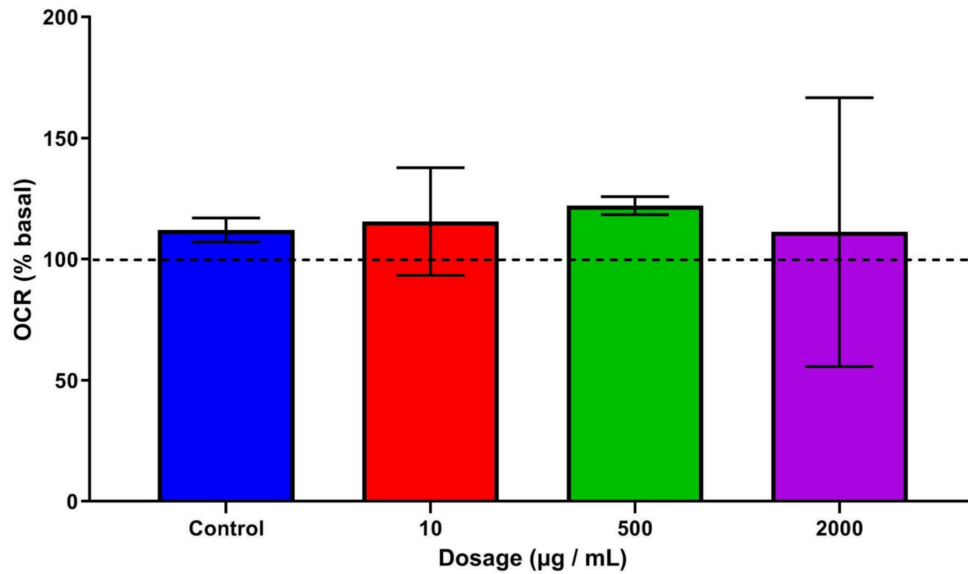


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

Maximal capacity calculated by subtracting OCR after administration of antimycin A and rotenone from OCR after administration of FCCP and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 96: Effect of Pre-extracted Liquorice Tea Extract 24 hour treatment on Mitochondrial Maximal Capacity in MCF7 Cells



MitoStress assay performed with SeaHorse XF $_e$ 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count.

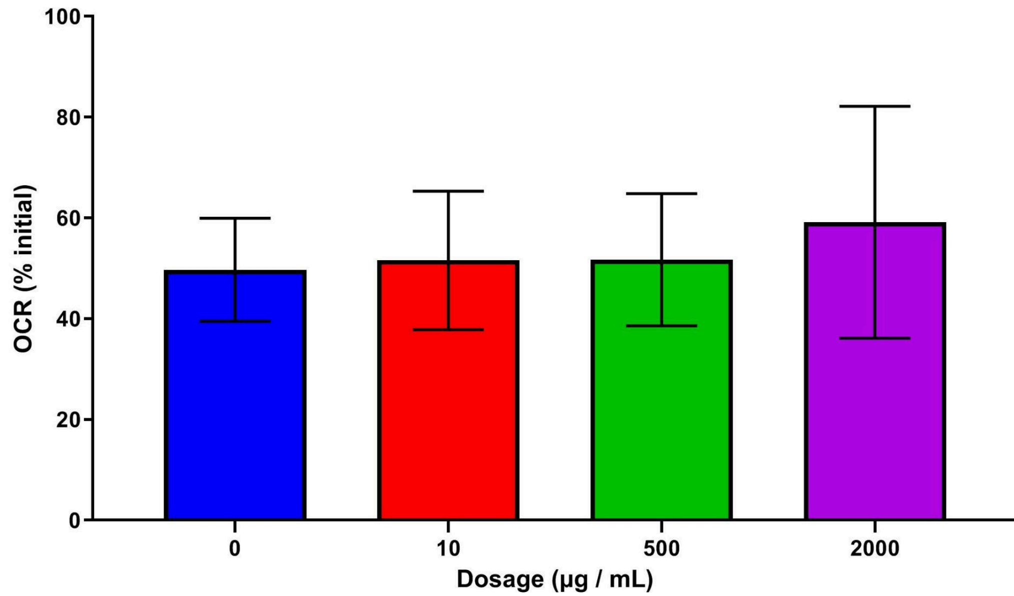
Spare capacity calculated by subtracting initial OCR from OCR administration of FCCP and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 97: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment on Mitochondrial Spare Capacity in MCF7 Cells

16.2. Interpretation of the Results of Pre-extracted Liquorice Tea Extract on Mitochondrial Respiration in MCF7 Cells as Protection Against Mitochondrial Modulating Drugs

24 hour treatment of MCF7 cells with XLTE showed no significant difference in terms of the effect the extract was having on the mitochondrial modulating drugs used in the Seahorse MitoStress assay (figures 98-100).

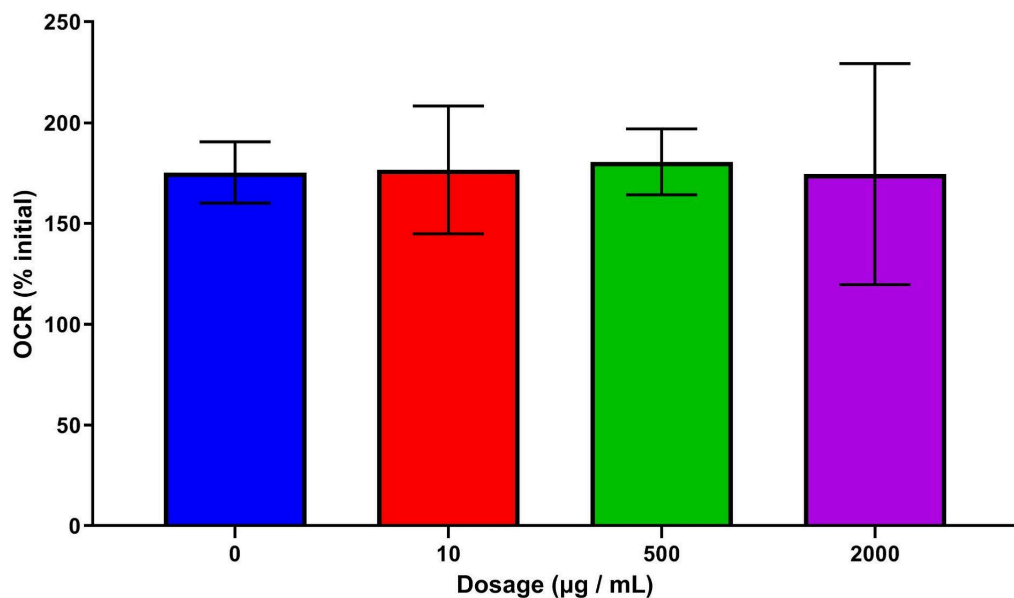


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 98: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment followed by Oligomycin on OCR of MCF7 Cells

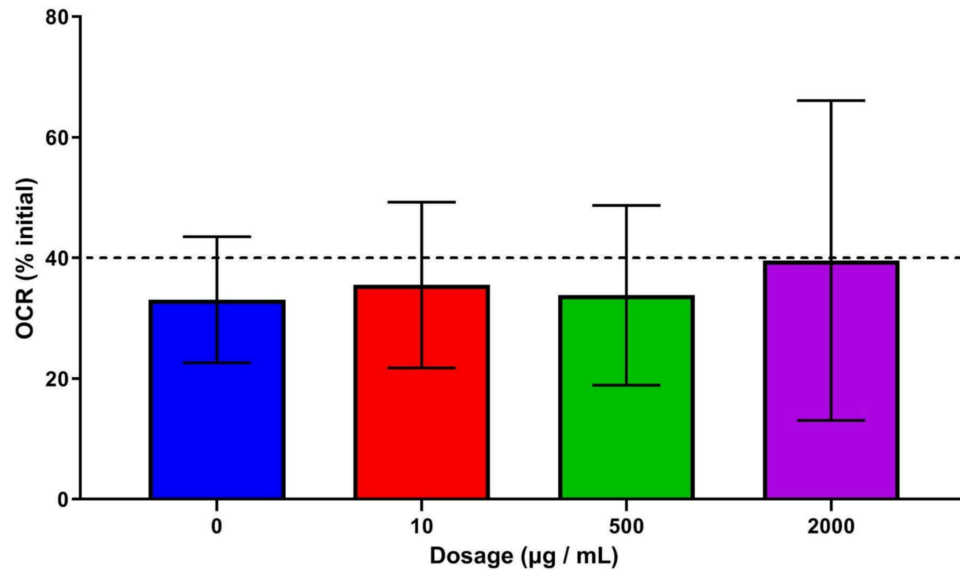


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 99: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment Followed by FCCP on OCR of MCF7 Cells



MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 100: Effect of Pre-extracted Liquorice Tea Extract 24 Hour Treatment Followed by Antimycin A and Rotenone on OCR of MCF7 Cells

16.3. Summary of Results from Respiratory Measurements of Pre-extracted Liquorice Tea Extract in MCF7 Cells

The results can be summarised as follows:

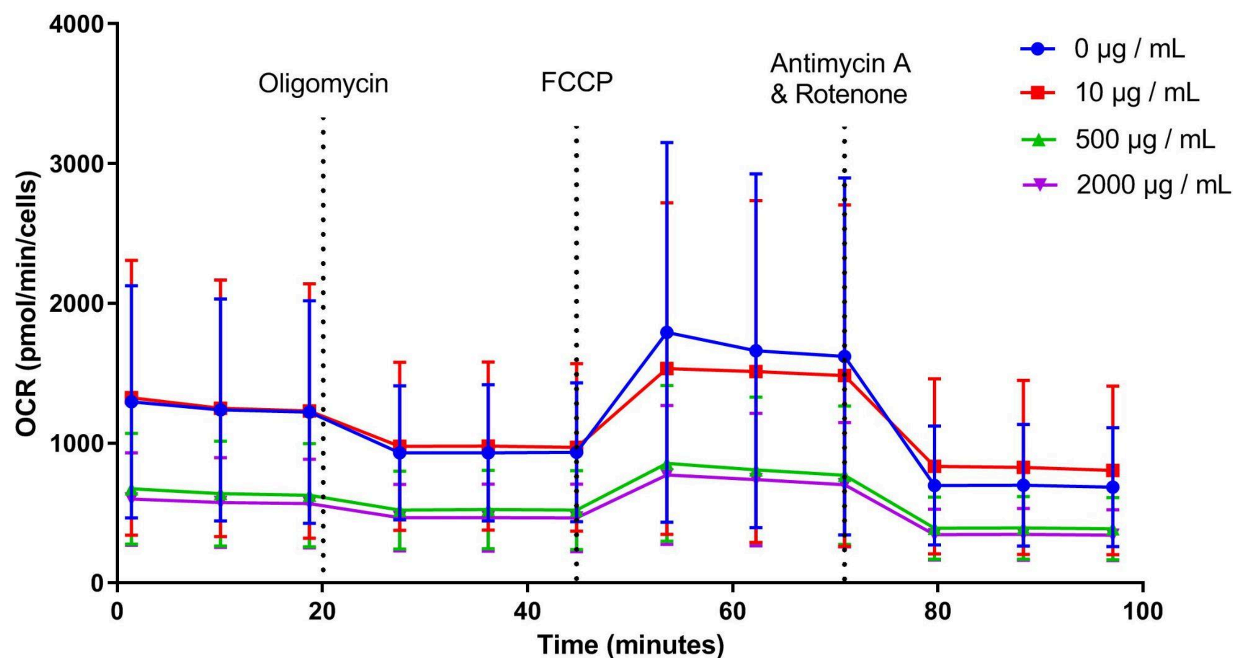
- 24 hours treatment with XLTE showed no significant effects on respiratory measurements in MCF7 cells.

Chapter XVII

17. Results from Respiratory Measurements of Pre-extracted Liquorice Tea Extract in MCF10A Cells

17.1. Effects of Pre-extracted Liquorice Tea Extract on Mitochondrial Respiration and its Measurement in MCF10A Cells

24 hour treatment of MCF10A cells with XLTE showed a reduction in basal OCR in all dosage groups (figure 101)

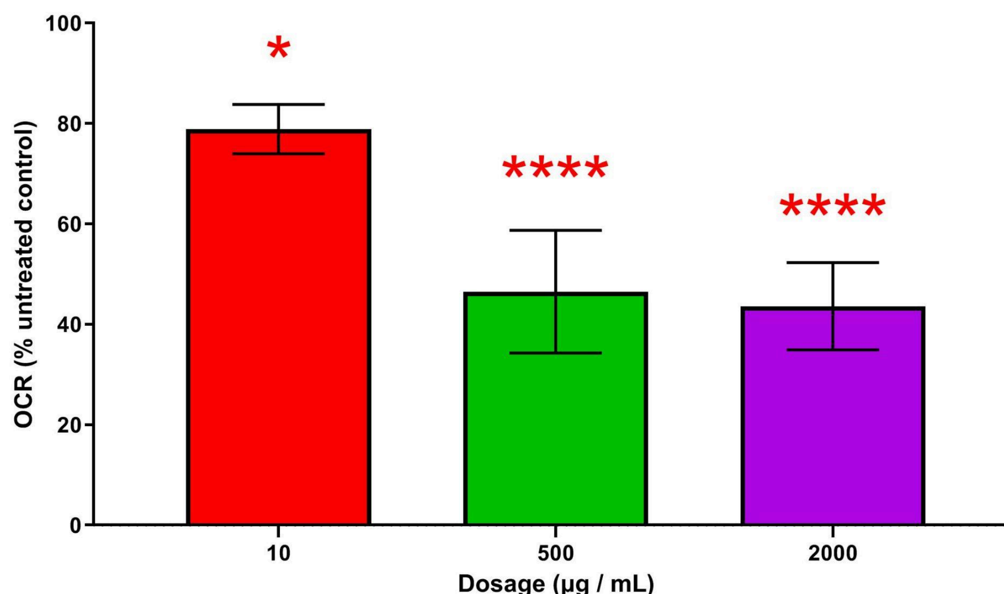


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content in measured with Pearce BCA assay to estimate cell count. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient to reveal maximal & spare capacity. Change at 70 minutes shows effect of administering antimycin A and rotenone to shut down mitochondrial respiration by inhibiting complexes I & III revealing non-mitochondrial respiration.

Figure 101: Results of Seahorse MitoStress Assay Showing Changes in OCR of MCF10A cells After Treatment with Pre-extracted Liquorice Tea Extract

24 hour treatment of MCF10A cells with XLTE saw a reduction in basal OCR compared as a percentage against the control (figure 102). At 10 µg / mL the basal OCR was reduced by 21.12% ($p < 0.05$); at 500 µg / mL the basal OCR was reduced by 53.52% ($p < 0.0001$) and at 2000 µg / mL the basal OCR was reduced by 56.41% ($p < 0.0001$).



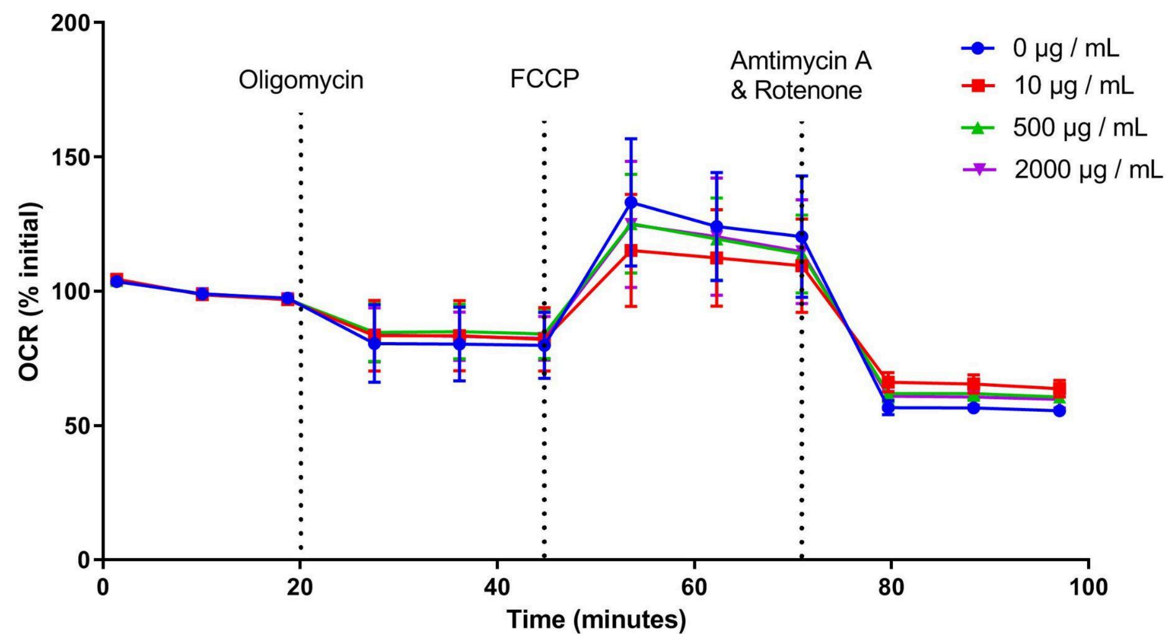
MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content in mg / mL measured with Bradford and BCA assays to estimate cell count.

Basal OCR calculated by subtracting OCR after administration of antimycin and rotenone from initial OCR. Data shown as mean average +/- SD compared to untreated control shown as dotted line at 100%. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.* indicates significance ($p < 0.05$) and **** indicates significance ($p < 0.0001$) compared against untreated control.

Figure 102: Effect of Pre-extracted Liquorice Tea 24 Hour Treatment on Basal OCR of MCF10A Cells Shown as Percentage in Relation to Control

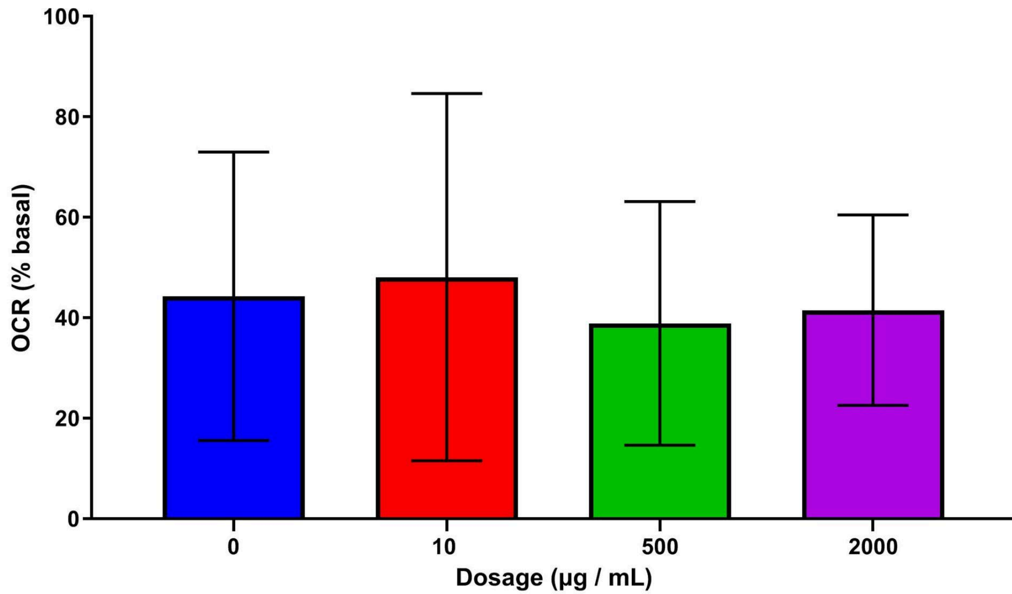
No significant effects were seen in any of the other respiratory measurements (figures 103 - 110).



MitoStress assay performed with SeaHorse XF_e24 Flux Analyzer, normalised using protein content in mg / mL measured with Bradford and BCA assays to estimate cell count shown as % of initial OCR. Points shown as mean average +/- SD. n = 3.

Change at 20 minutes shows effect of administering oligomycin to inhibit ATP synthase. Change at 45 minutes shows effect of administering FCCP disrupt proton gradient and reveal spare capacity. Change at 70 minutes shows effect of administering Antimycin and Rotenone to shut down mitochondrial respiration revealing non-mitochondrial respiration.

Figure 103: Results of Seahorse MitoStress Assay Showing Changes in OCR of MCF10A cells After Treatment with Pre-extracted Liquorice Tea Extract as a Percentage of Basal OCR

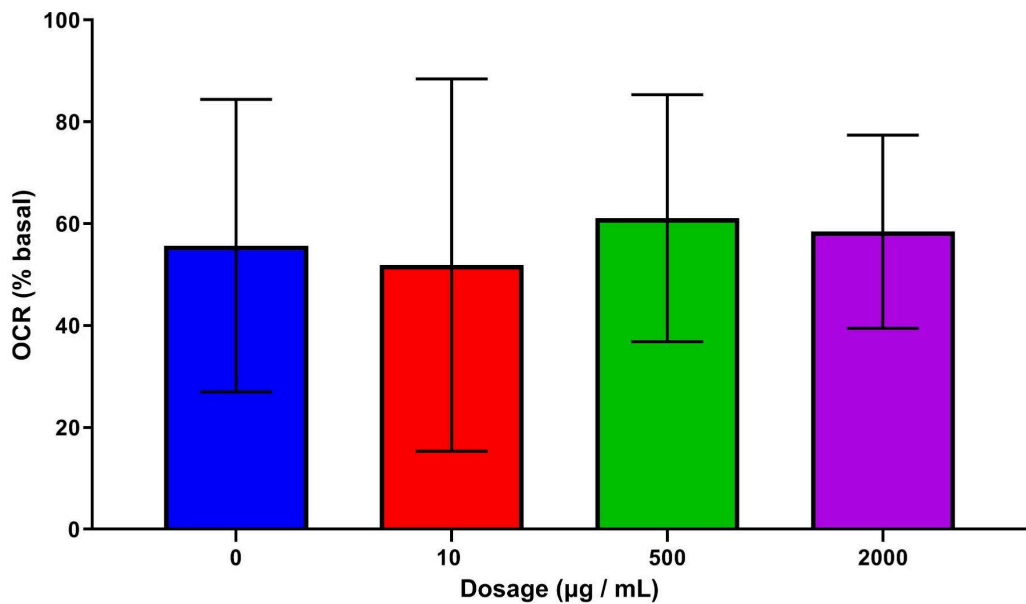


MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content measured with Pearce BCA assays to estimate cell count.

ATP related respiration calculated by subtracting OCR after administration of oligomycin from initial OCR and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 104: Effect of Pre-extracted Liquorice Tea 24 Hour Treatment on ATP Linked OCR of MCF10A Cells



MitoStress assay performed with SeaHorse XF $_e$24 Flux Analyzer, normalised using protein content with Pearce BCA assays to estimate cell count.

Proton leak calculated by subtracting OCR after administration of antimycin A & rotenone from OCR after administration of oligomycin and shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 105: Effect of Pre-extracted Liquorice Tea 24 Hour Treatment on Mitochondrial Proton Leak in MCF10A Cells

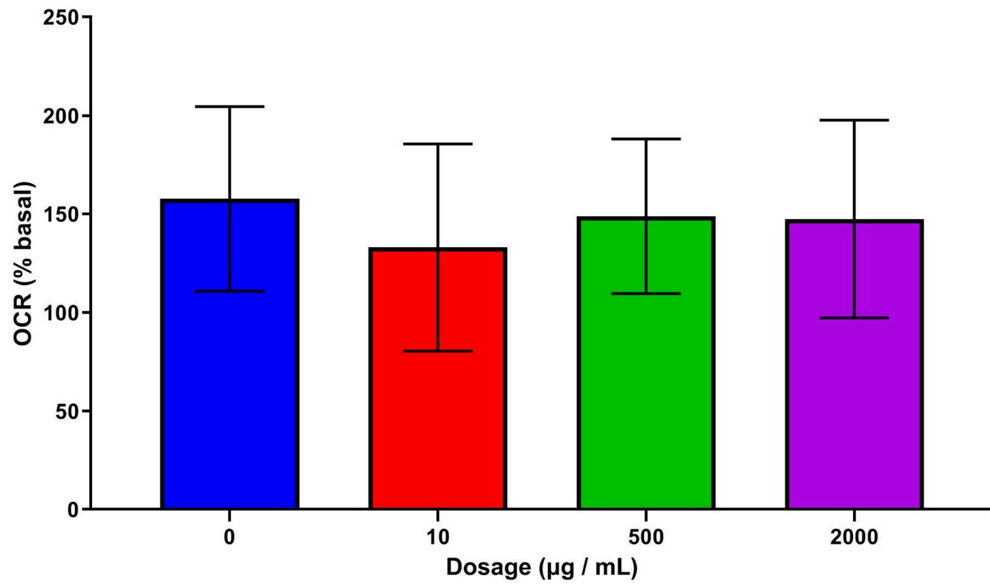


Figure 106: Effect of Liquorice Tea 24 Hour Treatment on Mitochondrial Maximal Capacity in MCF10A Cells

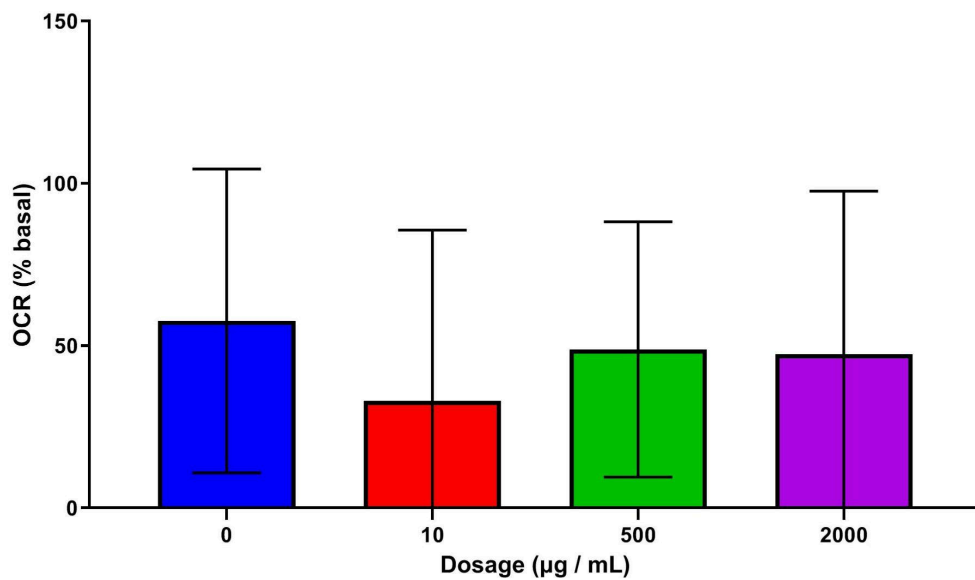
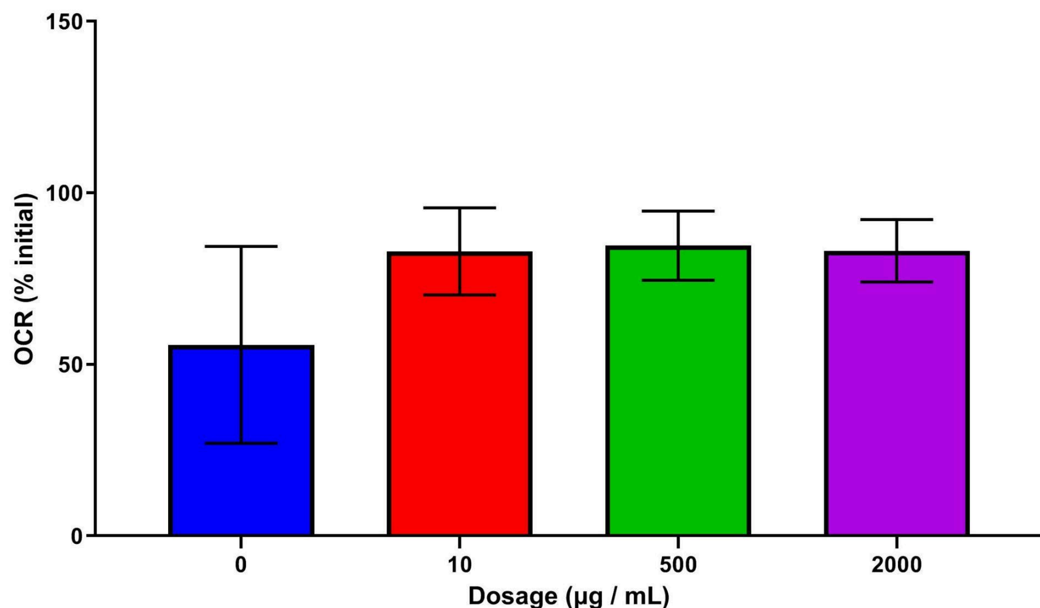


Figure 107: Effect of Liquorice Tea 24 Hour Treatment on Mitochondrial Spare Capacity in MCF10A Cells

17.2. Interpretation of the Results of Pre-extracted Liquorice Tea Extract on Mitochondrial Respiration in MCF7 Cells as Protection Against Mitochondrial Modulating Drugs

24 hour treatment of MCF10A cells with XLTE showed no significant difference in the effect of oligomycin and FCCP on OCR (figures 108-109).

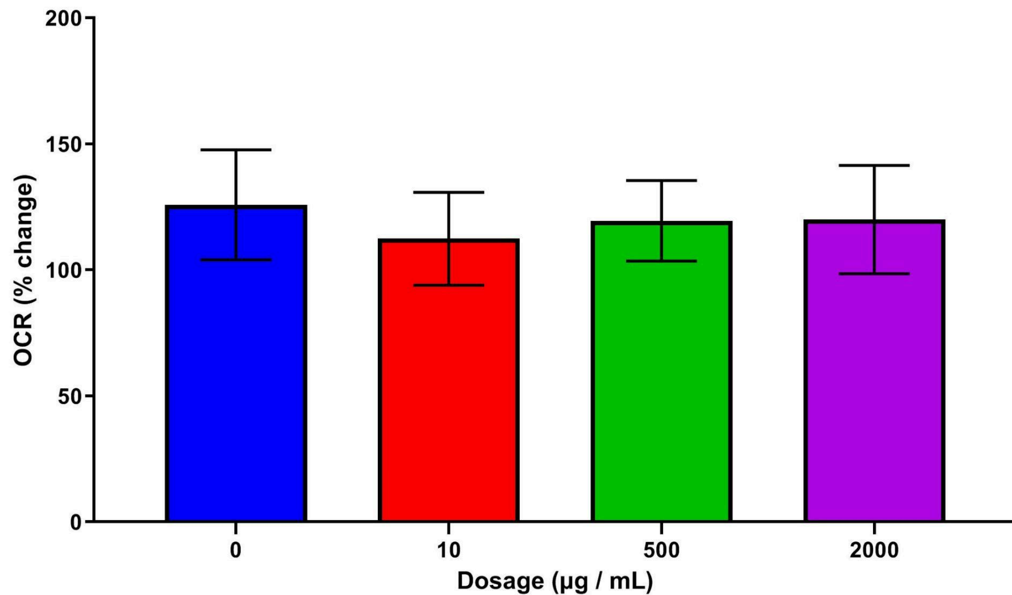


MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 108: Effect of Pre-extracted Liquorice Tea 24 Hour Treatment Followed by Oligomycin on OCR of MCF10A Cells



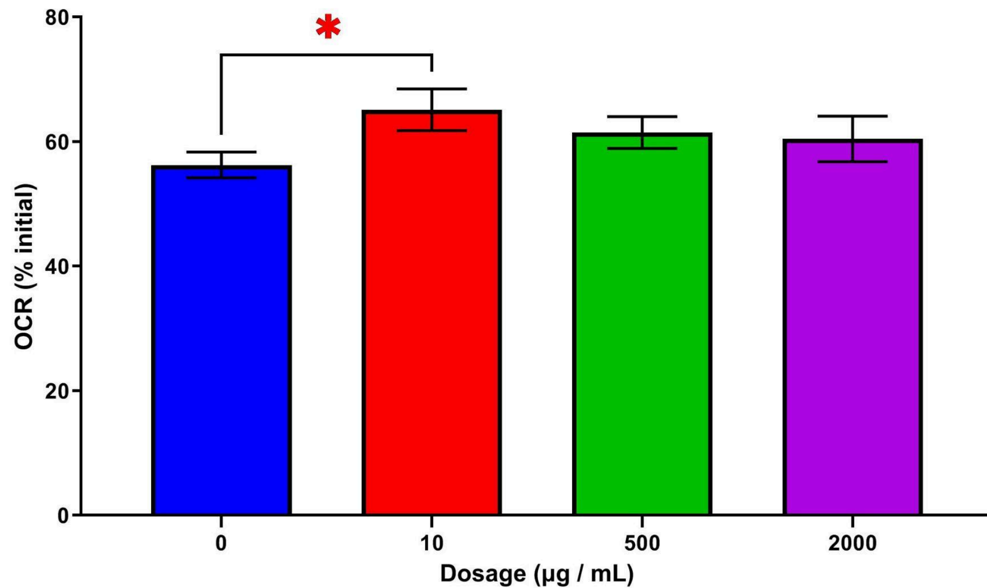
MitoStress assay performed with SeaHorse XF_e 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons.

Figure 109: Effect of Pre-extracted Liquorice Tea 24 Hour Treatment Followed by FCCP on OCR of MCF7 Cells

24 hour treatment of MCF10A cells with 10 µg / mL XLTE showed an 8.85% increase in OCR (65.12% compared to 56.27% in control; $p < 0.05$; figure 110) after administration of antimycin A and rotenone, implying a reduction in the effect of antimycin and rotenone to inhibit OCR.



MitoStress assay performed with SeaHorse XF$_e$ 24 Flux Analyzer, normalised using protein content measured with Pearce BCA assay to estimate cell count.

Data shown as mean average +/- SD. n = 3.

Significance assessed using one way ANOVA with Dunnett's multiple comparisons. * indicates significance ($p < 0.05$) compared against untreated control.

Figure 110: Effect of Pre-extracted Liquorice Tea 24 Hour Treatment Followed by Antimycin A and Rotenone on OCR of MCF10A Cells

17.3. Summary of Results from Respiratory Measurements of Pre-extracted Liquorice Tea in MCF10A Cells

The results can be summarised as follows:

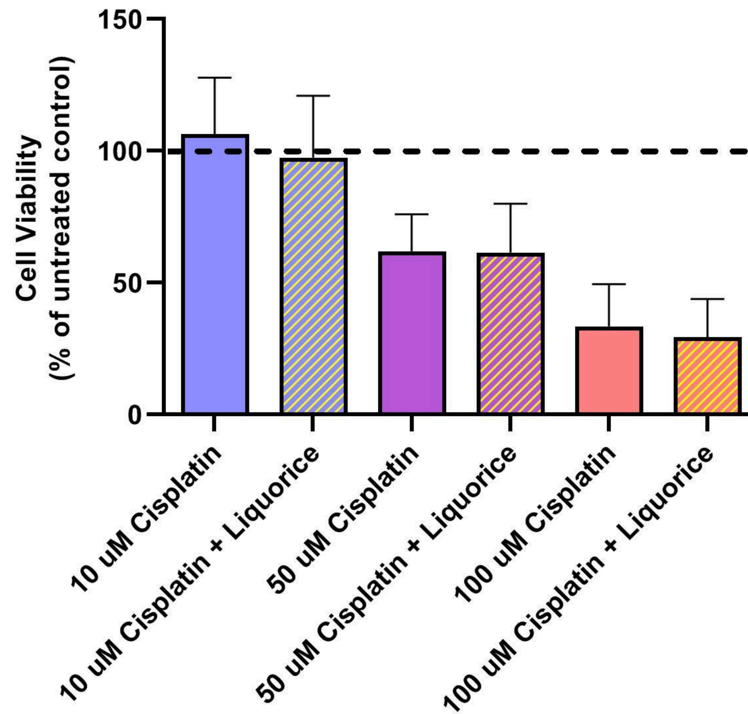
- 24 hours pretreatment with 2000 µg / mL XLTE showed a 56.41% decrease ($p < 0.0001$) in basal OCR of MCF10A cells
- 24 hours pretreatment with 500 µg / mL XLTE showed a 53.52% decrease ($p < 0.0001$) in basal OCR of MCF10A cells
- 24 hours pretreatment with 10 µg / mL XLTE showed a 21.12% decrease ($p < 0.05$) in basal OCR of MCF10A cells
- 24 hours pretreatment with 10 µg / mL XLTE showed a 8.85% reduction ($p < 0.05$) in the effect of antimycin A and rotenone to reduce OCR in MCF10A cells.

Chapter XVIII

18. Results from Pre-extracted Liquorice Tea Extract on Drug Resistance in MCF10A Cells

18.1. Effects of Pre-extracted Liquorice Tea Extract on Cisplatin Induced Reduction of Cell Viability in MCF10A Cells

No significant difference in cell viability was seen in MCF10A cells after 24 hours pretreatment with XLTE followed by 24 hours cisplatin treatment (figures 111 - 112).

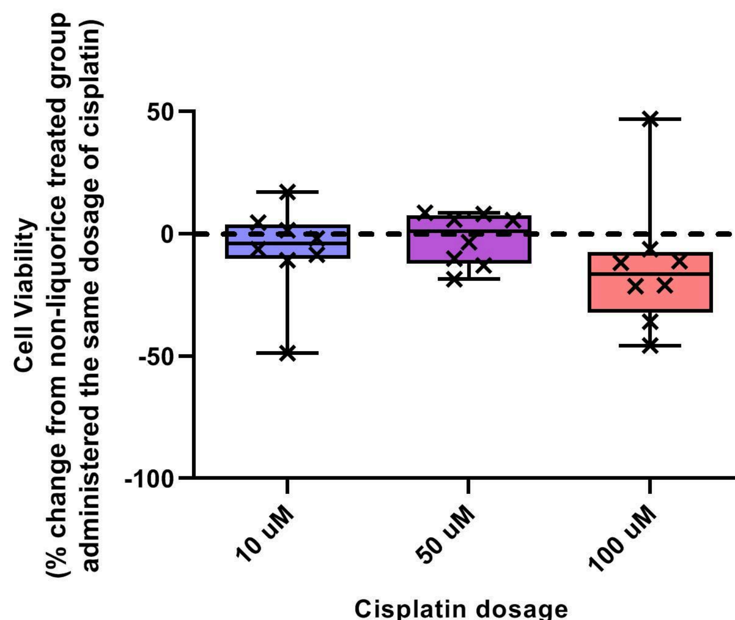


Liquorice groups pretreated with 2000 μg/ml freeze dried liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as the change in cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from the untreated control drawn as dotted line at 100%.

Data shown as shown as mean average +/- SD. n=8.

Significance assessed using unpaired t-test to compare each liquorice pretreated group to its untreated group given the same dosage of cisplatin.

Figure 111: Effect of Pre-extracted Liquorice Tea Extract Pre-treatment on Cytotoxic Effect of Cisplatin in MCF10A Cells



Liquorice groups treated with 2000 µg/ml liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as difference between the cisplatin only group and the liquorice & cisplatin groups.

Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from group not treated with liquorice and given same dose of cisplatin.

Data shown as box and whisker plot where the box represents the upper and lower quartiles around the median average shown as a line within the box and the whiskers represent the maximum and minimum values with all data points displayed. n=8.

Significance assessed using unpaired t-tests to compare liquorice treated and non-liquorice treated groups for each dosage of cisplatin.

Figure 112: Difference Between Pre-extracted Liquorice Tea Extract Pre-treatment and No Pre-treatment on the Cytotoxic effect of Cisplatin in MCF10A Cells

18.2. Summary of Results from Pre-extracted Liquorice Tea Extract in Drug Resistance

The results can be summarised as follows:

- 24 hours pretreatment with 2000 µg / mL XLTE did not produce any significant effect on the cytotoxic effects of cisplatin.

Chapter XIX

19. Results from Other Methods of Adulteration Detection

19.1. Visual Inspection

The rehydrated samples were subjected to visual inspection and revealed that the XLTE was slightly more clear and less yellow in colour than OLTE (figure 113, left). However, this could not be considered a definitive test as a different sample from the same batch under slightly different light made this difference lessen and even disappear entirely (figure 113, right)

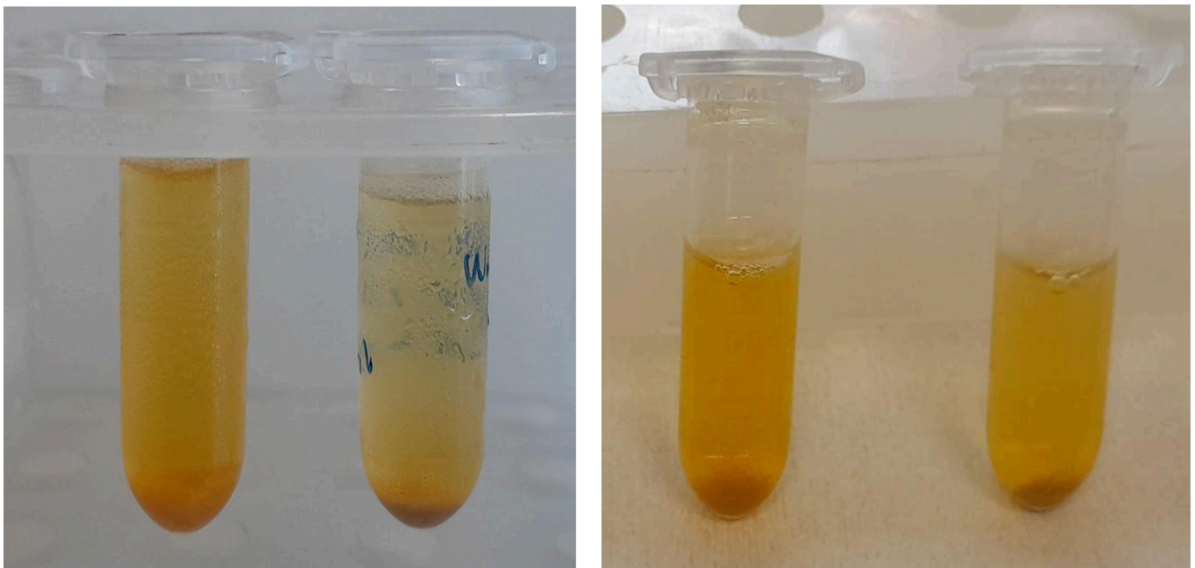


Figure 106: Visual Comparison of Original and Pre-extracted Liquorice Tea Extracts.
In each picture OLTE is on the left and the XLTE is on the right.

19.2. High Performance Thin Layer Chromatography

Two sets of each sample were analysed with HPTLC (figs 114 - 118). The individual numbered tracks represent:

1. Raw “3 Licorice Tea” extracted in 70:30 v/v ethanol:water as per HPTLC Association guidelines for analysis of liquorice.
2. *G. glabra* sample from the University of Westminster Polyclinic extracted in 70:30 v/v ethanol:water
3. *G. uralensis* geno-verified botanical reference sample of *G. uralensis* (Brion Research Institute, Taiwan) extracted in 70:30 v/v ethanol:water
4. OLTE sample
5. XLTE sample at equal strength to OLTE
6. XLTE sample at 20% strength of OLTE so simulate the strength of a second extraction from a pre-extracted teabag.

Two sets of tracks are displayed. The left set of tracks show results from using 2 μL of sample, while right tracks show results from using 5 μL of sample.

Figure 114: HPTLC Results After Development Under Visual Light.
No bands are visible prior to viewing under UV light or derivatization.

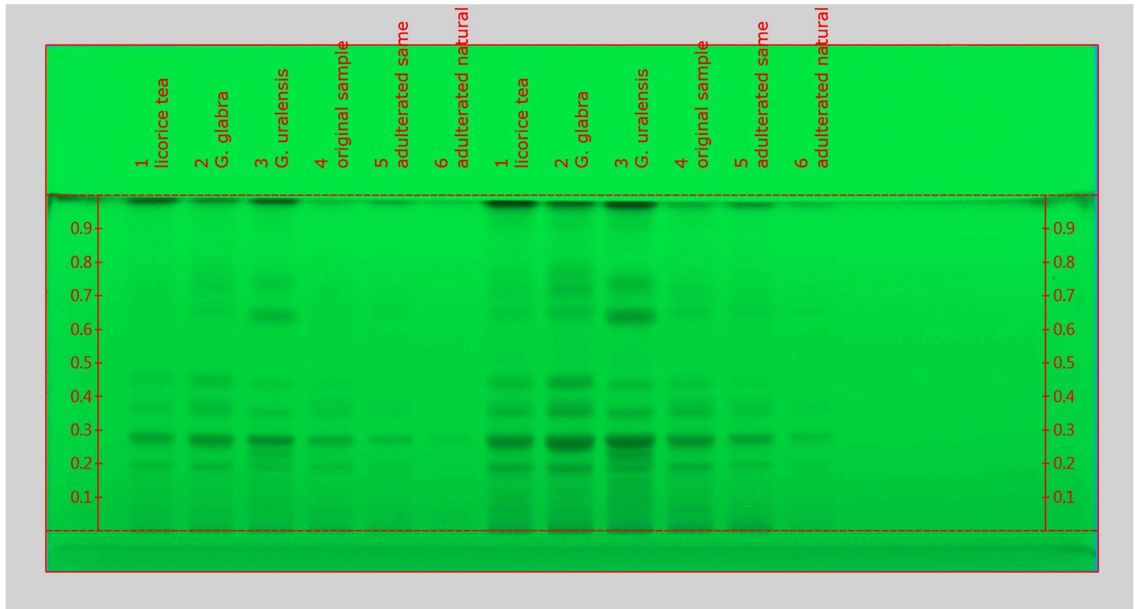


Figure 115: HPTLC Results After Development Under UV 254 nm

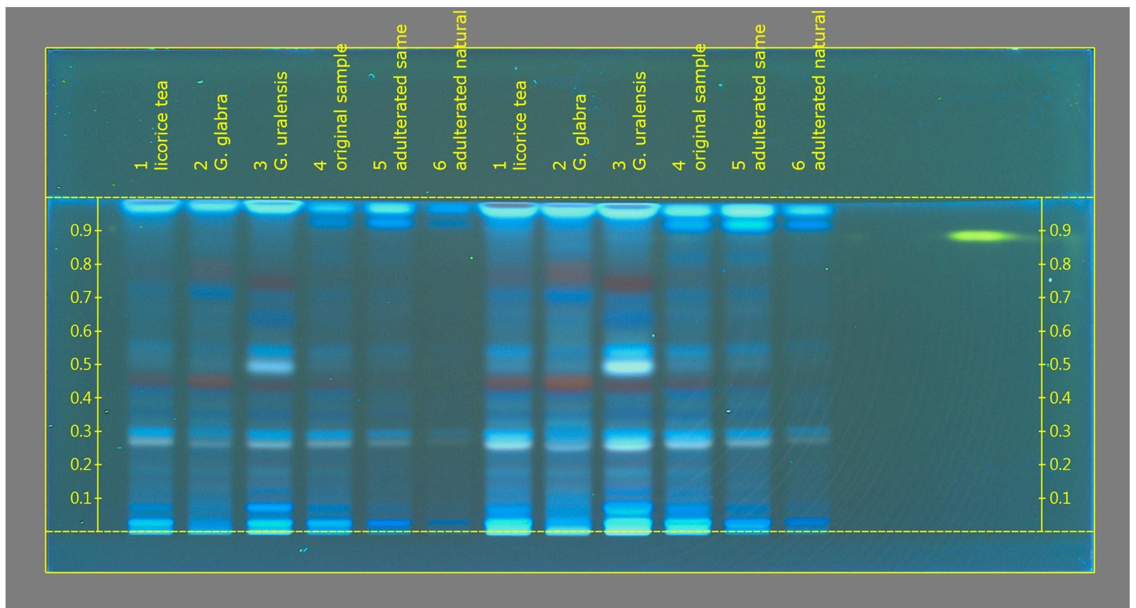


Figure 116: HPTLC Results After Development Under UV 366 nm

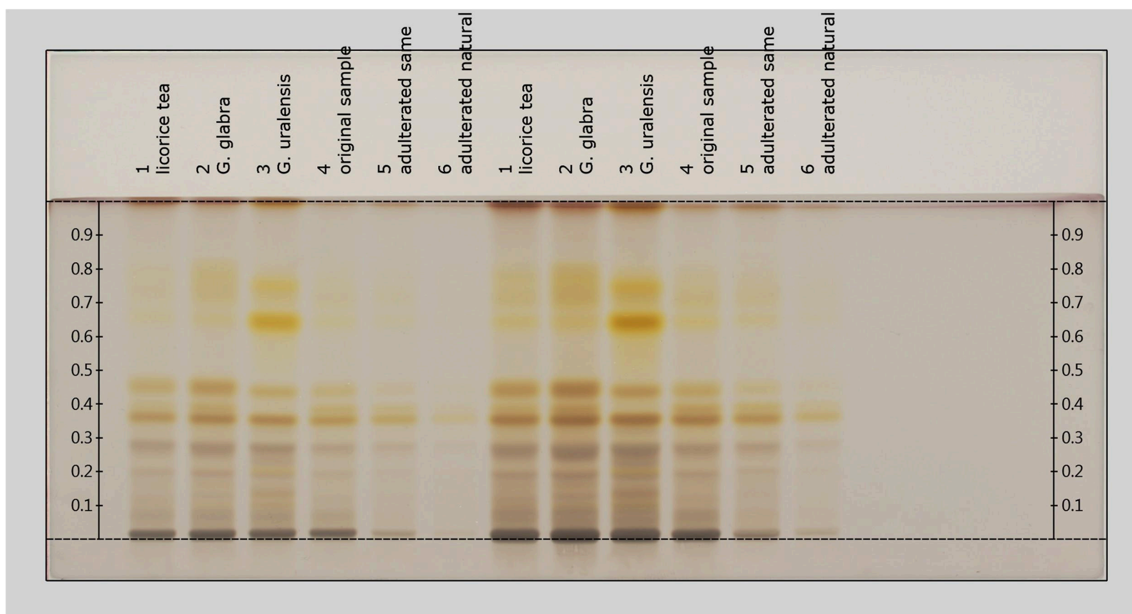


Figure 117: HPTLC Results After Derivatization Under Visible Light

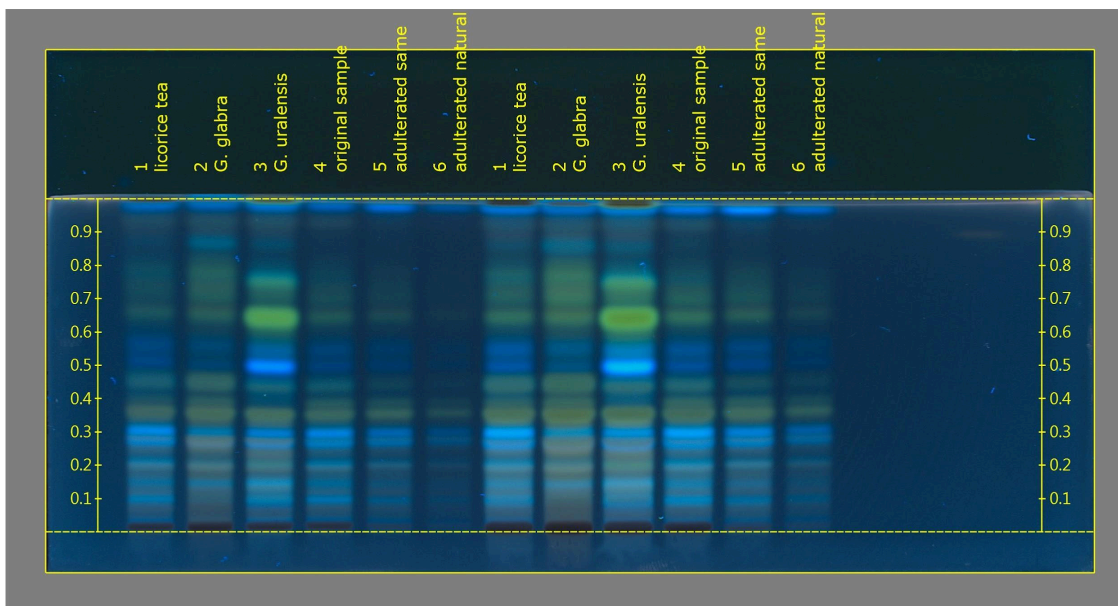


Figure 118: HPTLC Results After Derivatization Under UV 366 nm

19.2.1. HPTLC Comparison of Species

Specific marker compounds to differentiate *G. uralensis* and *G. glabra* (Kondo et al., 2007) could be used to identify the species and extraction method. *G. uralensis* (track 3), could be differentiated from *G. glabra* by a band at 0.5 Rf after development and viewing with UV 366 nm, and at 0.65 Rf after derivatization (figure 119) which could be conjectured to be liquiritin that is only present in *G. uralensis* (Rizzato et al. 2017).

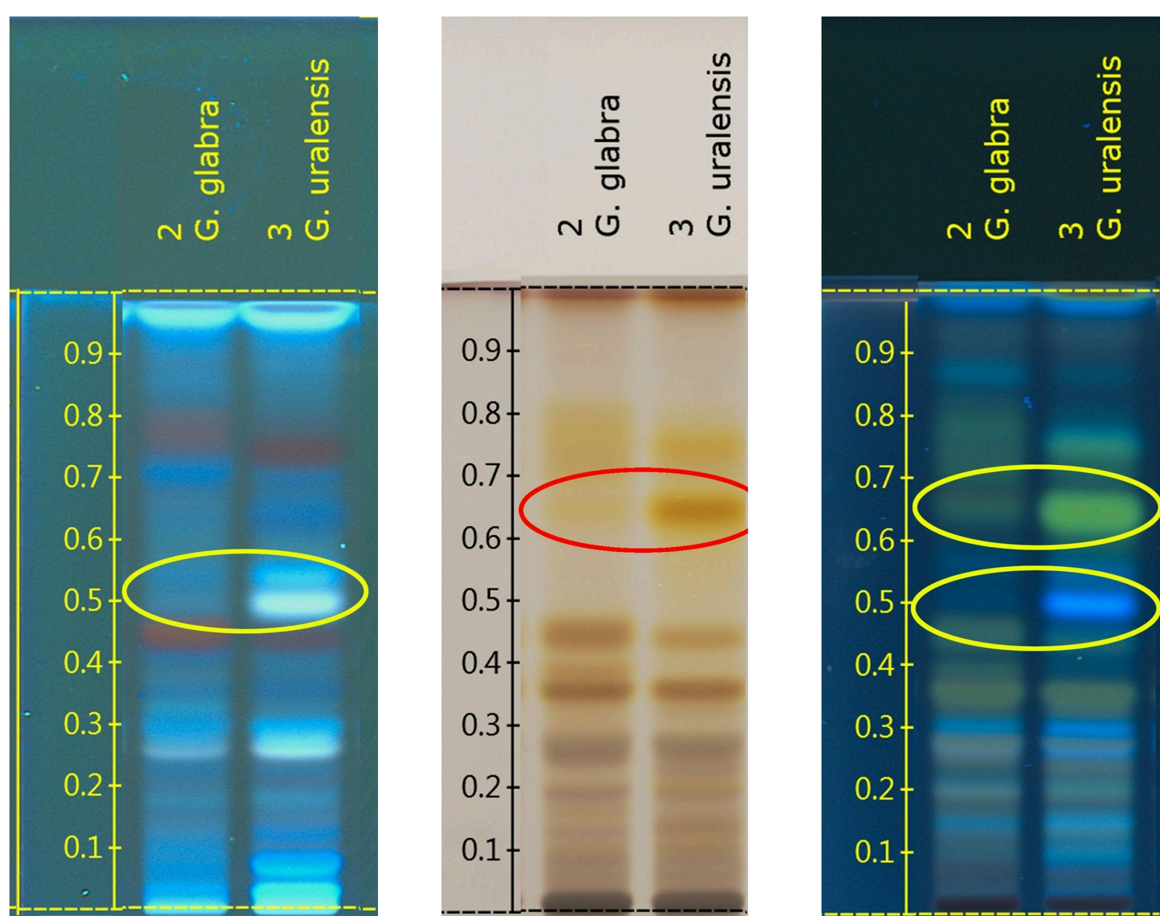


Figure 119: HPTLC Comparison of *G. glabra* (left) and *G. uralensis* (right)
Left: Developed and viewed under UV 366 nm
Middle: Derivatized and viewed under visual light
Right: Derivatized and viewed under UV 366 nm

19.2.2. HPTLC Comparison of Extraction Method

7:3 ethanol / water (v/v) extracts of *G. glabra* (tracks 1 & 2) produced a red band at 0.45 Rf after development and viewed under UV 366 nm, which could differentiate them from water extracts (tracks 4, 5 & 6). The most notable are tracks 1 and 4 (figure 120) because these used the exact same starting material, the Pukka “3 Licorice” tea bag, except track 1 was extracted using the ethanol solution, while track 4 was the freeze dried aqueous extraction sample used in the OLTE mitochondrial analysis. This band was speculated to be glabridin, a species-specific secondary metabolite of *G. glabra* and insoluble in water (Tian, Yan & Row, 2008).

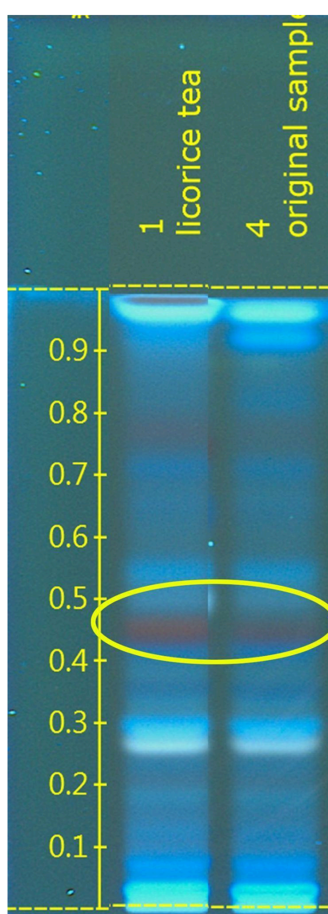


Figure 120: HPTLC Comparison of Ethanol (left) and Water (right) Extracts of *G. glabra* After Development and Viewed Under UV 366 nm

19.2.3. HPTLC Comparison of Source

It was even possible to differentiate between the sample taken from the University of Westminster Polyclinic dispensary (track 2), and the Pukka “3 Licorice” tea bags (track 1) due to the presence of many additional brown bands in the polyclinic sample after derivatization (figure 121).

Figure 121: HPTLC Comparison of *G. glabra* from a Commercial Tea Brand (left) and a Herbal Medicine Clinic (right) After Derivatization under UV 366 nm

19.2.4. HPTLC Comparison of Original and Pre-extracted Material

It was possible to differentiate the OLTE (track 4) from the XLTE, when prepared at the dosage that would be expected from the same amount of starting material (i.e. if the tea bag was reused, yielding approximately 20% of the freeze dried material). All bars were visibly faded (track 6) at all points (figure 122).

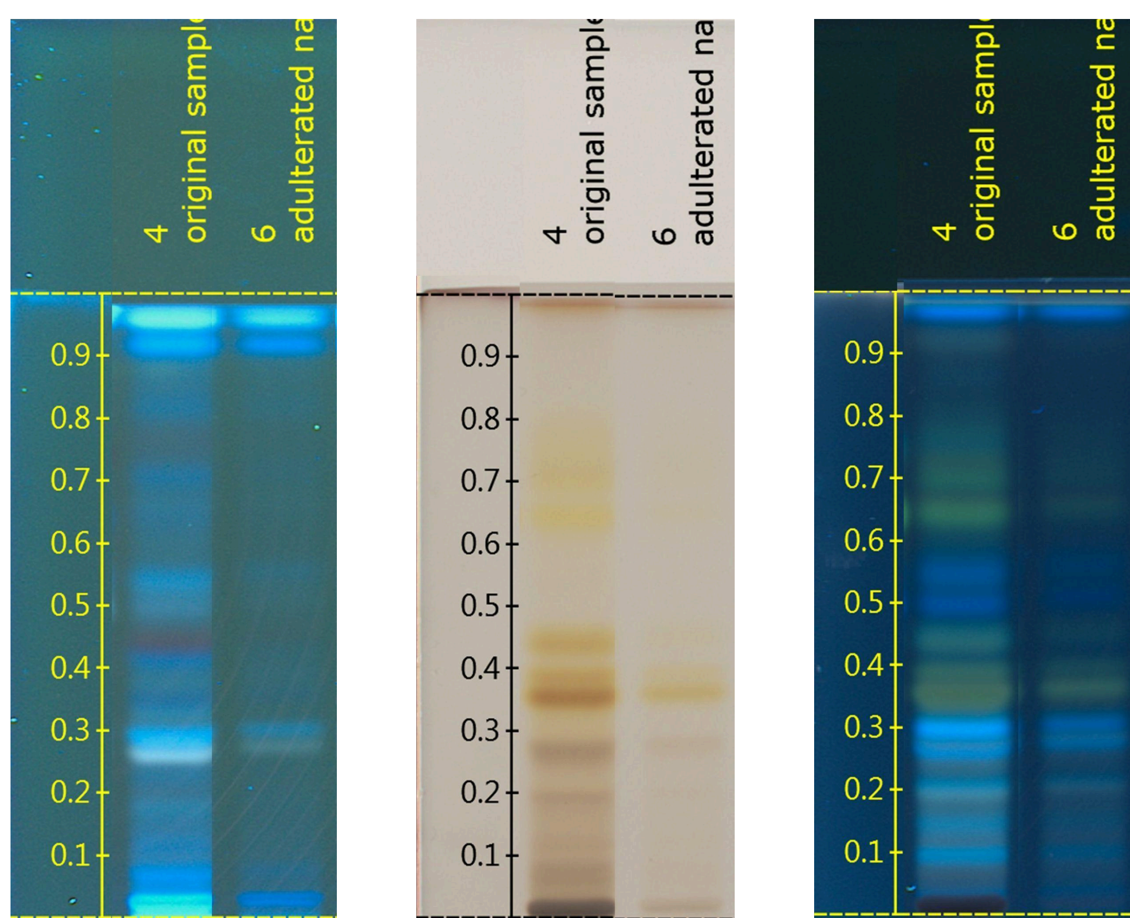


Figure 122: HPTLC comparison of Original (left) and Pre-extracted (right) Liquorice Tea Extracts at Natural Strength if Extraction was Taken Directly from an Pre-extracted Tea Bag

Left: Developed and viewed under UV 366 nm

Middle: Derivatized and viewed under visual light

Right: Derivatized and viewed under UV 366 nm

When OLTE (track 4) and XLTE prepared at an identical dosage of freeze dried material (track 5), as was used in the mitochondrial functional analysis, the differences were slight and may be within a normal range of variation and were even harder to differentiate after derivatization (figure 123).

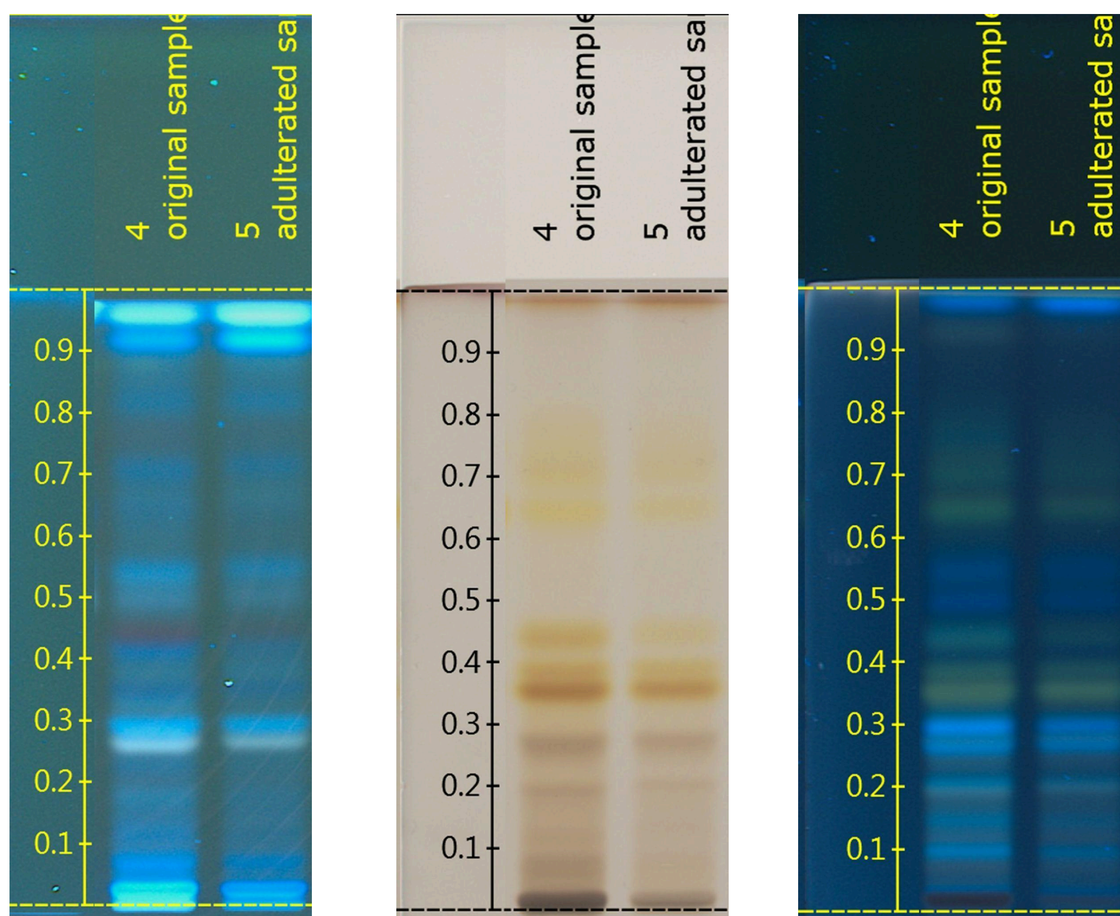


Figure 123: HPTLC Comparison of Original (left) and Pre-extracted (right) Liquorice Tea Extracts at the Same Dosage of Freeze Dried Material

Left: Developed and viewed under UV 366 nm

Middle: Derivatized and viewed under visual light

Right: Derivatized and viewed under UV 366 nm

Additionally, both OLTE and XLTE at equal strength showed a quenching zone at 0.27 Rf (figure 124) suggesting that both had contained glycyrrhizin, the main constituent commonly searched for in liquorice extracts. OLTE appeared stronger but not to a degree that may be immediately apparent.

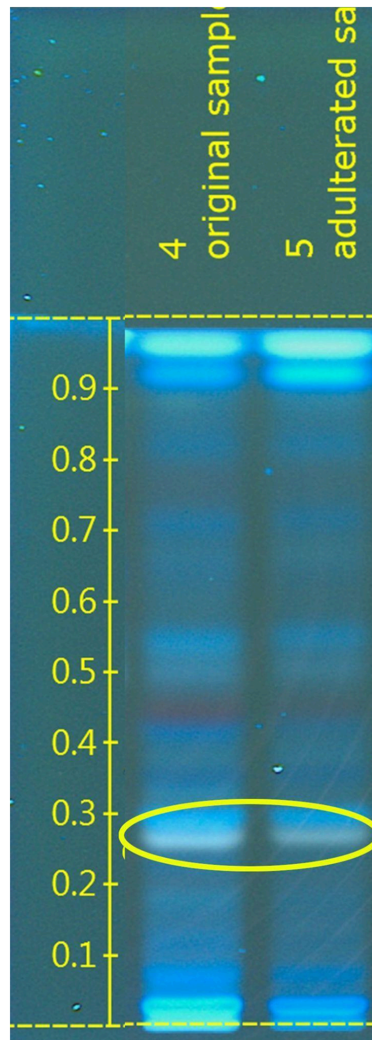


Figure 124: HPTLC Comparison of Original (left) and Pre-extracted (right) Liquorice Tea Extracts at the Same Dosage of Freeze Dried Material after Development Under UV 366 Showing Probable Glycyrrhizin Elution Zone

19.3. Summary of Results from Other Methods of Adulteration Detection

The results can be summarised as follows:

- Visual inspection could detect a slight difference but one that was subtle, dependent on lighting and easily overlooked
- HPTLC analysis easily differentiated between species and between an alcohol and water extracts but was much less effective at differentiating between the OLTE and XLTE when administered at the same dosage of freeze dried material
- The difference between the OLTE and XLTE was even less apparent after derivatization and observation under UV 366 nm

Discussion

Chapter XX

20. Discussion of the Results

The results of this study will now be discussed. First, the reasons for the effects observed in this study will be discussed, followed by how these relate to the detection of adulteration and the hypothesis being tested. An examination of the limitations of mitochondrial functional analysis in general and of this study in particular will then be undertaken. Next, the implications these findings may have for other fields of research and practice will be explored. Finally, future work that could be undertaken to expand mitochondrial functional analysis into a tool that can be used to develop an industry standard for functional quality evaluation will be suggested.

20.1. The Effects of Liquorice Tea Extracts on Reactive Oxygen Species

Three significant results were obtained in relation to ROS, the first being a rise in MC7 cells in the 500 µg / mL and 2000 µg / mL groups after 3 hours exposure to the OLTE, the second and third being significant reductions in ROS after 72 hours in both the OLTE and XLTE at 2000 µg / mL doses.

20.1.1. Increases in Reactive Oxygen Species

The first significant result obtained was an increase in ROS among the MCF7 cells treated for 24 hours with 500 µg / mL and 2000 µg / mL OLTE for 3 hours. ROS increased by 16.51% ($p < 0.01$) in the 500 µg / mL group and 14.88% ($p < 0.05$) in the 2000 µg / mL group (figure 55, section 11.2). This is unusual in

relation to the literature as very few studies report any components of liquorice increasing cellular ROS. Two notable exceptions are Fiore et al. (2004) and Singh, Pal and Darokar (2015).

Fiore et al. (2004) observed that glycyrrhetinic acid can cause an increase in H_2O_2 within the mitochondrial matrix in isolated rat liver mitochondria. It achieved this by interfering with the iron-sulphur clusters in Complex I which generates $O_2^{\bullet-}$ that is removed by SOD to become H_2O_2. In the presence of Fe^{2+} this become $^{\bullet}OH$ which are highly reactive and oxidise thiol groups in the presence of Ca^{2+} leading to the opening of the mPTP. This mechanism has been exploited in many attempts to utilise glycyrrhetinic acid to deliver chemotherapeutic drugs directly to mitochondria in liver cancer (Speciale et al., 2022). Glycyrrhetinic acid is the aglycone form of glycyrrhizin and primarily formed by enzymatic hydrolysis of the gut microbiota (Hattori et al., 1983) and present only in small amounts in the plant itself (Graebin, 2018) making a comparison complex, however some sources state that glycyrrhizin and glycyrrhetinic acid as being pharmacologically equivalent (Wahab et al., 2021) and if the dose is equivalent or greater then it may be possible that this effect is achieved.

Fiore et al. (2004) achieved their results based on 10 μM of glycyrrhetinic acid and, although it is not possible to get a precise molarity on the OLTE due to it being a whole root extract whose precise constituent proportions are unknown, an estimate can be attempted based on the fact that Tian, Yan & Row (2008) managed to extract 2.44 mg / g glycyrrhizin using a water extraction. The total yield of freeze dried material was 377.33 mg / g, making 2000 μg (2 mg) equivalent to 5.3 mg of raw material:

$$\text{Dose (mg)} \div \text{yield (mg / g)} = \text{initial starting material (g)}$$
$$2 \div 377.33 = 0.0053 \text{ g} = 5.3 \text{ mg}$$

which equates to 12.932 μg of glycyrrhizin:

$$\text{Extraction rate (mg / g)} \times \text{starting material (g)} = \text{glycyrrhizin content (mg)}$$
$$2.44 \times 0.0053 = 0.012932 \text{ mg or } 12.932\ \mu\text{g}$$

The molecular weight of glycyrrhizin is 822.9 g/mol (NCBI, 2023a), so by using a concentration conversion formula (Abcam, 2024) we can calulate:

$$\mu g / ml \div (1000 \div \text{molecular weight}) = \mu M$$
$$12.932 \div (1000 \div 822.9) = 10.641$$

Therefore, assuming our yields were the same as Tian, Yan & Row, (2008), we can estimate that approximately 10.6 μM glycyrrhizin was used, close to the concentration that Fiore et al. (2004) used to achieve an increase in ROS in rat liver mitochondria with glycyrrhetinic acid. This does not account for the fact that Tian, Yan & Row (2008) extracted using room temperature water for 240 minutes instead of using boiling water for 15 minutes as was used in this study. This was designed to make their results comparable to other solvents which would evaporate at higher temperatures but ignores the fact that most aqueous herb extractions occur under heated conditions. If we instead take the minimum glycyrrhizin content found in the root as our starting point, assuming that the insoluble material would offset the fact that not all the glycyrrhizin contained in the root would be extracted, then we can expect a minimum of 2% glycyrrhizin (Ministry of Health and Welfare, 2016, p.136), which equates to 7.55 mg / g in our extract, or 3x the amount used by Fiore et al. (2004) with some sources stating that glycyrrhizin content can be as high as 24% (Esmaili & Karami, 2022) increasing this much further. This would place our estimates for a glycyrrhizin molarity at anywhere between 30-360 μM. Either way, we should have had at least as much glycyrrhizin in our extract as Fiore et al. (2004) had glycyrrhetinic acid and maybe more.

Although the dosages used in this study may have been similar, or even higher that that used by Fiore et al. (2004), there are other dissimilarities between the studies. Fiore et al., (2004) used isolated rat mitochondria and so the cellular uptake of OLTE in MCF7 cells has to be accounted for too. Also, the fact that glycyrrhizin and glycyrrhetinic acid are often treated as pharmacologically equivalent (Wahab et al., 2021) refers to the fact that glycyrrhizin, once metabolised, becomes glycyrrhetinic acid which is pharmacologically active and

not that they have identical effects *in vitro*. The experiment of Fiore et al. (2004) has not been reproduced using glycyrrhizin in MCF7 cells and an important argument against this mechanism being activated in the current study is that the increase in H_2O_2 was accompanied by a drop in ΔΨm which was not observed at any time point in this study.

Singh, Pal & Darokar (2015) studied the effects of glabridin on multidrug-resistant *Staphylococcus aureus* finding an antibacterial effect at higher concentrations (up to 25 µg / mL) through the generation of ROS and increased NO leading to lipid peroxidation, detected by malondialdehyde (MDA), and DNA fragmentation. Although glabridin is present in *G. glabra* and considered one of its marker compounds (Rizatto et al., 2017; Avula et al., 2022), it has poor water solubility (Tian, Yan & Row, 2008) so little would be present in the water extracts used in this study. This can be speculatively confirmed by the HPTLC chromatograms under UV 366 nm (figure 116) that revealed a red band present at 0.45Rf in the 70% ethanol extracts (tracks 1 & 2) which is absent in the aqueous extracts used in the mitochondrial studies (tracks 4 & 5). Small amounts of glabridin are extracted in water, equating to 0.18 mg / g compared to 0.93 mg / g in ethanol (Tian, Yan & Row, 2008), which implies that if 0.18 mg of the 377.33 mg extracted from 1 g of liquorice tea is glabridin, then, using the formula above, in 2000 µg / mL of extract:

$$\text{mg glabridin in 2 mg of extract} = (0.18 \div 0.0053) \times 1000 = 0.00094$$
$$= 0.954\ \mu g$$

If we can expect to find 0.95 µg / mL of glabridin if our 2000 µg / mL extract, it is unlikely to have exerted a pro-oxidant effect, and may even have exerted an antioxidant effect, as was found by Singh, Pal & Darokar (2015) at lower doses. A complete breakdown of the proportions of all components obtained from an infused aqueous extraction of *G. glabra* could not be found and so these doses can remain only estimates, but a full spectrum quantitative analysis of the OLTE using HPLC, MS, NMR or a hyphenated technique could be undertaken to gain better quantification of the individual constituents.

20.1.2. Decreases in Reactive Oxygen Species

Another possibility emerges from the subsequent results. After 24 hours treatment with 2000 µg / mL OLTE, ROS measurements with the MCF7 cells had returned to levels equal to control, and after 72 hours treatment with 2000 µg / mL OLTE, ROS measurements had reduced by 14.73% ($p < 0.05$) (figure 57, section 11.2). This effect was also seen among the MCF7 cells treated with the adulterated liquorice tea extract, where 2000 µg / mL treatment for 72 hours saw an even greater reduction in ROS of 18.7% ($p < 0.0001$) (figure 90, section 15.1). This concurs with the majority of literature on liquorice having an antioxidant effect in cancer cells, primarily due to its phenolic content (Pastorino et al., 2018, Sharma, Katiyar & Agrawal, 2018) but with some activities attributed to glycyrrhizin too (Ageeva et al., 2022).

In the same study that researched pro-oxidant effects of high doses of glabridin, antioxidant effects were found from low doses of 0.78 to 3.12 µg / mL (Singh, Pal & Darokar, 2015), for which the estimated proportion of glabridin present in the tea samples of 0.95 µg / mL would correspond. This has been supported by *in vivo* research which showed a 20% reduction in oxidised low density lipoproteins in blood samples taken from human volunteers supplemented with 60 mg glabridin for 12 months (Carmeli & Fogelman, 2009). In the present study, the enhanced antioxidant activity of XLTE could be explained by this effect. The initial water extraction will have removed the most polar compounds from the raw root pieces, meaning that the second extraction may have proportionally higher amounts of non-polar substances like glabridin than the initial extraction, thus exerting a stronger antioxidant effect. If the initial extraction was approximately 0.95 µg / mL, then the second extraction would be closer to the optimal range (1.56 µg / mL) that Singh, Pal & Darokar (2015) found for antioxidant activity.

Studies of the whole root extract through different extraction methods have shown that the antioxidant effects are not solely dependent on glabridin. A

comparison of different solvent extracts of *G. glabra* showed that aqueous extractions had comparable activity on DPPH, NO radicals, $O_2^{\bullet-}$ and H_2O_2 to less polar solvent extractions, despite achieving a phenolic extraction rate of 2.58 ± 0.014 mg gallic acid equivalents / g, compared to 79.297 ± 0.776 achieved from methanol (Hejazi et al., 2017). This suggests the antioxidant effect in aqueous may be due to components with a greater affinity for water.

Licochalcones have also been implicated in the antioxidant potential of liquorice. Sources differ as to which licochalcones are present in *G. glabra* with some finding only licochalcone B (Rizatto et al., 2017; Avula et al., 2022), while others find only licochalcone A (Cerulli et al., 2022) and others find both (Fu et al., 2013). Licochalcones A and B have been reported to have antioxidant effects against lipopolysaccharide induced oxidative stress in RAW 264.7 cells with licochalcone B having the superior action in a DCFDA cellular assay, but licochalcone A having a superior radical scavenging potential in an $ABTS^+$ assay (Fu et al., 2013). ABTS is a peroxidase substrate that, in the presence of H_2O_2 is oxidised to yield $ABTS^+$ whose colour can be measured by spectrophotometry at 600–750 nm (Siddeeg et al., 2021). The reduction in absorbance reflects the ability of the antioxidant to donate a hydrogen atom and neutralise the radical, implying that licochalcone A scavenges ROS through the hydrogen atom transfer method (HAT) as opposed to a single electron transfer (SET) method. This is confirmed by an UPLC-MS/MS evaluation of a licochalcone A DPPH assay which found that licochalcone A favours a HAT mechanism of ROS scavenging in weakly non-polar solutions which approximate organic conditions (Liang et al., 2018). Meanwhile licochalcone B seems to operate through a cellular mechanism, activating the keap1-Nrf2 pathway to raise endogenous antioxidant defences including SOD and GSH (Zhou, B. et al., 2021; Lv et al., 2018) and HO-1 (Huang et al., 2023; Su et al., 2018).

Another flavonoid that could be implicated in the antioxidant capabilities of *G. glabra* is liquiritin apioside. There is very little research on this specific molecule but it has been shown to increase levels of SOD and GSH in a mouse cigarette smoke induced lung disease model (Guan et al., 2012). Some further studies enforce this result by studying liquiritin apioside in relation to cough and finding it

likely to be the primary constituent for the antitussive effect (Kuang et al., 2018; Wei et al., 2020) but these did not investigate antioxidant mechanisms. There is more research on liquiritin, suggesting an inhibitory action on NF-κB through the AMPK/SIRT1 pathway (Tang et al., 2022) and the ERK1/2 pathways along with activation of Nrf2 (Li et al., 2020), restoring deficits in SOD, GSH and CAT (Sun et al., 2010; Zhou et al., 2017). However, liquiritin and liquiritin apioside are not identical molecules and so any comparison of their mechanism should be tentative.

Glycyrrhizin, the main constituent of liquorice water extracts, has also been implicated in antioxidant mechanisms despite being a triterpenoid saponin instead of a phenol. Although there is wide consensus that it has antioxidant potential, there is no consensus on its mechanism. Some argue it is unable to scavenge $^{\bullet}OH$ and $O_2^{\bullet-}$ but is able to scavenge DPPH (Imai et al., 2013; Račková et al., 2007; Polyakov et al., 2006), while others argue that it can scavenge $O_2^{\bullet-}$ and $^{\bullet}OH$ but not DPPH (Kato et al., 2008; Cheel et al., 2010). Others still observed cellular mechanisms such as the increase in GSH and the inhibition of NOX in neutrophils (Beskina et al., 2007). One mechanism that may connect these conflicting results is presented by Ageeva et al. (2022) who, based on earlier findings by Ghandi et al. (2004), used a chemically induced dynamic nuclear polarisation technique to demonstrate that the ROS scavenging effects of glycyrrhizin may be due an ability to capture solvated electrons created by UV irradiated naproxen before they bind to O_2, creating a glycyrrhizin radical anion. They also suggest that glycyrrhizin may act as a trap for harmful short-lived radicals. This implies glycyrrhizin is acting in a different way to most antioxidants that deradicalise ROS through the donation of H or electrons to create stable compounds, or at least less harmful radicals (Leopoldini, Russo & Toscano, 2011) and would explain its inconsistent results in antioxidant assays that depend on HAT or SET from the antioxidant to neutralise the assay reagent (Munteanu & Apetrei, 2021).

20.1.3. A Combined Explanation for Modulations in Reactive Oxygen Species

The concept of hormesis could connect these modulations in ROS. The available literature examining the effects of liquorice extracts to raise ROS remains unconvincing when applied to the quantities of compounds likely to be available in the extracts, but there is much more evidence for an antioxidant effect of liquorice extracts. Cells require a certain amount of oxidative stress in order to maintain cell signalling, proliferation and differentiation functions, with cancer cells tending to favour an environment of slightly raised oxidative stress (Hayes, Dinkova-Kostova & Tew, 2020). A sustained drop in ROS induces a state of reductive stress where these functions can be compromised (Xiao & Loscalzo, 2020). One of the hormetic mechanisms proposed is overcompensation in response to a perturbation in cellular homeostasis (Calabrese et al., 2016). The introduction of a large quantity of antioxidant material which threatens to reduce this optimal environment may initiate an attempt to overcompensate, seen in the rise in ROS in MCF7 cells after 3 hours treatment and returning to homeostasis at 24 hours. Over time this compensation cannot be maintained and after 72 hours treatment with 2000 µg / mL OLTE it succumbs and ROS is reduced, although at 72 hours treatment with 500 µg / mL OLTE, homeostasis appears to be maintained.

The lack of an increase in ROS after 3 hours treatment with XLTE but subsequent drop after 72 hours treatment with 2000 µg / mL XLTE to below that of the OLTE group may indicate that there is proportionally more of the antioxidant substance in this batch. This could prevent the MCF7 cells from being able to initially overcompensate and only maintain their basal rate of ROS. Further assays at time points between the ones chosen could elucidate when the rise and decline in ROS happens in each extract, and if it happens in the XLTE at all, but at a time point not measured in this study.

The lack of changes in MCF10A cells at any time point suggests that this antioxidant effect applies primarily to sources of ROS generated by cancer. In ordinary cells the majority of ROS is generated as a byproduct of mitochondrial

respiration which is initially generated as $O_2^{\bullet-}$ within the mitochondrial matrix before being transformed by SOD to H_2O_2 where it can diffuse across the IMM and OMM into the cytoplasm (see sections 3.1.1 & 3.1.2). Cancer cells have mDNA mutations that cause them to be metabolically reprogrammed and favour aerobic glycolysis as their preferred energy source (Kang, Lee & Lee, 2015). In order to maintain the redox balance optimal for proliferation and growth, additional ROS is generated through an increased expression of NOX which use heme groups to transfer electrons across membranes and transform O_2 into $O_2^{\bullet-}$ (Moloney & Cotter, 2018). This is then rapidly converted into H_2O_2. Although primarily associated with being located on the plasma membrane and generating extracellular ROS, NOX are also located inside the cell too: on the ER, mitochondrial membranes, the nuclear membrane and specialised domains within the cell (Skonieczna et al., 2017). This makes them them the main source of $O_2^{\bullet-}$ within the cytoplasm and in the external cellular environment. If the theory presented by Ageeva et al. (2022) is correct and glycyrrhizin can act as an electron acceptor and trap for short-lived radicals then we can speculate that the drop in ROS seen only in MCF7 cells is due to glycyrrhizin being able to trap the short-lived $O_2^{\bullet-}$ generated by NOX in this cell line, thus preventing its conversion to H_2O_2 and subsequent modifications. This mechanism would also have little effect on H_2O_2 itself, not being a radical itself and so having no electron to donate and which will be the main form of ROS that reaches the cytoplasm from mitochondrial sources in MCF10A cells.

The lack of changes in ΔΨm would suggest that the mitochondrial function is largely unchanged and that this is primarily due to a ROS scavenging effect. Upregulation of cellular antioxidant defences could be possible too, but these tend to only be observed in studies of liquorice extracts where an initial stressor has depleted them (Zhou et al., 2021; Lv et al., 2018; Huang et al., 2023; Su et al., 2018; Sun et al., 2010; Zhou et al., 2017).

Further experiments could elucidate this mechanism. Assays more specific to particular species of ROS could help determine the relative balance of ROS species. If glycyrrhizin is capable of capturing electrons from $O_2^{\bullet-}$ then an assay specific to this form of ROS such as mitoSOX to measure the mitochondrial $O_2^{\bullet-}$

(Invitrogen, 2022) and a general $O_2^{\bullet-}$ detection kit (Abcam, 2022b) could determine if this ROS species is affected and whether it is from mitochondrial sites or other sources. Since ROS is an important signalling molecule for proliferation, with higher ROS levels associated with hyperproliferation in cancer cells (Hayes, Dinkova-Kostova & Tew, 2020), future tests examining this mechanism could look at the cell viability of MCF7 cells after 72 hours treatment with 2000 μg / mL liquorice tea extracts, as well as longer time frames, to determine if this antioxidant mechanism can have an anti-proliferative effect. Although ROS is a stimulator for cancer cell proliferation, antioxidant therapies have been paradoxically associated with increasing proliferation due to a reduction in ROS, DNA damage and inactivation of the pro-apoptotic function of p53 (Sayin et al., 2014).

20.2. The Effects of Liquorice Tea Extracts on Respiratory Measurements

Some of the most striking results were from the Seahorse Flux Analyzer which produced a reduction in the effect of the mitochondrial modulating drugs used in the assay among the MCF7 cells, and a reduction in basal OCR among the MCF10A cells. After 24 hours treatment with OLTE a significant reduction in the effects of the mitochondrial modulating drugs was seen in the MCF7 cells (see chapter 10) and the basal OCR was observed among the 500 μg / mL (72.6%; $p < 0.01$) and 2000 μg / mL (85.16%; $p < 0.01$) groups in MCF10A cells. This was surprising since there was no change in cell viability, ΔΨm or Ca^{2+} in either cell line and no change in ROS in the MCF10A cells (see section 11). Furthermore, this effect was also seen in the MCF10A cells treated for 24 hours with XLTE which appeared to be dose dependent with a 21.12% ($p < 0.05$) decrease in OCR at 10 μg / mL, 53.52% ($p < 0.0001$) decrease in OCR at 500 μg / mL and a 56.41% ($p < 0.0001$) decrease at 2000 μg / mL suggesting this effect was not exhausted by the second extraction.

A search of the literature revealed that despite liquorice having the third most articles written about it in relation to mitochondria (table 8), there was no analysis of the whole root extract using the Seahorse Flux Analyzer.

PubMed Search Term : Mitochondria AND “ … ”	Results
Ginkgo biloba	99
Panax ginseng	84
Glycyrrhiza	66
Astragalus	64
Scutellaria baicalensis	52
Withania somnifera	37
Zingiber officinale	36
Panax notoginseng	36
Camellia sinensis	31
Cinnamomum	29
Sargassum	28
Centella asiatica	26
Garcinia mangostana	20
Coptis	19
Momordica charantia	19
Andrographis	18
Scutellaria barbata	14
Zingiber officinale	13
Piper longum	12

Table 8: Papers Discussing Mitochondria and Specific Herbs.
Searches were conducted on 18th September 2023. List is based on those in Mani, Swargiary & Singh (2020) and modified to include searches for herbs not directly involved in cancer prevention or treatment. Where multiple species or species names are used interchangeably, only the genus was searched for. Where only one specific species has therapeutic uses, or where different species have very different effects, the genus and species name was used.

Both herbs that have more published data than liquorice have had Seahorse evaluations of at least some of their components, with one study on gingko (*G. biloba*; Wang, Z. et al., 2021) and four on asian ginseng (*P. ginseng*; Huang et al., 2019; Liu, W. et al., 2021; Huang et al., 2023; Shin et al., 2021). Other herbs which have attracted far less mitochondrial interest have also been assayed with the Seahorse Flux Analyser including astragalus (*Astragalus mongholicus*, Bunge, *Fabaceae*; Huang et al., 2018), cinnamon (*C. cassia*; Li, X. et al., 2021), gotu kola (*Centella asiatica*, L. Urban, *Apiaceae*; Gray et al., 2018) and bitter gourd (*Momordica charantia*, L., *Cucurbitaceae*; Sur et al., 2019). It was speculated that other researchers may have encountered the same issues and assumed they were erroneous, as was initially thought in this study, and decided not to publish.

Of the data available, some individual compounds found in liquorice have been examined using the Seahorse Flux Analyzer. Jin, X. et al., (2022) found that 12 hours exposure to 100 µM glycyrrhetinic acid down-regulated OXPHOS and fatty acid β-oxidation in in HeLa cervical cancer cells, producing a similar Seahorse curve with inhibited basal, ATP-related and maximal respiration (ibid.: figure 1, graph D). This is similar to the high dose of liquorice tea extract used in this study (figures 63, 74 & 101) with a lower basal rate and flatter curve. They identified the mechanism as inhibition of mitochondrial serine hydroxymethyltransferase 2 (SHMT2) by targeting the folate-binding pocket of SHMT2 and competing with tetrahydrofolic acid, resulting in inhibited proliferation but did not repeat the experiments on normal cell lines. This could explain some of the results in the MCF7 group which had similar reduced ATP-related and maximal OCR but the lack of comparison to normal cells presents a problem because this study found an even greater reduction of basal OCR in the non-cancerous MCF10A cells (figures 74 & 101), yet without any apparent inhibition of function. While SMHT2 is present in both cancerous and non-cancerous cells, its expression supports cancer growth and elevated expression is associated with poor prognosis (Zeng et al., 2021) making this an unsatisfactory model for explaining the drop in basal OCR in MCF10A cells.

Searching beyond liquorice studies, another study looking at Ginsenoside compound K from *P. ginseng* in an oxygen-glucose deprivation / reperfusion (OGD/R) was found with a similar flattened curve and lower basal OCR to that found in this study (Huang et al., 2023). This used rat adrenal medulla pheochromocytoma (PC12) cells subjected to OGD/R to imitate IRI during stroke. The aspect to note is that the group that shows a pattern similar to the high doses of liquorice (figures 63, 74, 91 & 101) is the OGD/R model without any treatment, and to a lesser extent, the low dose group (ibid.; figure 1, graph B). This could also imply that high doses of liquorice are having an inhibitory effect on mitochondrial respiration, similar to OGD/R, but, as a form of IRI, this should induce mitochondrial dysfunction leading to increases in ROS and Ca^{2+} as well as loss of $\Delta\Psi m$ and cell death (He, Z. et al., 2020), none of which were observed in either cell line.

20.2.1. Possible Explanations from Clinical Uses of Liquorice

The literature does include some reports that liquorice has protective effects against IRI. Licochalcone B and D have been reported to have a cardioprotective effect against IRI in rat hearts (Han et al., 2014; Yuan et al. 2015), while glycyrrhizin and magnesium isoglycyrrhizinate have been attributed hepatoprotective roles in IRI models (Kou et al. 2020; Huang, Qin & Lu, 2014). Liquiritin has also been investigated for its potential in preserving the blood brain barrier during stroke (Li, M. et al., 2021). All these studies found reduced infarct size, apoptosis, inflammation and oxidative stress mediated by different pathways depending on the investigations being performed but usually sharing an increase in SOD and GSH, and reduced proinflammatory markers such as MDA and TNF-α.

This relates directly to a clinical usage of liquorice where it is the chief herb in the CHM formula “Prepared Liquorice Decoction” (*Zhi Gan Cao Tang* 炙甘草湯) which has been used since the Han dynasty for irregular pulse and palpitations, and remains one of the most popular formulas for cardiovascular disease in

Taiwan today (Wang, L. et al., 2022), with its reported protective effects against IRI attributed primarily to glycyrrhizin through the inhibition of apoptosis in cardiomyocytes injured by high glucose and hypoxia, and reduction of infarct size in a diabetic myocardial infarction mouse model (Hu, M. et al., 2023). Cardiovascular disease may have been indicated in ancient Europe too with one the few indications given for liquorice in Anglo-saxon England being “chest pain” (van Arsdall, 2002, p.213), or “breast pain” (Pollington, 2008, p.269). This indication survived into the 17th century with Culpeper indicating liquorice for “griefs of the breast” (Culpeper, 1653). Pretreating with mitochondrial targeted antioxidants has been reported to be an effective method of cardioprotection from IRI through reduction in cytochrome c release and subsequent apoptosis (Adlam et al., 2005) but these do not reduce OCR. This finding could suggest that liquorice is able to induce a form of hypoxia that preconditions the cells in order to improve survival in future hypoxic states. Preconditioning the heart against IRI is considered one of the most potent forms of protection from IRI, achieved by inhibition of the mPTP, thus preventing apoptosis (Cadenas, 2018), a mechanism similar to that induced by mitochondrial targeted antioxidants.

20.2.2. Possible Explanations from Mechanistic Research

During hypoxia ROS increases. This is due to metabolic stress which triggers Ca^{2+} and causes NO release from NO synthase which acts as a vasodilator to return normal perfusion to the cells during brief periods of hypoxia but also suppresses Complex IV of the ETC, thereby reducing the use O_2 as a terminal electron acceptor (Burtscher et al., 2022). This causes accumulation of electrons within the ETC which result in increased $O_2^{\bullet-}$ from complex III, succinate dehydrogenase at Complex II being changed to fumarate reductase to enable fumarate to act as an electron acceptor from reverse electron flow (Paddenberg et al., 2003) and bursts of H_2O_2 being generated from Complex I of the ETC which act as an O_2 sensitive signalling molecule (Okoye, Koren & Wojtovich, 2023). This triggers further cellular responses to hypoxia such as Ca^{2+} release from the ER (Desireddi et al., 2010) to initiate more cellular responses, or organism wide

response if the tissues involved form part of the carotid body. HIF-1α, which is stabilised in hypoxic conditions, induces the mitochondria to undergo alterations in protein composition of the complexes in the ETC, a loss of mitochondrial mass through fission and mitophagy and changes in morphology including lower cristae density to compensate for the reduced O_2 as a terminal electron acceptor while maintaining essential ROS signalling functions (Fuhrmann & Brüne, 2017).

The lack of change in ROS or Ca^{2+} at 24 hours and the eventual drop in ROS argue against hypoxia occurring. Normal ΔΨm was maintained at all time points, and ROS and Ca^{2+} was also equal to control at 24 hours (figures 56, 59 & 61). Even if the cells were surviving, the lower metabolic activity induced by these changes should result in less MTT being converted to formazan crystals and therefore the appearance of lower cell viability in this assay (Zhang & Cox, 1996; Liu & Dalgleish, 2009) but this was not observed (figure 54). Instead, the drop in OCR among the MCF10A cells and the reduction of effect in OCR in response to the mitochondrial modulating drugs in MCF7 cells in the Seahorse assay, combined with no significant changes in ROS, ΔΨm, cell viability and Ca^{2+} implies that the mitochondria are respiring normally but using less O_2. This is only possible if there is an alternative terminal electron acceptor, or if the O_2 used as a terminal electron acceptor can be recycled.

The most obvious explanation would be that liquorice increases CAT activity which converts $2H_2O_2$ into $2H_2O + O_2$ which could then diffuse back across the IMM be reused by the cells, thus reducing the amount of O_2 consumed. Whole liquorice root extract has shown increased CAT in *Caenorhabditis elegans* (Maupas, 1900), *Rhabditidae* (Reigada et al., 2019) and Licochalcone A administration has shown increased CAT *in vitro* (Chen et al., 2017) but the majority of assays only show increased CAT in relation to a stressor that has depleted it. Glycyrrhizin has been observed to restore levels of CAT that are depleted in an allergic rhinitis mouse model (Li et al., 2011) and glabridin has been found to raise levels of CAT in monocytes, but only after their depletion through glucose stress (Yehuda et al., 2011). Liquorice may also reduce levels of CAT, as was seen in a rabbit model of vibration stress (Oganesyan, 2002). Since the literature is conflicting, the most direct method of testing this would be to measure

CAT activity in MCF7 and MCF10A cells after exposure to liquorice using a CAT assay kit (Cayman Chemical, 2023).

Another explanation would be that the liquorice is able to provide an alternative electron acceptor to O_2. This seems unlikely but is not impossible. Alternative electron acceptors exist in anaerobic organisms and even mammalian mitochondria can use alternative substances such as fumarate as final electron acceptors in limited O_2 environments (Paddenberg et al., 2003; Spinelli et al., 2021). This is due to them retaining some of the anaerobic ancestral architecture in their respiratory complexes (Glass, Elbon & Williams, 2023). Some exogenous substances, such as methylene blue, have also been demonstrated to act as electron bridges, bypassing complexes I and III of the ETC by acting as electron receivers and donors, rerouting electrons directly from NADH to cytochrome c (Wen et al., 2011; Tucker, Lu & Zhang, 2018; Sváb et al., 2021) and the antioxidant study of glycyrrhizin by Ageeva et al. (2022) demonstrated the possibility that glycyrrhizin could act as an electron acceptor and prevent the binding of solvated electrons to O_2.

The main challenge for this model is that the IMM is almost impermeable so it would be necessary for glycyrrhizin to enter the mitochondrial matrix, or the electrons to leave. This could be made possible by the mechanism proposed by Fiore et al. (2004) that glycyrrhetinic acid can trigger the opening of the mPTP. Although generally associated with permanent opening to release cytochrome c and initiate apoptosis, a transient opening of the mPTP for physiological regulatory functions has been suggested (Endlicher et al., 2023), enabling the release of Ca^{2+} and ROS into the cytosol which can then act as signalling molecules, while also accelerating electron flow and lowering mtROS to restore homeostasis. This could enable the transition of electrons, or $O_2^{\bullet-}$ to glycyrrhizin outside the mitochondria whereupon it could act as an electron acceptor, or receive the electron from $O_2^{\bullet-}$, returning it back to O_2. It is even possible that glycyrrhizin could enter the mitochondrial matrix, or at least bind to the IMM through its observed ability to incorporate itself into membranes and enhance their permeability (Selyutina & Polyakov, 2019) enabling the direct capture of electrons leaving complex IV (figure 125). Mitochondrial isolation could be used to detect whether this activity is

occurring from liquorice compounds in the cytoplasm or the mitochondria themselves but this process often leads to contamination (Liao et al., 2020). Alternatively, radioactive isotopes have been used for a long time to detect interaction of compounds with mitochondria (Siekevitz, 1961), so a radioactive isotope of glycyrrhizin could be synthesised which can then be traced.

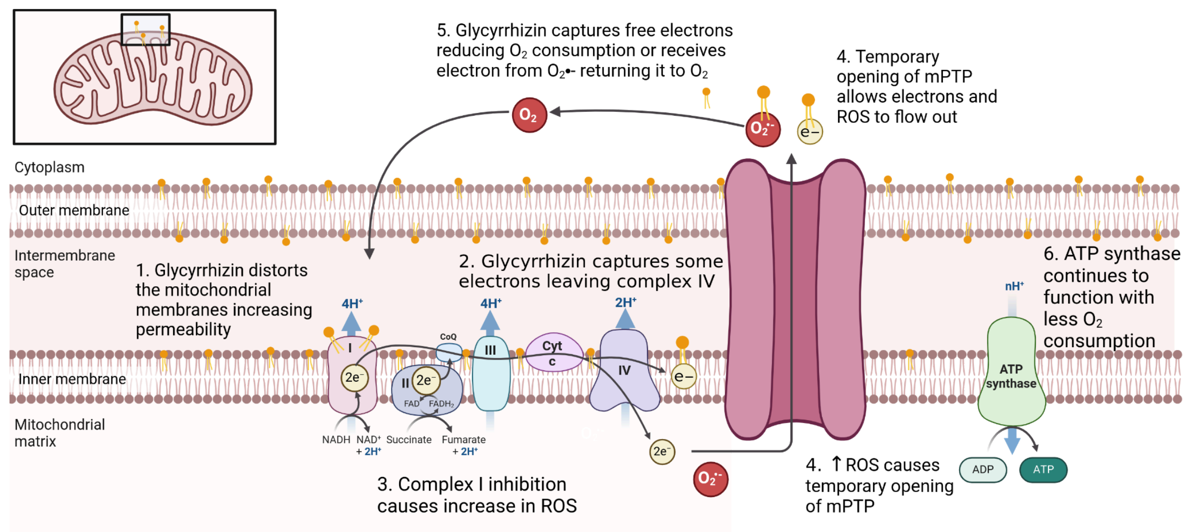


Figure 125: Theoretical Mechanisms to account for Lower Oxygen Consumption in High Dose Liquorice Treated Cells (created with BioRender.com)

This would explain how the only measure affected was OCR and the response to drugs that should be detectable through modulations in OCR, and why the decrease in OCR is only seen among the MCF10A cells. As non-cancerous cells, MCF10A use OXPHOS more predominantly than the cancerous MCF7 cells which prefer glycolysis even in the presence of oxygen (Hanahan & Weinberg, 2011), enabling them to adapt to the hypoxic environment of tumours (Zheng, 2012). This would also explain why the cells do not appear to be suffering the damaging consequences of reperfusion: if the liquorice were inducing hypoxia, then washing and returning them to normal media would induce IRI and be detected in rising Ca^{2+}, ROS and apoptosis from the sudden reinitiation of OXPHOS (figure 31, section 3.1.6.2) but if the cells have been functioning normally on reduced O_2 and the compounds from liquorice responsible for this change were still present within the cell, then only OCR would be affected.

These could explain the unusual results of the SeaHorse experiment due to the measure being OCR and the cells using an alternative substance for their respiration or being able to recycle some of their O_2. One might expect this to produce a drop in ROS as measured by DCFDA but the fluctuation between increasing ROS which causes the opening of the mPTP to restore mitochondrial homeostasis (Endlicher et al., 2023) and the antioxidant function of glycyrrhizin acting on the ROS leaving the mPTP, or directly capturing electrons, could cancel each other out, or at least produce inconsistent changes making significant results difficult to obtain.

This could also explain the drop seen in ROS among the MCF7 cells at the 2000 μg / mL dose in both extracts due to differences in this cell line. Cancer cells produce additional $O_2^{\bullet-}$ through NOX (see section 20.1.3) and glycyrrhizin may be neutralising this ROS through electron capture after 72 hours but not affecting the glycolytic favouring metabolism of MCF7 cells. Meanwhile in the OXPHOS favouring MCF10As, the electrons captured may be primarily from complex IV of the ETC or recycling $O_2^{\bullet-}$ into O_2 resulting in lowered OCR. This would also explain the improvements seen in IRI models and the clinical use of liquorice in cardiovascular disease: if the cells are able to function closer to normal in a hypoxic environment by consuming less O_2 then they would experience less reperfusion injury when O_2 is returned.

20.2.3. Testing the Theoretical Mechanism

Many experiments can be designed to test this theoretical mechanism. One of the first should test the obvious explanation, that liquorice is interfering with the Seahorse sensors. The cells are washed twice before entering the Seahorse Flux Analyzer so there should only be what is inside the cells and extruded after washing or attached firmly enough to the outside to resist washing and diffuse into the media after soaking. Performing a spectroscopic analysis of the media after assaying could reveal how much of each component of liquorice is in the media after the assay is complete. Following this the assay could be repeated once more but with some wells filled with media spiked with liquorice at the concentration that

was found in the Seahorse media during the assay and comparing the O_2 levels with blank controls that receive only media. The Seahorse Flux Analyzer measures OCR, which must be by a comparison of two O_2 readings, so either the device would require recalibration to just take a single O_2 reading, or untreated cells could be used that are given the Seahorse media spiked with the traces of liquorice. A simpler alternative could be to use the media from some treated cells after washing and preparing for the Seahorse assay. To do this, two Seahorse plates would be prepared, one with treated and one with untreated cells. The treated cells would be washed and prepared as in preparation for the Seahorse assay. After a period of incubation equal to the time taken to perform the assay (1-3 hours; usually 45 minutes to 1 hour in a non CO_2 incubator while the MitoStress assay drugs are prepared and 2 hours to perform the actual assay), their media could be removed, filter-sterilised to avoid contamination with any detached treated cells, and applied to the active arm of the washed and untreated cells. These then undergo the MitoStress assay and any unusual fluctuations in the OCR compared to the control group must then be connected to liquorice components that have been effluxed into the media in the time taken to perform the assay.

Once it is certain that the results are not equipment failure, the next test should be an alternative way to measure mitochondrial respiration to compare with the OCR readings, through the direct measurement of the respiratory end product, ATP. Alternative tests to measure ATP concentrations exist (Rajendran et al., 2016) but one of the most sensitive and reliable techniques is the bioluminescent luciferin–luciferase reaction (Morciano et al., 2017; Morciano et al., 2019). Luciferase is an enzyme derived from the North American firefly, *Photinus pyralis* L. *Lampyridae*, which uses ATP to generate its characteristic yellow-green light. When applied to cells, along with the substrate D-luciferin and magnesium ions, it oxidises the substrate into oxyluciferin to produce a luminous flash at a peak emission of 560 nm, whose brightness is proportional to the amount of ATP present and can be detected with luminescence detectors. The limitation of this technique is that it does not differentiate between the ATP of the cells and those from other substances, which may include those from the herbal materials, and which may also have their own luminescence (AIB Staff, 2013). It will also not

differentiate between ATP generated by glycolysis and OXPHOS. This could also be determined through a glycolysis stress test using the Seahorse Flux Analyzer. This assay works in the same way as the MitoStress assay but by injecting different drugs in the sequence and measuring the acidification rate from the generation of lactic acid (Agilent Technologies, 2019b). Initially, antimycin A and rotenone are injected to inhibit OXPHOS and the rate of acidification from glycolysis is measured. This is then followed by an injection of 2-deoxy-D-glucose which inhibits glycolysis giving a set of values that can be used to calculate the glycolytic acidification from other potential sources (figure 126).

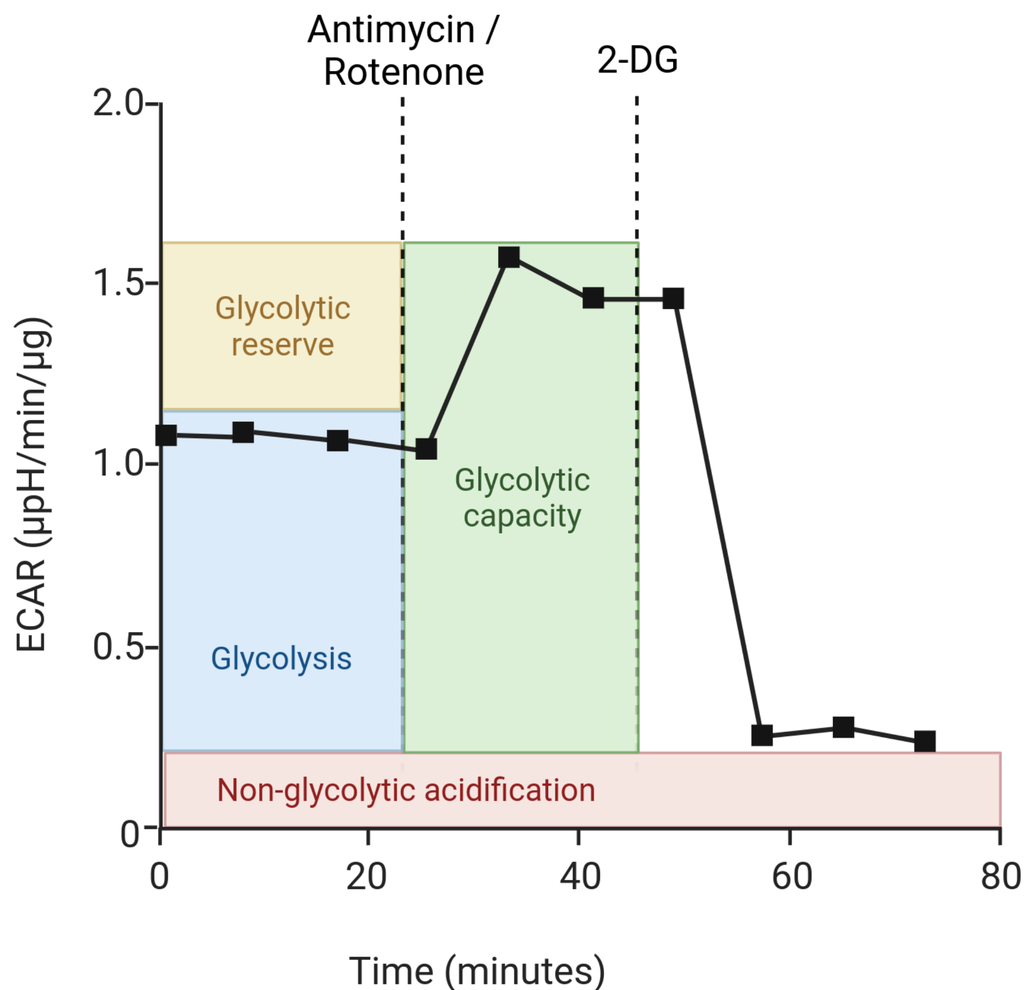


Figure 126: Seahorse Glycolytic Rate Test Example (created with BioRender.com)

This could suffer from the same problems as the MitoStress assay, since it utilises antimycin and rotenone which could be affected by components in liquorice (see the following section 20.3.1). Alternatively, glucose and lactose levels can be measured with a spectrophotometer or bioanalyzer, or the uptake of a glucose analogue such as 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxy-glucose (2-NBDG) (TeSlaa & Teitell, 2014). These may be useful if interference with the Seahorse sensors or drugs from high levels of liquorice is suspected, although the glycolysis stress test is using different sensors than the OCR assay.

One of the final ways that could be employed to test this mechanism would be actual exposure of cells to hypoxia to see if liquorice treated cells can survive for longer. The research into IRI discussed above can be brought to bear indirectly on this, but the mechanisms involved are more complex than simply O_2 deprivation (see section 3.1.6.2 and figure 31). Attempting to analyse the cells under continuous hypoxic conditions would be challenging and a proxy measurement, such as measuring the activation of HIF-1α could be problematic due to the possibility of components of liquorice inhibiting this transcription factor (Park et al., 2021; Zhou et al., 2022). An *in vitro* model of IRI cell injury is presented by Li, M. et al. (2021) which involves culturing cells in a basic media, without FBS under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) for 12 hours, before replacing the media with a complete medium containing FBS and culturing under normal conditions (5% CO_2, 95% air) for a further 8 hours. By exposing cells to various dosages of liquorice for varying times and observing the effect when exposed to hypoxic conditions and subsequent reperfusion, it would be possible to evaluate if liquorice has a genuine effect on IRI and determine its mechanisms more precisely.

20.3. Drug Resistance Assays

The effect of 2000 µg / mL OLTE to protect MCF10A cells from 24 hours treatment with cisplatin was measured through an improvement in cell viability by 8.186% ($p < 0.001$) among the 100 µM cisplatin group and 11.79% ($p < 0.05$) in the 50 µM cisplatin group (figure 88, section 14.3). This assay was developed after

receiving the first respiratory results from MCF7 cells (chapter 12) which produced such unusual results, including a spare capacity of -9.59% ($p < 0.01$) (figure 69, section 12.1), that they demanded an alternative explanation.

20.3.1. Detoxifying Effects of Liquorice

The results obtained from the MCF7 respiratory assays were reminiscent of the traditional CHM use of liquorice as a detoxifying agent (Bensky et al., 2004, p.733; Chen & Chen, 200, p.868). This led to a reinterpretation of the OCR data as modulating the effect of the drugs on the cells (section 12.3). This resulted in discovering that 24 hours treatment with 2000 µg / mL OLTE reduced the decline in OCR caused by oligomycin by 29.94% ($p < 0.01$; figure 71), reduced the increase in OCR caused by FCCP by 43.2%% ($p < 0.01$; figure 72) to remain close to the basal rate (99.82%) and reduced the effect of antimycin A and rotenone by 32.09% ($p < 0.01$; figure 73). Investigation for the cause of this effect revealed both chemical and biological mechanisms in the literature.

Chemical interactions at the stage of preparation and decoction have been investigated. Liquiritin, found primarily in Chinese liquorice (*G. uralensis*) was shown to form a complex with aconitine from aconite (*A. carmichaelii*) reducing the free aconitine in decoctions (Peter et al., 2013). A review of studies (Jiang et al., 2020), including Chinese literature, confirmed this finding that liquorice reduces the toxicity of aconite (*A. carmichaelii*) decoctions by forming chelate precipitates with the the toxic ester-series alkaloids while also reviewing the interactions of liquorice on several other herbs. Strychnine and brucine concentrations of strychnine nut (*Strychnos nux-vomica*, L., *Loganiaceae*) preparations were reduced, also by forming insoluble precipitates. Not all herbal compounds were reduced though, and liquorice increased concentrations of certain therapeutic agents from peony root (*Paeonia lactiflora*, Pall., *Paeoniaceae*) including paeoniflorin, oxidized paeoniflorin, benzoyl paeoniflorin and albiflorin. In ginger (*Z. officinale*) both effects were observed together, with liquorice increasing the extraction of 8-gingerol and 6-shogaol from ginger while reducing 6-gingerol that is considered a slightly toxic component and responsible for the “acrid and drying”

nature of ginger in CHM. Liquorice also increased the ephedrine methylephedrine of ephedra (*Ephedra sinica*, Stapf, *Ephedraceae*) extractions while reducing pseudoephedrine and hydrochloride isomers of ephedrine by forming complex salts. Two other studies found that liquorice interacted with the berberine content of Chinese Goldenthread root (*Coptis chinensis*, Franch., *Ranunculaceae*), forming a sediment that lowers intestinal absorption, both reducing the efficacy of this component but also reducing the digestive side effects associated with it (Zhang, H. et al., 2019; Zhang, J.Q. et al., 2020). While these are of relevance to clinical practice, the drugs used in the Seahorse assay were not prepared with the liquorice extract and so are not able to explain the results found in this study.

Biological mechanisms for a detoxifying effect of liquorice can be divided into four types. Effects were reported on Cytochrome P450 (CYP450) liver enzymes, glucuronidation, drug transporter proteins and direct effects on mitochondria and their associated pathways.

Reviews of the evidence for interaction with CYP450 enzymes suggests that various components of liquorice do modulate several CYP450 enzymes but that the effect is inconsistent and contradictory depending on the administrations used in different studies (Wang et al., 2013). This mechanism is also not relevant to the effects seen in this study since the liquorice does not undergo metabolism by CYP450 enzymes before being introduced to the media.

Glucuronidation remains a possible mechanism but available literature showed that while glycyrrhizin and glycyrrhetinic acid could induce glucuronidation, it could also show inhibitory activity under different circumstances, with glycyrrhetinic acid having more potent inhibitory action (Feng, Ding & Qiu, 2015). This may be due to glycyrrhetinic acid being a substrate for hepatic uridine 5'-diphospho-glucuronosyltransferase that converts it to glycyrrhetinic acid 3-O-glucuronide, responsible for its toxic effects of pseudohyperaldosteronism (Koyama et al., 2017) by competing for aldosterone binding sites. Since the main site of this glucuronidation reaction is also the liver, it is not applicable to the cultures of breast cells that were used in the mitochondrial functional analysis.

The evidence related to drug transporter proteins are the most relevant to this study. They can be expressed on a variety of cells, potentially affecting cell culture work where the substrates are not metabolised by liver enzymes before being introduced to the cells. The review articles had contradictory findings on the effects on drug transporter proteins with Wang et al. (2013) concluding that glycyrrhizin inhibits P-glycoprotein (P-gp) and glycyrrhetinic acid activates it, while Feng, Ding & Qiu (2015) reported the opposite. The most recent study found that several compounds in liquorice (glycyrrhetinic acid, liquiritin, liquiritigenin, isoliquiritin, isoliquiritigenin and licochalcone A) upregulated ATP Binding Cassette (ABC) efflux transporters P-gp, BCRP, and MRP2 and improved the transport of fluorescent Rhodamine 123 from the cells via P-gp, suggesting this to be a generic mechanism by which liquorice could provide protection against a variety of toxins (He et al., 2019). Although these are usually expressed on the cell membrane, they are dependent on ATP to function and on mitochondria for maximum efficiency (Giddings et al, 2021) and have been detected on mitochondria where they engage in biosynthesis, management of oxidative stress and chemoresistance (Schaedler et al., 2015; Zutz et al., 2009), including BCRP (Kobuchi et al., 2012; Zhang, H. et al., 2021). However, of the substances studied by He et al. (2019), glycyrrhetinic acid is the metabolised form of glycyrrhizin so suffers from the uncertainty that glycyrrhizin will have the same effect, while liquiritin and isoliquiritin are found only in *G. uralensis* (Avula et al., 2022), leaving only liquiritigenin, isoliquiritigenin and licochalcone A as potentially directly comparable.

Liquorice appears to have direct effects on mitochondrial pathways that attenuate the toxicity of other substances. Zhang, M. et al. (2019) discovered that liquorice reduced brucine induced nephrotoxicity in rats given *Semen Strychnine* (*Strychnos nux-vomica*, L., *Loganiaceae*) by reducing oxidative stress and activating STAT3 which combined, suppressed the mitochondrial apoptosis pathway. Upadhyay, Mantha & Dhiman (2020) also discovered that liquorice extract was able to protect cardiac cells from doxorubicin induced toxicity. Kim et al. (2004) and Lee et al. (2009) found that liquorice had a protective effect against cadmium poisoning by inhibiting the translocation of pro-apoptotic Bad proteins to the mitochondria. Rashedinia et al. (2019) found a similar effect with aluminium

toxicity in nerve cells, suggesting liquorice could maintain mitochondrial function, reducing ROS and increasing ATP production and induce biogenesis. Yan et al. (2016) also found an indirect effect of glycyrrhizin on mitochondrial ROS induced apoptosis of hepatocytes induced by paracetamol overdose through the inhibition of TNF-α. However, the interaction of liquorice with mitochondria and other drugs can also have the opposite effect. Chen et al. (2020) found the reason for the traditional incompatibility between liquorice and *Radix Euphorbiae Pekinensis* (*Euphorbia pekinensis*, Rupr., *Euphorbiaceae*) was because the pair increased ROS production and activated apoptosis through the intrinsic pathway.

Some studies also revealed a specific protective effect on some of the drugs used in the Seahorse Flux Analyzer. Glabridin was shown to alleviate the toxicity of antimycin A in MC3T3-E1 cells by scavenging ROS and $O_2^{\bullet -}$ generated through the inhibition of Complex III, prevented nitrotyrosine increase and thioredoxin reductase inactivation and restored phosphoinositide 3-kinase, promoting cell survival (Choi, 2011). However, this component was likely not extracted well in the tea samples due to its relative insolubility in water (discussed in section 20.1.1). Glycyrrhizin demonstrated a protective effect *in vivo* against a rotenone-induced model of Parkinson's disease by restoring SOD, GSH and CAT, thereby reducing MDA, and lowering pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Ojha et al., 2016). This was subsequently investigated *in vitro* (Karthikkeyan et al., 2020; Karthikkeyan, G., Prabhu et al., 2021; Karthikkeyan, G., Pervaje et al., 2021; Karthikkeyan et al., 2022) finding the signalling pathway involved to be primarily inhibition of ERK-1/2 hyperphosphorylation preventing the activation of Bax and Bak and subsequent intrinsic apoptosis (see figure 26, section 3.1.4). Both of these drugs are used in the Seahorse assay, suggesting the possibility that the high doses of liquorice used may be having a protective effect against the drugs being used, confounding the results, and raising the possibility that it could be affecting oligomycin and FCCP too.

20.3.2. Development of the Drug Resistance Assays

The discovery of these potential mechanisms prompted the development of the drug resistance assays. Only the 2000 µg / mL dose of liquorice was used due to this producing significant modulations of the drug effects in the MCF7 Seahorse assay. Initial investigations utilised the same drugs employed in the Seahorse assay, but measured with DCFDA, to acquire a broad measure of changes in oxidative stress from which to investigate further but did not produce any significant results (figure 84) and gave the appearance that liquorice enhanced the reductive stress induced by the drugs. However, there were several flaws in this assay which could be responsible for these results. The first is the complexity of the assay itself, involving the treatment of 8 different groups (four drugs plus four drugs after liquorice exposure), in addition to controls, which had to all be washed in order to ensure the liquorice was not chemically interacting with the drugs, within 10 minutes. Several repeats failed to generate any results, decided by a lack of any effect from the drug-only groups and were presumably from user error due to this tight timeframe.

The second confounding factor is the antioxidant effects of liquorice (see section 20.1) which could create the illusion of having an additive effect to the drugs when measured using DCFDA. Antimycin A inhibits complex III and rotenone inhibits complex I, which are the main producers of ROS in the ETC (Bleier & Dröse, 2013). Meanwhile, FCCP is known as an uncoupling agent and uncoupling is a physiological mechanism used to reduce the production of ROS (Hass & Barnstable, 2021). Therefore, the antioxidant properties of liquorice could give the appearance of enhancing the effects of these drugs when measured with DCFDA when it is actually functioning as an antioxidant.

Another reason is that a “one size fits all” approach to the timing of the drug administrations may not have been appropriate. Oligomycin has been reported to have no effect on neuronal survival after 30 minutes (Chen et al., 2020) and inhibits OXPHOS after 1 hour (Hao et al., 2010). Therefore, a 10 minute window to observe effects may be sufficient to produce changes in OCR but it may not be

sufficient to see effects from other assays and the same could be true of antimycin A and rotenone. A preferable approach would be to use simpler individual assays optimised for each drug. The first of these could have been to use FCCP as recommended as a positive control for the detection of $\Delta\Psi m$ modulations in a TMRE assay (Abcam, 2022) compared to a liquorice and FCCP group. This could be followed by separate assays for oligomycin, antimycin A and rotenone optimised to detect their specific effects based on examples found in the literature.

Instead, it was decided to use cisplatin, a drug with known cytotoxic effects through induction of mtDNA lesions (Oliveira et al., 1995), causing increased ROS (Choi et al., 2015) and initiating apoptosis primarily through the mitochondrially mediated intrinsic pathway (Ghosh, 2019), whose toxicity could be easily measured through an MTT assay to assess cell viability after 24 hours exposure. Previous and current members of the laboratory team had experience using this drug and its reliability in measuring mitochondrial apoptosis (Mould, 2019; Henley, 2015; Henley et al., 2017) so advice could be sought on its execution. These previous studies had researched "priming," a mechanism whereby increasing oxidative stress in cancer cells without affecting their non-cancerous analogues enhanced the chemotherapeutic potential of cisplatin without increasing overall cytotoxicity. This assay would test for an opposing effect: that liquorice pretreatment would reduce the cytotoxicity of cisplatin treated cells. The dose of OLTE remained at 2000 µg / mL due to this producing a protective role against the drugs in the Seahorse assay.

20.3.3. Cisplatin Resistance Assay Results

Pretreatment with 2000 µg / mL OLTE followed by 24 hours treatment with cisplatin produced no effect in MCF7 cells (figure 86) but revealed a small but consistently higher cell viability in the MCF10A cells treated with 50 µM and 100 µM cisplatin than those not pretreated with 2000 µg / mL OLTE (figure 88). Those treated with 50 µM cisplatin saw an 11.79% increase ($p < 0.05$) and those treated with 100 µM cisplatin saw a 8.186% increase ($p < 0.001$). The difference between the protection conferred at 50 µM and 100 µM cisplatin was not significant (3.6%;

$p < 0.5465$), suggesting that this cytoprotective effect is more likely to activate as the dosage of the cytotoxic agent increases but once it is activated, the result is the same.

This result was the opposite of that discovered in the Seahorse respiratory assays where the MCF7 cells displayed a resistance to the drugs while the MCF10A cells showed only a drop in basal OCR. It can be speculated that this is connected to the reasons behind the drop on OCR among the MCF10A cells discussed in 20.2 potentially distorting the respiratory readings and making them an unreliable marker of the true changes in mitochondrial activity.

The cisplatin resistance effects only being seen among the MCF10A cells can be explained through the mechanisms described above. The study by He et al. (2019), revealing an increased expression of ABC efflux transporters induced by several components of liquorice, provides the most likely explanation. Cisplatin resistance has been associated with expression of P-gp (Li et al., 2018), BCRP and MRP2 (Herraez et al., 2012) that were upregulated in response to liquorice constituents. Additionally, the efficiency of ABC transporters is connected to mitochondrial ATP (Giddings et al, 2021) which is the favoured system of ATP generation in non-cancerous cells. In contrast, cancer cells favour glycolysis (Hanahan & Weinberg, 2011), suggesting a less efficient functioning of ABC transporters. The assays conducted by He et al. (2019) to measure expression of ABC proteins including western blot and measurement of efflux using fluorescent dyes effluxed by particular transporters, could elucidate which ones are being expressed.

The antioxidant potential of glycyrrhizin discussed in section 20.1 could also provide another mechanism for the chemoresistant properties of the liquorice treated cells, lowering the elevation in ROS that leads to apoptosis in cisplatin induced cell death (Choi et al., 2015). However, modulations in ROS were only noted in MCF7 cells in the DCFDA assay which were unaffected by pretreatment with liquorice with regards to resistance to cisplatin, although potential cellular mechanisms that restore depleted levels of endogenous antioxidant enzymes but do not initiate reductive stress in ordinary conditions is possible. Further DCFDA

analysis after treatment with liquorice and cisplatin at different time points could reveal whether an antioxidant effect is responsible for the enhanced cell viability observed in MCF10A cells after 24 hours pretreatment 2000 µg / mL OLTE followed by cisplatin.

Chapter XXI

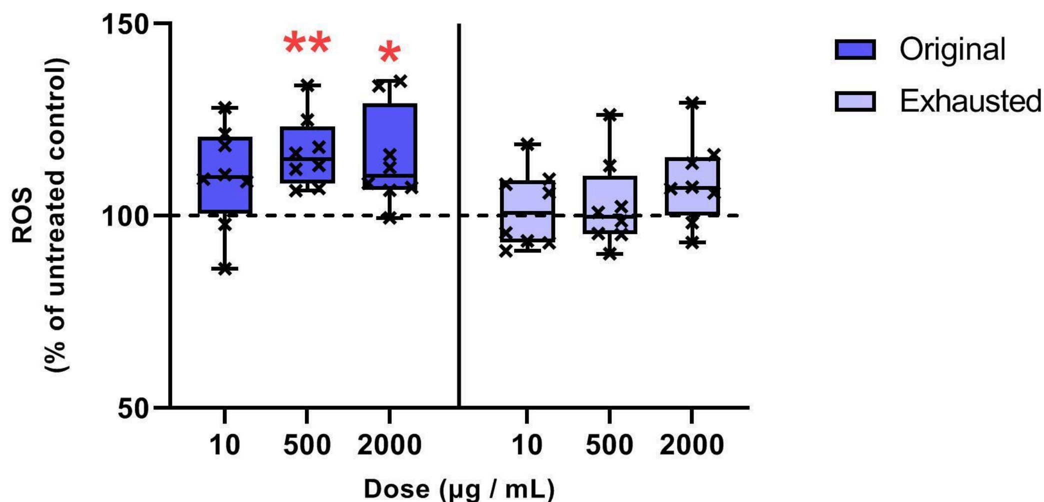
21. Adulteration Detection

21.1. Ability of Mitochondrial Analysis to Detect Functional Adulteration

The alternative hypothesis was that a medicinal herbal tea (liquorice) will induce specific mitochondrial modulations that enable a measurement of functional quality and identification of adulteration (section 5.3). In relation to detecting adulteration and potency, the most important factors are whether these results are unique to one of the liquorice tea samples and reproducible enough to provide a reliable evaluation of quality or adulteration. The areas where there were significant differences induced by the OLTE included the production of ROS in MCF7 cells, the OCR measured by the Seahorse Flux Analyzer, and the cytoprotective effect conferred against cisplatin-induced cytotoxicity in MCF10A cells. Some of these modulations were not replicated by XLTE enabling differentiation between OLTE and XLTE, even when administered at the same dosage of freeze dried material, and which were difficult to differentiate through visual inspection and HPTLC analysis. A discussion of each of these tests and its suitability for adulteration detection will now be undertaken.

21.1.1. Modulations in ROS

The first significant change to be observed that could differentiate the two extracts was the rise in ROS in the MCF7 cells after 3 hours of exposure to 500 µg / mL OLTE (16.51%; $p < 0.01$) and 2000 µg / mL OLTE (14.88%; $p < 0.05$) which was not reproduced by the XLTE at any dose (figure 127).



Reactive Oxygen Species (ROS) measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) represented as % change from untreated control drawn as dotted line at 100%.

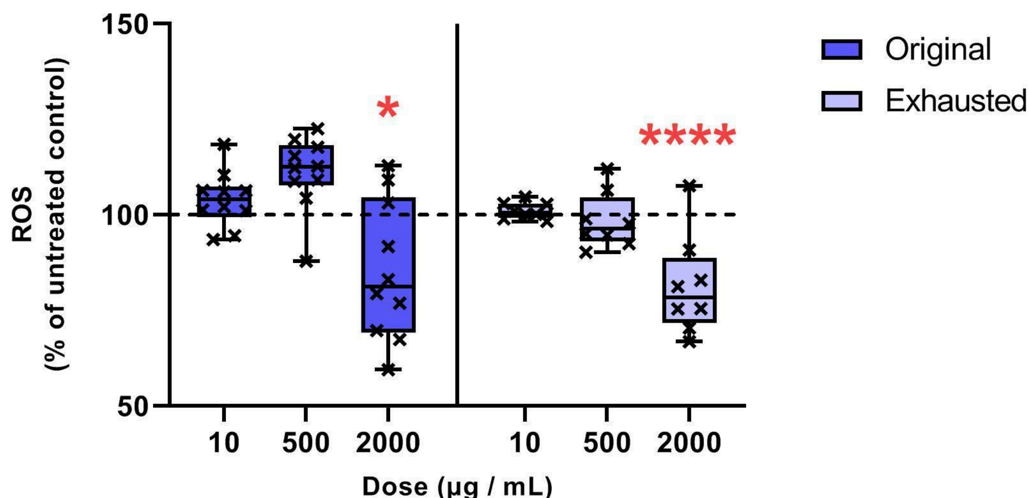
n=8.

* indicates significance ($p < 0.05$) & ** indicates significance ($p < 0.01$) compared against control using one way ANOVA with Dunnett's multiple comparisons.

Figure 127: Comparison of Original Liquorice Tea Extract and Pre-extracted Liquorice Tea Extract on ROS Production in MCF7 Cells after 3 Hours

The short time point and ease of this assay would make this rise in ROS among the medium and high dosages appealing for a rapid test. However, many herbs can have pro-oxidant effects on cancer cells while having antioxidant or no effect on normal cells due to their differing metabolism (Mahanta & Challa, 2022; Fernando, Rupasinghe & Hoskin, 2019) making this rise in ROS at 3 hours unlikely to be unique to liquorice. Therefore, this marker used alone, would not suffice for the identification of potency or adulteration in liquorice.

This was followed by returning to levels equal to control after 24 hours and then dropping significantly in the 2000 µg / mL dosage group in both the OLTE group (14.73%; $p < 0.05$) and the XLTE group (18.7%; $p < 0.0001$) (figure 128). The mechanisms for this are discussed in section 20.1.



Reactive Oxygen Species (ROS) measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) represented as % change from untreated control drawn as dotted line at 100%.

n=8.

* indicates significance ($p < 0.05$) & **** indicates significance ($p < 0.0001$) compared against control using one way ANOVA with Dunnett's multiple comparisons.

Figure 128: Comparison of Original Liquorice Tea Extract and Pre-extracted Liquorice Tea Extract on ROS Production in MCF7 cells After 72 Hours

The drop in ROS was seen in both groups, to a similar degree (14.73% OLTE; 18.7% XLTE) despite the XLTE being far more significant ($p < 0.001$ compared to $p < 0.05$). This would also make differentiation between the two extracts problematic using this test alone. If the combined effect of raising ROS at 3 hours, returning to normal at 24 hours and dropping at 72 hours can be consistently reproduced with other authenticated samples of liquorice and the same pattern is not seen from an adulterated batch, or from other herbs once the data expands, then this combined result could be considered a potential marker using redox status as a fingerprint for *G. glabra*.

21.1.2. Respiratory Measurements

The apparent interference of liquorice with the drugs used in the Seahorse Assay (see section 20.3.1) presents a significant obstacle in the use of the Seahorse MitoStress assay to assess functional potency and adulteration in herbal

medicine. The injection of mitochondrial modulating drugs is essential to the Seahorse MitoStress assay (Agilent, 2019a), which relies on calculations between the effects of all four drugs to determine the mitochondrial parameters being measured. If a single drug fails to work, then the results cannot be used, so if liquorice reduces the effectiveness of any of the drugs, then the results of this assay may be compromised. Since liquorice is included in around half of all Chinese formulas (Guo et al., 2014) and many commercially available tea blends (Rolfe, 2023; Yogi Tea, 2023) and supplements (Aura Nutrition 2023a), evaluations using the Seahorse assays may be problematic when being used to evaluate herbal tea blends. This problem may also extend beyond adulteration research which will be discussed fully in section 23.2.

The use of the Seahorse Flux Analyzer may have other issues when used as a method of detecting adulteration too. Firstly, it is a specialised piece of equipment that requires training and may not be available in the laboratories available to many herbal supply companies. In the UK, medicinal teas often come under food regulations that only require the correct ingredients listed and do not require a proof of efficacy, provided they do not make medical claims. When they do, it is possible to be registered under the Traditional Herbal Regulation scheme which only requires evidence of traditional use and not efficacy (Heinrich, 2015). This means that many small companies who supply herbal teas exist and are unlikely to have this specialist piece of equipment, or the expertise in its use, restricting this kind of analysis to larger corporations. Simple assays that only require a sterile hood and incubator, and tests that can be easily performed may be more accessible to these smaller businesses. Ease and affordability will likely appeal to many larger companies too. Second, It also takes approximately 2 hours to take Seahorse readings, during which time the equipment cannot be used by anyone else, with additional preparation before and the protein assay after, placing a bottleneck on the speed of the results compared to assays where many repeats using different populations of cells by multiple members of staff can incubated at the same time. This will be a major obstacle in quality control where industry will want a rapid turnaround in order to confirm the quality of their products for their intended use before sale. However, the Seahorse Flux Analyzer did provide some

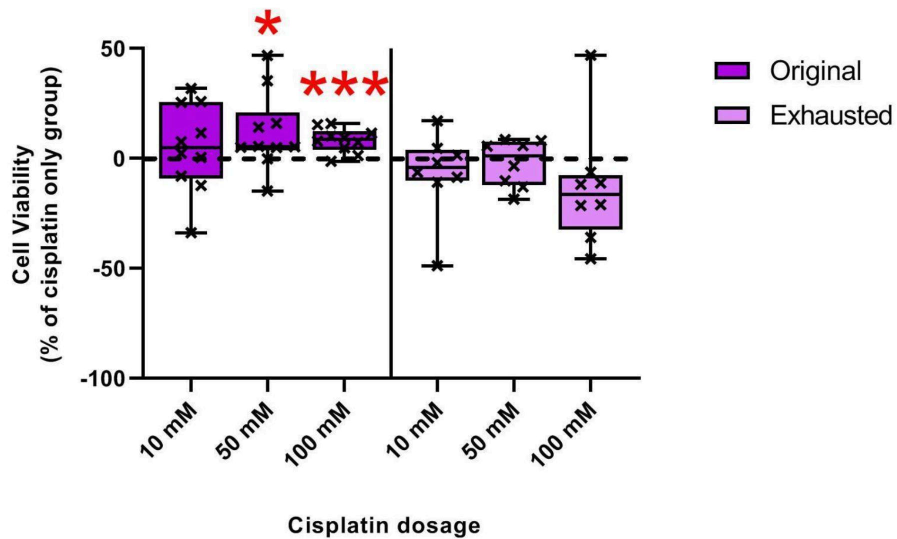
valuable insights that inspired simpler assays which obtained results that could be used for functional evaluation making it a useful tool for basic research.

One such assay was the cisplatin resistance assay inspired from the MCF7 respiration results (see section 20.3) and the results from the MCF10A respiratory assays could also inspire other simple tests. The proposed mechanism behind the drop in OCR amongst MCF10A cells without any apparent inhibition in function could also be used to devise some functional mitochondrial assays unique to liquorice. Should liquorice prove to be an effective protective agent against IRI, as proposed in section 20.2, then this model could be used to evaluate adulteration. The *in vitro* model of IRI cell injury presented by Li, M. et al. (2021) described in section 20.2.3, involving FBS and O_2 deprivation and return to normal media to model IRI, could present another test by which to functionally evaluate liquorice.

21.1.3. Cisplatin Resistance

The protective effect of the OLTE against cisplatin induced cytotoxicity occurred only in MCF10A cells and was the best indicator of its functional potency for several reasons. These include its replicability and the fact that it was directly related to a clinical indication.

The effect of OLTE to protect MCF10A cells from cisplatin exposure was highly replicable with only one result out of ten repeats in the 100 μM cisplatin group being below the non-liquorice treated group, and this only 1.29% below (figure 129). This gave it a significance of $p < 0.001$, suggesting a strong likelihood of it being repeated in future experiments. Conversely, there was no significant difference in the XLTE treated MCF10A cells, suggesting this result is highly replicable and specific to OLTE, making it the optimal quality indicator found in this study.



Liquorice groups treated with 2000 µg/ml freeze dried liquorice tea extract for 24 hours prior to exposure to cisplatin for 24 hours and presented as difference between the cisplatin only group and the liquorice & cisplatin groups. n=10 for original liquorice sample, n=8 for washed out liquorice..

Cell viability measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represented as % change from group not treated with liquorice and given same dose of cisplatin.

* indicates significance ($p<0.05$) compared to equivalent dose of cisplatin only and *** indicates significance ($p<0.001$) compared to equivalent dose of cisplatin using a students t-test.

Figure 129: Comparison of Original and Pre-extracted Liquorice Tea Extract on Cell Viability When Used as Pre-treatment for 24 Hours Before 24 Hour Exposure to Cisplatin in MCF10A cells

This action of protecting MCF10A cells from cisplatin was directly derived from a traditional use (Bensky et al., 2004, p.733; Chen & Chen, 200, p.868) in contemporary clinical practice where it is actively used to counter the side effects of chemotherapy and radiotherapy regimens (Wang, K. et al., 2022; Jain et al., 2022). This makes it representative of a functional effect in relation to a clinical application. It is also reassuring for clinicians that the cytoprotective effect of liquorice tea extract is achievable with a water extract, and hence applicable to commonly used decoctions, and that protection was not conferred to the cancerous MCF7 cell line. The implications of these results for cancer research will be discussed further in section 23.3.

21.1.4. A Question of Dose

One criticism of applying these results to clinical use is the dosage used in the study. The dosage of 2000 µg / mL equates to 10 g of extracted material in the bloodstream of a person with 5 L blood, the average estimated amount in an adult human (Sharma & Sharma, 2023). Since the extraction rate was 377.33 mg / g, this makes it the equivalent of 26.5 g of fresh liquorice, far beyond the safe daily amount of 0.2 mg / kg / day, estimated at 6 g / day by van Gelderen (2000), although doses occasionally reach this level in CHM for short periods (Bensky et al., 2004, p. 732). Furthermore, as no metabolization occurred, this is the equivalent of 26.5 g liquorice being extracted and injected. Although an unrealistic comparison to *in vivo* dosages, parallels between human and animal *in vitro* and *in vivo* trials are still the optimal way to determine mechanisms (Mattes, 2020) and so the parallel between the results of this study and a clinical indication represents a starting point for this kind of research. For quality evaluation purposes, a precise *in vivo* translation is not necessary. Provided that a measurable and predictable effect is seen in the original sample and not in the adulterated sample, it can be used as a quality marker, and the parallel to a clinical indication implies that the quality indicator may share some of its mechanism with its clinically intended purpose.

21.2. Comparison with Other Methods of Adulteration Detection

To determine whether there is any benefit to mitochondrial testing of herbal quality and potency, it is important to consider whether other methods can achieve the same result, especially if they are faster and more cost effective. In this study two alternative methods were utilised, a simple visual inspection of the two samples, and an HPTLC analysis.

21.2.1. Visual Inspection

Examination by visual inspection is important since there would be no need for further testing if the two extractions could be easily differentiated by visual inspection alone. The XLTE was paler than the original extract, but not to a degree that would cause alarm and this difference disappeared with only slight alterations in positioning and lighting conditions (figure 113).

Further organoleptic testing could have been undertaken. This is still used in the quality evaluation of medicinal herbal materials (Dentali, 2013), especially among herbalists themselves who may not have access to sophisticated laboratory equipment. A comparison of taste could have been undertaken, given that the primary sensory quality attached to liquorice is that of sweetness, but was not performed due to the limited amount of the XLTE material that was obtained after the sample boiled over in the freeze dryer (see section 7.2) and the ease of deceiving this method with added sugar and anise oil, a form of adulterating liquorice so common in confectionary that it accounts for the majority of "liquorice" products sold in the United States (NCCIH, 2020). In addition, the HPTLC analysis suggested that glycyrrhizin content, which is primarily responsible for the sweet taste of liquorice, was not substantially different between the OLTE and XLTE samples. The HPTLC Association (2019) guide shows glycyrrhizin eluting at 0.17 Rf and forming the first quenching zone in the samples of all species. In the HPTLC analysis presented in section 19.2, this zone corresponds to 0.27 Rf and is present in both samples, and only to a slightly faded degree in the XLTE (figure 124), suggesting that they would both likely have tasted sweet.

21.2.2. HPTLC Analysis

The HPTLC analysis (section 19.2) revealed some clear differences between species and extraction solvents but the crucial analysis, to differentiate the functionally adulterated sample from the original when prepared at the same strength, was difficult.

It was easily possible to differentiate between *G. glabra* and *G. uralensis* on the presence of liquiritin (figure 119) and the extraction method used for *G. glabra* by the presence of glabridin (figure 120). It was also possible to differentiate between two samples of *G. glabra* from different sources, with a number of additional brown bands appearing in the sample sourced from the University of Westminster Polyclinic dispensary (track 2), and the Pukka “3 Licorice” tea bags (track 1) due to the presence of many additional brown bands in the polyclinic sample after derivatization (figure 121). This may be due to one sample being medical grade while the other is food grade liquorice, or due to the different storage conditions. The Pukka “3 Licorice” tea bag had been stored in the laboratory inside its original box and individual envelope, while the dispensary sample was stored inside a large airtight glass jar. The author’s own previous experience as a dispensary technician at the University of Westminster suggested that Western herbalists tend to prescribe tinctures over raw herbs, and when they do prescribe raw herbs, it is usually leaves and flowers that infuse quickly, rather than roots and bark which need a longer decocting time for extraction (Jeanroy, 2022), implying that the raw liquorice root could have been stored and exposed to a relatively large volume of air for a long time, changing some of the components.

It was also possible to differentiate the OLTE from the XLTE, when prepared at the dosage that would be expected from the same amount of starting material (if the tea bag was reused, yielding approximately 20% of the freeze dried material). This was observed by a considerable fading of all bars (figure 122).

The most challenging was the comparison between OLTE (track 4) and XLTE when prepared at an identical dosage of freeze dried material (track 5), as was used in the mitochondrial functional analysis. The differences were slight and may be within a normal range of variation and were harder to differentiate after derivatization (figure 123). This may be due to the derivatization process making compounds more visible and masking the quantitative differences between the two extracts. This agrees with the assessment of Gafner et al. (2023), that HPTLC is mainly qualitative, and the detection of pre-extracted material is problematic for this kind of analysis.

Alternative forms of chromatography could have been explored. HPLC is more accurate for quantitative assessments and may have been better suited to detect alterations in the levels of specific compounds (Loescher et al., 2014) which might be able to differentiate the two samples. However, HPTLC is the accepted standard for determination of quality in pharmacopoeias (Cañigueral et al., 2018) and a sample that passes this analysis is likely to pass industry testing too.

21.3. Relationship to the Hypothesis

These results show that some of the mitochondrial tests did produce significant, measurable effects and that some of these effects were specific to the OLTE and not XLTE. Furthermore, comparison by HPTLC, the main method for routine testing of herbal materials today, was challenging whereas the mitochondrial tests produced clear differences. Therefore we can presently reject the null hypothesis and retain the alternative hypothesis that "a medicinal herbal tea will induce specific mitochondrial modulations that enable a measurement of functional potency and identification of adulteration" for further testing.

21.4. Issues Associated with Mitochondrial Functional Analysis to Detect Adulteration

One of the main drawbacks of mitochondrial functional analysis as a means of adulteration detection is the length of time it takes to perform. Each assay requires a day for seeding, the specified treatment time which was between 3 and 72 hours, and the time taken to perform the assay, usually 1-5 hours, and then each assay has to be repeated enough times to perform statistical analysis, a minimum of 3, although more is preferable (Morriswood, 2017). This means that, even if a lab is equipped to be running multiple assays on several distinct populations of cells simultaneously, it will still likely have a turnaround time of weeks instead of hours using chemometric methods.

A second major limitation to mitochondrial functional analysis is that it is impossible to prove adulteration through this method, only that the sample does not meet the standard criteria. While genetic profiling can reveal the presence of DNA that is not inherent to the target plant's DNA and chemical fingerprinting can forensically reveal the addition of foreign substances (see chapter 2), functional analysis can only indicate that a sample does not perform in the assay as expected when compared to a voucher specimen or standard. There can be other reasons for a crop failing to meet the standard such as environmental factors during the development of the plant which can affect the synthesis of secondary metabolites (Li, Y. et al., 2020). These may be deliberately introduced crops from poor growing regions or using substandard cultivation techniques, but they could equally be due to annual variations in weather producing a poor harvest, despite the best efforts of the farmers. Although mitochondrial analysis may not be able to provide a forensic analysis of how the adulteration occurred, it can still serve as a bar above which medicinal herbs are expected to reach or be rejected from use regardless of the cause.

Another potential issue is that mitochondrial functional analysis could be deceived by the addition of substances aimed to affect the mitochondria in similar ways. Quality evaluation and adulteration technologies tend to move together whereby novel technologies developed to assess the quality of herbs are soon matched by fraudulent dealers developing ways to deceive these technologies and make a substandard product appear correct. From the manufacture of wooden pips to make a fake "raspberry jam" appear like the genuine article reported by Sylvia Pankhurst in 1931 (Lawrence, 2003), to attempts to deceive chemometric testing by adding adulterants that mimic chromatography profiles of active or marker compounds (Booker, A., Frommewiler et al., 2016), there is an ongoing competition between those who adulterate supplies and quality assessors trying to detect them. This raises a possibility of adulterants being added that will mimic the mitochondrial activities of plants. For example, as discussed in section 3.2.5, the metabolic effects of cinnamon have been identified as potentially being due to an uncoupling effect which temporarily lowers ATP, followed by a hormetic response to stimulate mitochondrial biogenesis, leading to the browning of adipose tissue (Li, X. et al., 2021; Kwan et al., 2017). This could lead to the addition of other

uncoupling agents, including natural extracts and synthetic drugs which have been discovered to have uncoupling effects (Goedeke & Shulman, 2021). Some of these can cause acute toxic responses (Grundlingh et al., 2011) suggesting that this kind of adulteration could be as dangerous as other functional adulterations of herbs, such as sibutramine in weight loss supplements (Jairoun et al., 2021). This could be especially relevant if the mitochondrial effect being tested for was cytotoxicity against cancer cells that could be mimicked with chemotherapeutic drugs. However, the likelihood of being able to add an adulterant that both mimics the mitochondrial effects and is undetectable through chemometric analysis will be far more challenging than deceiving either method when used alone. This makes mitochondrial functional analysis an additional angle to the orthogonal approach already suggested by Gafner et al., (2023) involving visual, microscopic, organoleptic, chromatographic and spectroscopic methods to keep ahead of fraudulent suppliers. Unfortunately, they also report that fraudulent suppliers nearly always find ways to evade detection, even deliberately sending their adulterated material for quality evaluation to discover what passes the existing tests. Therefore, it is inevitable that methods of deceiving mitochondrial functional analysis will be created. The goal of using mitochondrial functional analysis in quality evaluation, like other methods before it, can only be to create an additional layer of complexity to adulteration, meaning fewer suppliers can engage in the practice unnoticed.

Chapter XXII

22. Limitations of this Study

There were several limitations to this study, including the method of adulteration used, that mixtures of genuine and adulterated supplies were not tested, and that only one herb ended up being tested. This section will only discuss the limitations, while the steps that can be taken to address these will be discussed in the section on future work (section 24).

22.1. Method of Adulteration

One of the main limitations of this study was the method of adulteration that was used. Despite requesting samples of herbs that failed quality evaluation from the supplier of the original herbal tea (Pukka, UK) and contacting another researcher in quality evaluation who was working on liquorice (UCL), no samples were obtained leaving us to devise a method of functional adulteration that we could generate in the laboratory. This means that the method is still only a proof of concept and remains to be tested on real-world samples of adulterated material. Although the form of adulteration chosen is reported in the literature with other herbs (see section 2.6.1), there is no evidence that pre-extracted material is specifically employed for liquorice. In addition, the concerns regarding glycyrrhizin raising blood pressure have created a market for deglycyrrhizinated liquorice (Öztürk et al., 2017), meaning that there is less incentive to sell pre-extracted liquorice as an adulterant when it can be openly marketed and promoted as safer by manufacturers.

Given more time and resources, it could have been possible to locate some real-world samples or create equivalents that more closely imitate how liquorice is known to be functionally adulterated. Liquorice is preferred to be foraged from the wild because cultivated roots lack the same potency (Brinckmann, 2020), which would make cultivated vs. wild foraged roots an excellent comparison. A search for

farmed liquorice did not produce any results and this kind of liquorice is probably not marketed as such due to its purported inferiority, but is simply lacking certifications like FairWild, that require products to be traceable to ensure their wild foraged origin (FairWild Foundation, 2010: principle 10.2). Products with this label have a marketing advantage to sellers, suggesting they are ethical and of superior quality, but there is no marketing advantage to advertising an inferior farmed sample as such. For a scientific study, absence of evidence should not be considered evidence of absence (Wright, 1888), so using a sample that simply has no certification should not be taken as an assumption that it is farmed or inferior. A farm could have been contacted directly and liquorice farms have even been reopened in Pontefract, once the home of UK liquorice, for use in sweets (Smithers, 2012). This could also suggest a comparison of liquorice “terroir” since the UK soil and environment should be expected to produce a different product than one from Kazakhstan, Georgia and Egypt, as the sample used in this study is said to come from (Pukka Herbs, 2020a; Rolfe, 2023). An even better comparison would have been if the researcher could have grown samples in different conditions, both ideal and sub-optimal, to reveal the pattern of efficacy and constituents of several different conditions. This would have taken time, resources and agricultural knowledge that were not available to the researcher during this study but could be options in the future, if working with a team.

A similar critique could be made with regard to storage techniques. If planned from the start, it would have been possible to deliberately store the same liquorice tea that was used for the initial tests in an inappropriate condition, e.g. opened and exposed, and when the time came to test the adulterated sample, the badly stored sample could have been used. It was not expected that the characterisation of liquorice would take so long, with the first year seeing laboratory access closed during most of 2020 and restricted for much of the following year due to Covid-19. The sudden nature of the lockdown, which occurred just as the protocol for the original extract was about to be executed, meant that the possibility of taking advantage of this time to naturally age some of the liquorice was not available. Only accelerated shelf-life testing, as used in the food industry, would have been possible but this must be validated against real-life storage conditions to define its accuracy (Betts & Bakhai, 2023), so would not

have been appropriate. The liquorice stored in the University of Westminster dispensary could also have been a potential candidate, especially if the additional brown bands seen on the derivatized HPTLC chromatogram (right column in figure 121) were evidence of excessive exposure to air for a long period, but this is only speculation. The dispensary records could be requested to determine how much time had elapsed since the sample had been purchased and opened, and the mitochondrial functional assays repeated on this sample alongside a recently acquired sample for comparison. The alternative explanation was that these differences could be due to food and medical grade liquorice, which could also be the basis of another test: acquiring samples from herbal medicine suppliers and others intended for use in the food or confectionary industries to compare their effects on mitochondrial function.

22.2. Mixing Adulterated and Original Extracts

Another limitation of the study was that it did not look at mixtures of the original and pre-extracted material to discover the proportion at which the effects disappeared. This is a significant limitation because companies that adulterate dried herbs with pre-extracted material, or employ the double extraction method of enhancing their yields for supplements, are likely to combine the two extracts to evade detection. The XLTE yielded approximately 20% of the original extract, so another set of tests, which examined a ratio of 5:1 OLTE / XLTE, would have provided some useful real-world data regarding the potential reduction of effects if the two extracts were combined. Other ratios could also have been tried to find out when the functional effects ceased to be significant. Finally, this study did not try extracting a third and fourth time, until no new material was produced. This could have been useful since any manufacturer that will try to enhance yields by double extraction is likely to try to continue extracting until the yield no longer provides a profitable return.

22.3. Other Herbs and Blends

The original aim of the study was to test several medicinal herbal teas but due to the loss of laboratory access during the coronavirus pandemic, it became only possible to test liquorice. Liquorice was selected because it is present in most herbal blends, both in CHM formulas and commercial infusions (Wang et al., 2013; Guo et al., 2014; Yogi Tea, 2023; Pukka Herbs, 2020a) and due to its presence as a stand-alone tea among the donors' range. Therefore, any effect of liquorice needs to be accounted for when examining blends. Even without the impact of coronavirus on laboratory access, the option of testing multiple herbs and blends may still not have been possible given how many potential avenues for research were revealed during the evaluation of liquorice. Instead, the current study stands as a proof of concept and the full development of a pipeline for many herbs and products may have to be developed over time with greater resources. A plan for this and a priority list of future herbs to profile and blends to be examined first is presented in the section on future work (section 24.4 and 24.5).

Chapter XXIII

23. Implications of the Results for Other Research

One of the great advantages of mitochondrial testing to functionally evaluate medicinal herbal teas is that it can operate reciprocally with other areas of science and medicine. The results gained from mitochondrial quality evaluation can initiate or inform research and practice in other fields, while the current interest in mitochondrial aspects of disease can inform quality evaluators on the parameters that they can look for. As part of the pipeline process to develop standards (see section 24.3), any tests chosen for quality evaluation need to be reproduced to ensure that they are reliable markers and not unique occurrences, since reproducibility in scientific literature can be as low as 10% (Baker, 2016) with estimates of 40% in cancer biology (Errington et al., 2020), a field closely related to mitochondrial functional testing due to the importance of mitochondria in cancer formation and apoptosis (see section 3.1.6.3). This can then feed back to the research community with rebuttals if the results cannot be reliably reproduced and adopted as a standard if they can.

23.1. Implications for Adulteration Research

For the first time, this study shows that the functional adulterations of herbal medicines described in the 5th century by Tao Hongjing may be detectable through mitochondrial profiling. This involves herbs that are the correct species but have been tampered with in ways that reduce their clinical efficacy and can occur through incorrect procedures when growing, harvesting, storing or handling but are still sold as medicines (Liu, 2021). The relative ease of detecting species through genomic or chemometric fingerprinting and functional adulteration through the detection of drugs (see chapter 2) has left a large gap in adulteration research regarding poor quality herbs. The current study suggests that efforts can go further, identifying the functional capability of herbs to achieve their intended result through *in vitro* assays. This marks a break from previous forms of functional

quality evaluation which have not changed since Galen’s testing of antivenom on chickens in 2nd century Rome (Karaberopoulos, Karamanou & Androutsos, 2012), or the testing of ginseng (*P. ginseng*) on runners in the 11th century (Shang & Gong, 1994). The costs and ethical considerations of animal and human testing have caused functional evaluation to disappear from routine quality evaluation protocols but *in vitro* protocols could allow them to return.

One important finding from this research is that the assays performed to evaluate functional testing should be relevant to clinical applications. In all the basic tests performed, only a rise in ROS after 3 hours was significant and different between the two samples (figure 127), a finding that returned to normal at 24 hours. The Seahorse results also led to a complex picture among both cell lines (figures 63-69 & 74-83) that remained unexplained in the follow up assays, despite inspiring the cisplatin resistance assay which achieved the most repeatable and clinically applicable result to date. This suggests that when designing functional adulteration tests, the assays employed should also be functional, inducing a cellular model of a pathological state and assaying if the herb can affect this condition. Besides direct applicability to the clinician, the advantage of this approach is that it is equally applicable to single herbs, blends, teas, powders or extracts. It also avoids the issues of substitution discussed in section 2.6.2 because a substitution that is able to achieve a functional effect on a specific cellular model can be considered a functionally correct substitution. Therefore, different substitutions aimed at different ends, like the substitutions for aconite (*A. carmichaelii*) in *Jin Gui Shen Qi Wan* made by Aura Herbs and GinSen, described in section 2.6.2, which are focused on replicating different aspects of the original formula, can also be evaluated using different models.

23.2. Implications for Research into Liquorice

These results could have an impact on research into liquorice using drug induced models of disease. The idea that liquorice may have a protective effect against drugs came partly from the research findings of the Seahorse experiment, partly from the traditional use of liquorice in TCM but also from the literature that

suggested liquorice could have a protective effect against Parkinson's disease though the use of a rotenone induced model of disease in rats (Ojha et al., 2016) and cell cultures (Karthikkeyan et al., 2020; Karthikkeyan, G., Prabhu et al., 2021; Karthikkeyan, G., Pervaje et al., 2021; Karthikkeyan et al., 2022). If liquorice can induce a protective effect against the drugs which are being used in these models then there is a risk that they are committing a type I error, rejecting the null hypothesis, when it is in fact true (Banerjee et al., 2009) due to not accounting for the possibility that liquorice could be interfering with the action of the drugs used to induce the model of disease. Rotenone is a compound found in several *Leguminosae* plants (Wood et al., 2005) and so not a natural part of the development of Parkinson's disease. It is used because its inhibitory action on Complex I of the ETC was found to cause dopaminergic neuron degeneration with Lewy body-like structures forming in the brain and Parkinson-like symptom appearance after intravenous administration to rats (Betarbet et al., 2000). This means that if liquorice can protect cells from the toxic effects of the drug, then it could interfere with the model and not have a protective effect against the natural pathogenesis of Parkinson's disease at all. This model has already come under criticism due to the high mortality associated with intravenous injection and the poor bioavailability by oral administration resulting in more gastrointestinal issues than induction of Parkinson-like effects (Niederberger et al., 2022). The results of the current study add an additional critique that can be applied to any experiments using drug induced models of disease to examine the effects of liquorice.

This detoxifying effect does not have to invalidate all research where a drug is used to initiate a disease state. Sometimes experiments look at endogenous molecules that induce toxicity at high doses. These include studies looking at liquorice having a protective effect on glutamate induced neurotoxicity (Wang, D., Guo et al., 2014; Lee et al., 2018; Yang, E. et al., 2012), or those investigating the effect of liquorice on corticosterone (Zhou et al., 2017; Li, X. et al., 2020) and 7-ketocholesterol (Kim et al., 2009) induced toxicity. These may still be valid since these substances are part of the pathophysiological mechanism under investigation, and a protective effect against them may still confer protection against their natural pathogenesis.

23.3. Implications for Chemotherapy Research

The results found in this study are most relevant to the actions of liquorice tea on chemotherapy. They suggest that if liquorice was to become an adjuvant therapy in cisplatin regimes, then the liquorice used should undertake an *in vitro* cisplatin resistance test to ensure its effectiveness. These findings also add to the current debates regarding liquorice as an adjunct to chemotherapy. Most chemotherapy utilises cytotoxic agents with the intention to raise the already elevated ROS in cancer cells above the threshold to induce apoptosis (Yang, H. et al., 2018). This is a tactic which can be resisted through the expression of ABC efflux transporters that promote chemoresistance (Sharom, 2008). It is therefore reasonable to question the use of a herb with antioxidant activity and which may have the ability to create chemoresistance by promoting the expression of ABC efflux transporters as an adjuvant to chemotherapy.

Some of the findings of this study support a view of caution when taking antioxidants with chemotherapy agents designed to induce apoptosis through ROS production (Lawenda et al., 2008; Harris & DeNicola, 2020). After 72 hours of exposure to 2000 µg / mL liquorice tea extract, ROS dropped 14.73% in the MCF7 cells compared to control (figure 57). While the natural environment of slightly raised ROS in cancer cells is associated with increased proliferation and metastasis, reductive stress is associated with increased cancer survival, drug resistance and stemness (Chun, Kim & Surh, 2021). As mentioned in section 21.1.3, the doses used in these assays are not realistic representations of the concentrations that would be found in the body. However, in the interests of caution, it does warrant further investigation and implies that regular consumption of liquorice based adjuvant therapies for more than 3 days before chemotherapy could potentially negatively affect the outcome of a regime that depends upon ROS activation.

Other findings in this study suggest that liquorice may actually have potential as an adjuvant in chemotherapy. First, after 3 hours of exposure to liquorice tea extract, ROS increased in the MCF7 cells by 16.51% in the 500 µg /

mL dosage group, and by 14.88% in the 2000 µg / mL group while leaving MCF10A cells unaffected (figure 55, section 11.2). This would suggest that the timing of liquorice administration could be important, adding nuance to the discussion of whether liquorice is simply good or bad during chemotherapy. Ingestion of liquorice just before, concomitantly, or shortly after chemotherapy could raise ROS in cancer cells enough to enhance the chemotherapeutic effect through "priming" (Mould, 2019; Henley 2015; Henley et al., 2017). The exact optimal time for ingestion will depend on the length of time it takes to metabolise the different compounds of liquorice and for it to reach the cells, as well as how long it takes to leave the body. Further research involving blood analysis for liquorice metabolites at different time points after ingestion could elucidate this. It is also important to determine whether the metabolised forms will have the same effect as those in the tea extract used in this study before hypotheses about clinical efficacy can be developed. However, it suggests that the intervals between consumption of liquorice and chemotherapy, and that duration of consistent consumption may be important factors to consider in future research aimed at reaching a stage of clinical trials to determine whether liquorice is beneficial or detrimental in chemotherapy.

The most important finding in relation to chemotherapy is that liquorice protected MCF10A cells from the cytotoxic effect of cisplatin, but did not offer any protection to MCF7 cells (figure 129). This suggests that the antioxidant effects of liquorice are not interfering with cisplatin and that, if this finding is consistent with further experiments in other cell lines and *in vivo* activity, that liquorice may exert a protective effect on non-cancerous cells while conferring no benefit to cancerous ones. It could even be speculated that the increased survival rate of the liquorice treated MCF10A cells of 8.186% at 100 µM and 11.79% at 50 µM cisplatin treatment imply that a greater dose of cisplatin could be tolerated, making the regime even more effective with the same side-effects. Before any conclusions can be drawn, these results would need to be replicated among other cell lines to determine whether this effect is seen only amongst breast cells or in other cells, especially those which limit therapeutic dosing guidelines.

Nephrotoxicity is often the limiting factor in cisplatin therapy, forcing clinicians to balance tumour toxicity against kidney failure (Miller et al., 2010; Volarevic et al., 2019), so determining the cytoprotective effect of liquorice on cisplatin treated kidney cells is necessary to inform clinical trials using liquorice as an adjunct to cisplatin therapy. Some experiments have been performed on this exact question. *In vitro* studies found that liquorice extracts reduced cisplatin induced cytotoxicity in human kidney cells (HK-2 and HEK-293) by reducing ROS, inflammatory and apoptosis markers, including caspase-3 and p53/p21 expression (Ju et al., 2017; Basist et al., 2022). *In vivo* mouse studies support these findings, with glycyrrhizin rescuing cisplatin induced nephrotoxicity and delayed cisplatin induced injury (Arjumand & Sultana, 2011; Wu, Chen & Yen, 2015). This rescue is achieved by preventing oxidative stress, reducing inflammation, restoring antioxidant enzyme levels, decreasing DNA fragmentation and restoring kidney function markers, possibly due to upregulation of Nrf2 and downregulation of NF-κB.

The toxicity of cisplatin also means that it is often used in reduced dosage along with other chemotherapeutic agents (Dasari & Tchounwou, 2014), so it would be prudent to assess the effect of liquorice in conjunction with other chemotherapeutic agents and common combinations. Several studies have found protective effects of various compounds in liquorice against doxorubicin induced cardiotoxicity through different mechanisms with the common effect of reducing ROS induced apoptosis with restoration of antioxidant enzymes (Cheng et al., 2022; Wang, M. et al., 2023; Xu, Z. et al., 2022; Zhang et al., 2011). Only Zhang et al. (2011) also evaluated the tumour inhibitory capacity to confirm that *G. uralensis* extract did not reduce the efficacy of doxorubicin. One study investigated the interaction of liquorice and paclitaxel (Ha et al., 2020) which found that 14 days pretreatment with *G. uralensis* decreased systemic exposure and increased clearance in rats, potentially due to upregulation of CYP450 enzyme 3A4 and P-gp, resulting in potential decreased effectiveness. They also reported that a single dose of liquorice did not affect parameters, recalling the findings of this study that the timing of liquorice consumption may be an important factor.

Other herbal formulas have been promoted as adjuvants in chemotherapy routines and could be quality tested for clinical use with similar models. In particular, CHM formulas TJ-41 (*Bu Zhong Yi Qi Tang*, 補中益氣湯, "Strengthen the Middle and Augment the Qi Decoction") and PHY906 (*Huang Qin Tang*, 黄芩湯, "Scutellaria Decoction") have entered phase II and III trials as adjuvants in cancer treatment (Wang, K. et al., 2022). Both of these formulas contain liquorice which may be responsible for some or all of the alleviation of side effects from chemo- and radiotherapy, making the results of this study potentially relevant to these projects. These formulas also claim to have chemosensitizing effects raising the possibility that, if they were to be used in routine clinical practice, it would be advisable to test the quality of each batch. This testing should include assessing for its chemopreventive effects in normal cells and chemosensitizing properties in cancer cells to ensure that batches have the optimal effect. Although the number of potential assays to test this could be immense, a test on the closest available cancer cell line to the one being treated, a healthy analogue for comparison and any cells that present a dose limiting factor with regard to toxicity could be suggested as an initial choice.

Clinical trials relating to the use of liquorice as an adjunct to chemotherapy can also feed information back to the adulteration testing data. It would be useful to know if the liquorice or other herbs being used in a clinical trial also pass the mitochondrial functionality test in order to determine if the two results are linked and whether the data gathered from mitochondrial functional testing relates to real-world applications. As discussed in section 20.1.3, the dosage used in the cisplatin assay is unrealistic for a clinical environment but finding parallels between human and animal *in vitro* and *in vivo* trials is still the optimal way to determine mechanisms (Mattes, 2020). Therefore, these two experimental methods can support each other. In designing this approach, it would be unethical to give volunteers a herbal material that had failed quality testing, so the only direction the research could take would be to test the herbs for quality first and then use those which gained a positive result to compare against the clinical trial results to see if this corresponds with a positive clinical trial outcome. This would inevitably bias the results as not all trials will be successful and only herbs that passed mitochondrial functional testing are used, inevitably resulting in failed clinical

results appearing to invalidate mitochondrial functional testing. A better option would be to acquire herbs that had already been used in clinical trials, or in trials that are ongoing and subject them to mitochondrial functional analysis to compare the results with the clinical trials. This would depend on the original herbs being stored by the researchers in the case of past trials, and potential degradation from storage that could still compromise the results, especially if the trials were from years ago. Currently ongoing trials would be a more valuable resource but are likely to be few in number.

Chapter XXIV

24. Future Work

Several avenues for potential future work are possible. These include further profiling of liquorice and the profiling of other herbs. The development of a pipeline process is necessary to facilitate the more efficient development of future mitochondrial functional profiles. This will enhance the scientific rigour and efficiency through standardisation, yet it should still address the practical concerns of the herbalists who may use the herbs and the public, especially those buying a medicinal herbal tea for the purported benefits attributed its ingredients.

24.1. Further Profiling of Liquorice

Further profiling of liquorice can be divided into work on other cell lines and models of disease, comparisons between different species of liquorice and the examination of the effects of other forms of adulteration for which mitochondrial functional analysis may be appropriate.

24.1.1. Other Cell Lines

One of the most obvious areas where this work could progress is other cell lines to find out if the same results are obtained or if the results of this study are unique to breast cells. The present findings would indicate that this should be correlated to clinical actions and so the priority cell lines should reflect the tissues liquorice is most likely to affect. Although liquorice is said in CHM to enter all bodily systems (Bensky et al., 2004, p.732), which would suggest it has an effect on all cell lines, there are a few indications that are shared across traditional medical systems which could be used to identify some preferential cell lines.

The main activity of liquorice in WHM is for the treatment of coughs (Bone & Mills, 2013: 719; Hoffman, 2000, p.555), a property it shares with CHM (Bensky et

al., 2004: 732; Chen & Chen, 2000, p.867), Ayurveda (Pole, 2012, p.220) and Unani medicine (Saad & Said, 2011, p.264). This action is also supported by the EMA (2012), suggesting that it is a clinical action that would be valuable to test through mitochondrial functional analysis. An evaluation of the effects of liquorice on several lung cell lines would therefore seem an obvious selection. Since primary cells are harvested directly from living donors, it would be unethical to routinely extract these cells, so only immortalised lines should be used. These can include established epithelial cells (BEAS-2B; American Type Culture Collection, 2023) and the use of human telomerase reverse transcriptase technology which has created immortalised lung fibroblasts (American Type Culture Collection, 2022) and airway smooth muscle (Burgess et al., 2018).

Antitussive drugs primarily act on the nervous system, either peripherally, by suppressing the responsiveness of sensory receptors that initiate the cough reflex, or centrally, at the brainstem, where the neural circuitry for coughing is located (Bolser, Hey & Chapman, 1999), making neuronal cell lines a possibility for research too. PC12 cells are an immortalised cell line often used in the study of nerve physiology and pharmacology due to them sharing common features with neurons such as neurosecretion, ion channels and neurotransmitter receptors, and can be differentiated into sympathetic ganglion neurons by culturing with nerve growth factor (Wiatrak et al., 2020). This could make PC12 cells another potential cell line to observe mitochondrial modulations in response to liquorice. Ayurveda's attribution of liquorice to calm the nervous system (Pole, 2012, p.220) and act as an aphrodisiac (Aronson, 2018) may also suggest that an effect could be observed on neuronal cell lines. Some research has already identified carbenoxolone, a synthetic analogue of glycyrrhetinic acid, as a gap junction blocker in epilepsy and pain research through the inhibition of connexins (Connors, 2012; Bell et al., 2021) which could serve as the basis for mitochondrial functional assays on nerve cells whose activity, when stimulated, should be reduced by exposure to liquorice compared to controls.

Another use for liquorice is to help with digestive ulcers. This indication is found in WHM (Bone & Mills, 2013, pp.721-722), Unani (Saad & Said, 2011, p.264) and Ayurveda (Pole, 2012, p.220). The EMA (2012) monograph does not

concur with this use but does suggest a traditional use for dyspepsia and burning sensation and both glycyrrhizin and carbenoxolone been have used as medicines for ulcers since 1946 with other components also being researched for anti-ulcer activity (Kaczor, 2015). CHM gives liquorice a broader digestive function, attributing its primary action to enhancing digestion (*Pi Qi*, 脾氣, "Spleen *Qi*") and treating symptoms of fatigue with decreased appetite and loose stools (Chen & Chen, 2000, p.867) but also includes indications for internal or topical use, singularly or within formulas, for a variety of "sores, swellings and carbuncles" (Bensky et al., 2004, p.733). This is particularly noticeable in the formula Liquorice Decoction to Drain the Heart (*Gan Cao Xie Xin Tang*, 甘草瀉心湯) where a higher than usual dose of unprocessed liquorice is used in the treatment of ulceration around the mouth, anus and genitals, symptoms which have been interpreted as syphilis or Behçet's Syndrome (Fruehauf 2010), a variable vessel vasculitis that involves ulceration of mucocutaneous tissue around the mouth and genitals (Karadag & Bolek, 2020). This protective activity on gastric mucosa would make immortalised gastric cell lines, such as KMU-CS12 (Applied Biological Materials, n.d.) which are derived from gastric stem cells but have the potential to be tumorigenic (Yang et al., 2009), or an immortalised primary gastric cell line, such as JOK-1 (Okayama et al., 2000), suitable for testing this activity.

Cardiomyocytes would be another useful cell line to study. In relation to this study, the drop in OCR among MCF10A cells after 24 hour treatment with 2000 µg / mL liquorice tea extract (chapters 13 & 17), despite no perturbations being detected in the basic mitochondrial function assays at this time point (chapter 11), make liquorice an interesting herb to study in relation to IRI (see section 20.2). Other research in this field has also suggested glycyrrhizin is an agent responsible for the protective effects in IRI (Hu et al., 2023) and its use in cardiovascular disease in CHM (Wang, L. et al., 2022). Usually, rat myocytes are used in cardiovascular research but human immortalised atrial myocytes are now available which have been transduced with a lentiviral vector to grow in the presence of doxycycline and differentiate into cardiomyocytes when the drug is withdrawn (deVries, 2022). This rapidly replicating human heart cell line could offer a promising target for developing mitochondrial functional analysis protocols for quality evaluation based on the protective effects of liquorice against IRI.

The recent development of induced pluripotent stem cells, which can be differentiated into any cell type (Jimenez-Tellez & Greenway, 2019), offer another possibility for mitochondrial functional adulteration research. These could potentially replace all the limitations of using existing cell lines by creating personalised cellular models. More optimisation is needed before this becomes a realistic opportunity but it suggests that an exciting new era of cellular research may be on the horizon. Organoid models are another recent innovation which more closely approximate the complexity of an organ and its natural physiological processes *in vitro* (Hagen, 2021) and could be used to examine the effects of liquorice on whole organ systems in greater detail.

24.1.2. Actions on Other Disease Models

The findings from this study suggest that cells being exposed to liquorice and measuring their response is not the optimal way to detect adulteration but that the effect of liquorice on clinically relevant models should be used. This implies that as well as testing on a variety of cell lines, the effects of liquorice on cellular models of commonly treated pathologies should be investigated too.

The use of liquorice in the treatment of coughs can be modelled in a number of ways. Infection of immortalised cells with viruses is difficult and primary cell cultures are normally used in researching pathogenesis studies for their similarity to natural disease responses to respiratory tract infections (Rijsbergen et al., 2021). Lung inflammation models are rare in the literature but have been developed using multicellular models composed of macrophages, dendritic cells, and epithelial cells and using lipopolysaccharide to induce the macrophages to shift to their proinflammatory M1 phenotype (Drasler et al., 2021). Although this uses primary macrophages and dendritic cells, they are derived from monocytes isolated from the buffy coats of blood samples from a transfusion centre and so are not as invasive as other primary cells which may need harvesting from biopsy. Pulmonary inflammation involves an overproduction of ROS from both mitochondrial and non-mitochondrial sources. Endogenous sources include

phagocytes which destroy pathogens with a "respiratory burst" of $O_2^{\bullet-}$ generated by NOX, while exogenous sources include medicines, air pollution or cigarette smoke (Zuo & Wijegunawardana, 2021). This increase in ROS initiates pro-inflammatory signalling cascades that attract more phagocytes which release more ROS and result in endothelial damage if not curbed. This would make an assay like DCFDA or MitoSOX a useful tool to measure the levels of ROS in a cellular inflammatory model and compare the protective effects conferred by liquorice, even though it would not be measuring mitochondrial ROS exclusively but also that produced by NOX.

The use of liquorice in the treatment of stomach ulcers can also be modelled in several ways. The protective effect of liquorice against gastric ulcers was observed by Revers in 1946 and led to both the extraction of glycyrrhizin, the subsequent synthesis of carbenoxolone, as well as the observation of its hypertensive side effects (Kaczor, 2015). This led to attempts to treat ulcers using deglycyrrhizinated liquorice with mixed results but is still the main marketing drive for deglycyrrhizinated liquorice, despite the glycyrrhizin derived compounds being the component that was used as prescription medicine for gastric ulcers until its replacement with newer non-steroidal anti-inflammatory drugs that did not affect blood pressure (Aronson, 2018). Once the role of *Heliobacter pylori* was established as the cause of gastric ulcers by Marshall and Warren in the 1980s, triple therapy with two antibiotics and a proton pump inhibitor became the first line of treatment (Marshall & Adams, 2005). The use of liquorice for gastric ulcers has been discontinued from UK clinical guidelines (National Institute for Health and Care Excellence, 2023) and remains only as a herbal or nutraceutical alternative. Much of the anti-ulcer activity of liquorice is attributed to increased gastric mucosal secretion but antioxidant mechanisms have also been proposed (Bafna & Balaraman, 2005). The pathomechanism of ulcer formation by *Heliobacter pylori* has been associated with the Duodenal ulcer-promoting gene A (Sharndama & Mba, 2022), whose effector proteins have been shown *in vitro* to induce the secretion of IL-8 and IL-12 by gastric epithelial cells (Dadashzadeh, 2017). Although its mechanism is not completely understood (de Lima Silva et al., 2021), this could be developed into a similar model to the lung inflammatory model described above, except utilising the induction of inflammation by the effector

proteins encoded by *H. pylori* instead of lipopolysaccharides and measuring the changes in ROS among liquorice treated cells against controls. However, if gastric secretions are the main protective mechanism, then it could be measured by the secretions produced by gastric cells exposed to liquorice compared to controls, which would be simpler than mitochondrial functional assays in this instance.

Although investigating the effects of liquorice from the perspective of traditional indications will reveal the most valuable information to herbalists, many indications have been produced from modern research that would simply require a reproduction of the original experiments, or another assay that measures the same outcome.

One of the most common of these is the induction of apoptosis in cancer cell lines. This has been reported for licochalcones in human bladder cancer (Yuan et al., 2013; Hong et al., 2019), breast cancer (Kang et al., 2017), liver cancer (Wang et al., 2019), oral squamous cell cancer (Oh et al., 2018), oesteosarcoma (Lin et al., 2019) and lung cancer cells (Kim et al., 2015); for glycyrrhizin in breast cancer (Lin et al., 2017) and leukaemia cells (Chueh et al., 2012); glycyrrhetinic acid for lung cancer (Luo et al., 2021), pituitary adenoma (Wang et al., 2014), ovarian cancer (Lee et al., 2010; Yang, J. et al., 2012), leukaemia (Huang et al., 2016) and breast cancer cells (Sharma et al., 2012); and for isoliquiritigenin for cervical cancer (Hirchaud et al., 2013; Hsu et al., 2009), gastric cancer (Ma et al., 2001), prostate cancer (Jung et al., 2006) and melanoma cells (Chen et al., 2019). These would be easy to replicate in mitochondrial functional tests using an MTT assay to measure cell viability. It should be noted that the liquorice extract used in this study had no effect on cell viability, even in the cancerous MCF7 cell line (figure 54), and at doses that are unrealistically high for a clinical comparison (see section 21.1.3), suggesting that these experiments are not representative of a clinically applicable use for liquorice as a whole extract but may be applicable to isolated extracts only.

A number of studies have also reported protection against apoptosis. These are often created *in vitro* using drug models of pathogenic processes and so intersect more directly with the findings of this study that unadulterated liquorice

offers a protective role in preventing apoptosis in non-cancerous MCF10A cell lines while offering no protection to the cancerous MCF7 cell line (figures 86 & 88; chapter 14). In a similar finding with clear parallels to this study, Cheng et al. (2022) found that glycyrrhetinic acid conferred a protective effect against cardiotoxicity induced by doxorubicin which would make another simple test based on replicating the same results. Most of the other studies which looked at the cytoprotective effects of liquorice against toxicity induced by other substances looked at neuronal cells and include a glutamate model of excitotoxicity (Lee et al., 2018; Wang, D., Guo et al., 2014; Yang, E. et al., 2012; Teng et al., 2014), corticosterone model of stress induced neurotoxicity (Zhou et al., 2017; Li, X. et al., 2020), 7-ketocholesterol model of oxidative stress induced neurotoxicity (Kim et al., 2009) and the rotenone induced models of Parkinson’s disease discussed previously in section 23.2 (Ojha et al., 2016; Karthikkeyan et al., 2020; Karthikkeyan, G., Prabhu et al., 2021; Karthikkeyan, G., Pervaje et al., 2021; Karthikkeyan et al., 2022). Each of these could be tested to find the protective effect which is easiest to replicate and differentiate adulterated products from the original sample.

24.1.3. Comparison Between Species

All of the cultivars used in the present study were from the *G. glabra* species of liquorice so further profiling of other species would potentially reveal different mitochondrial modulating effects, potentially enabling differentiation of species with mitochondrial functional analysis. As noted in section 4.2, different compounds are found in different species with glycyrrhizin common to all, glabridin almost exclusively present in *G. glabra*, liquiritin and glycycoumarin only present in *G. uralensis*, and licochalcones being a dominant characteristic of *G. inflata* (Kondo et al., 2007; Rizzato et al., 2017; Avula et al., 2022). This would suggest that these species may have different therapeutic effects, which could explain the different activities attributed to them in various herbal medical traditions. It is also possible that this may be observable in their ability to modulate mitochondrial activity differently.

The specific findings of this study observed an activity of *G. glabra* to protect non-cancerous MCF10A cells from cisplatin, whereas the protective effect of liquorice against toxic substances is an action found in Chinese herbal medicine where *G. uralensis* is predominantly utilised (Bensky et al., 2004: 732; Chen & Chen, 2000: 866). This detoxification property is not commonly recognised in WHM or Ayurveda which primarily use *G. glabra* (EMA, 2012; Pole 2012: 220), suggesting that a unique component amongst *G. uralensis* is responsible for this effect. The literature to date reflects this with the study by He et al. (2019), which found that components of liquorice could upregulate ABC efflux transporter expression, mainly looking at liquiritin, its precursors and isomers, predominantly found in *G. uralensis*. The present study suggests that this activity may be more common across species than previously assumed and possibly due to the actions of common components like glycyrrhizin or liquiritin apioside. Previous research on the role of these compounds as activators or inhibitors of ABC efflux transporters has returned contradictory results (Wang et al., 2013; Feng, Ding & Qiu, 2015), so this study lends strength to the argument that it might increase their expression. Furthermore, it suggests that further study into the detoxifying effects of *G. glabra* are warranted. When *G. glabra* is investigated for its protective effects against toxic substances, they are usually investigating drug induced models of disease, discussed in section 23.2, and not testing directly for the effect of *G. glabra* on administered drugs. This could be a significant oversight in the potential uses for *G. glabra* but also an invitation for researchers to compare the protective effects of *G. glabra* against *G. uralensis* to determine if additional substances in *G. uralensis* confer additional benefit.

24.2. Other Forms of Adulteration

How liquorice can be adulterated was discussed in section 4.8 and the limitations of the chosen method in section 22.2. Expanding on this, further work could be undertaken to determine if other forms of adulteration could be amenable to mitochondrial analysis.

24.2.1. Storage Conditions

Poor storage conditions can introduce a number of potential factors that affect the quality and potency of medicinal herbs. Storage for up to five years has been reported while farmers wait for market values to adjust to sell their crop for greater profit, at the expense of degradation with loss of metabolites, and fungal and weevil infestation (Booker et al., 2014).

Fungal cultures can affect the quality of liquorice. This is more commonly reported in liquorice intended for food markets than herbal medicine, but likely due to a lack of analyses of herbal medicine products (Ałtyn & Twarużek, 2020). Fungal contamination could make a suitable candidate for detecting functional adulteration with mitochondrial analysis. Several mycotoxins have mitochondria modulating effects that may be detectable with the analyses described in this study. The most common types of mycotoxins affecting liquorice are aflatoxins and ochratoxin A. Ochratoxin A is produced by several species of *Aspergillus* and *Penicillium* and has been researched for its mitochondrial dysregulatory activity since the 1970s (Moore & Truelove, 1970; Meisner & Chan, 1974). This research continues today regarding the role of mitochondrial dysfunction in ochratoxin induced neurotoxicity (Pei et al., 2021). Aflatoxins are also produced by *Aspergillus* species and induce hepatotoxicity through damage to mitochondrial structure and function among other mechanisms (Hua et al., 2021). This would make mitochondrial functional analysis a useful tool for detecting fungal contamination, especially as it could detect whether levels are sufficient to be causing mitochondrial dysfunction, determining safe levels. However, aptasensors can detect mycotoxins at concentrations in the ranges of fg / mL and more rapidly than chromatographic techniques (Shkembi et al., 2021), so they will be able to detect mycotoxins at levels far below the European Food Standards Agency recommended safe dose of 0.4 µg / kg bw / day (Schrenk, 2020). Another issue that could arise if liquorice does protect mitochondria from a range of toxic substances, is that liquorice may mask some of the effects of the mycotoxins, potentially making liquorice a poor candidate for detecting mycotoxin contamination, and perhaps other toxic adulterants.

An area where functional mitochondrial analysis would be useful is in the degradation of herbs stored for extended periods without fungal contamination. Stafford, Jäger & van Griggs (2001) followed up a study by Taylor et al. (1995) to evaluate the bioactive properties of herbs after 6 years of storage and found that, of 19 plants tested for antimicrobial and antifungal activity, 3 lost all activity, 6 retained all activity, and 10 had partial activity. Of the 13 that had been found to have light enhanced activity in the earlier study, all but one had lost this ability. This study is particularly interesting because it used the same herbs as tested 6 years ago and stored in cotton bags at room temperature, the method common in a traditional Nepalese dispensary, so the results were not influenced by potential variations in harvest. Stafford et al. (2005) used a 1 year accelerated ageing model of 9 medicinal herbs, discovering an increase in their antimicrobial capacity but reducing their cyclooxygenase-1 inhibiting capacity, suggesting that some storage may be advisable depending on the intended use of the herb. Laher et al. (2013) compared materials stored for 3 years in airtight containers against those picked fresh from the same site and found changes in their composition but not always to their detriment. *Senna petersiana* (Bolle) Lock, *Fabaceae*, had higher levels of all measured content (phenols, flavonoids, tannins and iridoids) than the recently picked dried plant and they concluded that effects were species-specific and that, as well as potential reduced efficacy, there could be an issue with enhanced potency and therefore toxicity among stored materials. However, their use of materials picked from the same site 3 years later could also be influenced by variations in the two harvests and an examination of the stored materials upon collection should also have been undertaken. Ali et al. (2018) looked at the effects of storage on the phenolic content of *Piper betle* L., *Piperaceae* and found that dark storage at 5°C affected the content of eugenol and isoeugenol and completely degraded 4-allyl-1,2-diacetoxybenzene with a half-life of 62 days. Only hydroxychavicol was unaffected. Booker et al. (2014) analysed samples of turmeric (*C. longa*) from a number of storage facilities in India and found significantly lower levels of volatile tumerone in stored samples. In contrast, Rowshan, Bahmanzadegan & Saharkhiz (2013) found that storage of *Thymus daenensis* Celak., *Lamiaceae* for 3 months at room temperature caused a decrease in compounds with lower boiling temperatures such as α-pinene,

α-terpinene and myrcene, but also caused a transformation of γ-terpinene and p-cymene into carvacrol and thymol, which are important quality indicators, resulting in an overall increase of 23-26%. Despite these contradictory findings, the one consistent thread that emerges is that storage does affect medicinal herb constituents and may affect their clinical efficacy in unknown ways.

This would make mitochondrial analysis a suitable method of evaluating the effects of storage on potency because it would bypass the issue of which precise compounds are causing the increase or decrease in efficacy, and instead assess the potency of the herbs on a model of their eventual therapeutic action in living cells. This could also elucidate which components are responsible for their therapeutic effects if combined with chemical analyses to compare which components increase in the more potent herbs. In terms of this study, it would be useful to evaluate the effects of various storage conditions on the mitochondrial modulating effects of liquorice to determine what effect, if any, storage has. This could also be a limiting factor on the existing results, as the freeze dried material was stored at -20℃ for the duration of the study, while the adulterated material was prepared from the same tea sample almost 2 years later that had been stored at ambient temperature in the laboratory, inside its original packaging. The samples were also from a commercial product instead of being obtained directly from a plantation, so their previous storage conditions cannot be verified, although Pukka's "Fair for Life" scheme involves paying their farmers a basic minimum wage (Anastov, 2023), reducing the likelihood that herbs are stored for long periods, waiting for market price increases. In order to test this, the liquid from the first extraction of the XLTE could also have been tested for its mitochondrial modulating functions to see if these had been compromised by the storage, or the double extraction.

24.2.2. Wild Harvested vs. Cultivated Liquorice

The superiority of wild liquorice compared to cultivated varieties (Brinckmann, 2020; Wang, H. et al., 2021) introduces the prospect of deliberate or accidental adulteration of the wild harvested crop with a cultivated one. As

demand for liquorice grows and excessive harvesting of wild liquorice reduces its natural habitat (Khaitov et al., 2022), the incidences of suppliers adulterating their wild harvested stock with cultivated specimens are likely to increase. This is precisely the functional adulteration described by Tao Hongjing in the 5th century China (Liu, 2021), where herbs may look good to the untrained eye, but lack efficacy due to their growing conditions not meeting the strict requirements of *Daodi* (section 2.6.1). This would be impossible to detect by genetic analysis and challenging with HPTLC but mitochondrial functional analysis could be an appropriate test to use to determine whether a cultivated crop is capable of producing the same biological effect as genuine wild-harvested liquorice at an equivalent dose.

The first step would be to acquire some cultivated liquorice samples, discussed in section 20.2.1 as potentially weaker functional adulterants, to determine if mitochondrial functional analysis could detect the difference between the two samples and can be adopted as a method of detecting this form of adulteration. If no difference between the wild and cultivated samples can be found, then the superiority of wild harvested liquorice could be called into question and cultivated crops considered as an acceptable substitution to preserve the habitat of the remaining wild plants. Another possibility is that a difference is found in certain tests and not others, in which case the cultivated crop could be adopted for certain purposes where no difference is found, and the wild harvested plants used for the actions where mitochondrial functional analysis found it superior.

24.2.3. Mixing of Adulterated and Genuine Samples

This study shows that mitochondrial testing could be used to detect a pre-extracted liquorice sample from an original sample but in the real world, adulteration is not usually so clearly defined. Most often, an adulterant filler is mixed with the genuine product in order to increase the size and weight of the product being sold, while minimising the risks of detection (Newmaster et al., 2013). This presents an important question for quality evaluation of medicinal herbal teas using mitochondrial functional profiling: what proportion of OLTE:XLTE

is detectable with mitochondrial analysis? This question could be addressed by spiking the OLTE with the XLTE (or samples adulterated in other ways) to see when the effect disappears.

24.2.4. Real-World Sampling

The ultimate purpose of this study was to demonstrate that mitochondrial functional analysis could be used in quality evaluation within the herbal tea industry, and this means that it would eventually have to be tested on some real-world samples. As already mentioned in the limitations (section 22.2), attempts were made to acquire some real-world samples that had failed other forms of adulteration testing, but these may not have been the ideal samples to use. Having already been detected by another, faster method, they would have already been rejected and so mitochondrial analysis would only be confirming that the adulterated herbs also lack potency. The main strength of mitochondrial functional analysis is that it may be able to detect herbs that were genetically and chemically close enough to pass existing inspections, but lacking in potency. Therefore, the aforementioned methods of creating adulterated samples in the laboratory and confirming what forms and levels of adulteration can be detected by this method that other methods cannot, and then applying this to real-world samples would be a superior sequence. However, it could be useful to note if some real-world samples that had been adulterated were capable of passing mitochondrial functional analysis, for example, through the addition of drugs to an incorrect species that could pass mitochondrial functional tests. This could represent an important development in adulteration technology of herbal supply chains should this method become frequently adopted, as already discussed in section 21.3.

24.3. Building a Pipeline

In order to build a fast, efficient method of developing mitochondrial functional quality tests, it is necessary to develop a pipeline. Based on the results found to date, this can be divided into a few necessary stages:

A. Literature Review

The first stage in developing a mitochondrial functional profile of any herb or formula is to review the literature on the specific actions of the herb or herbs. This should draw upon scientific literature on the subject and common uses from herbal medical traditions. There is a precedent for using herbal medicinal traditions to generate hypotheses for scientific testing. Artemisinin, the primary treatment for malaria around the world today, was discovered through the study of a 4th century Chinese text by alchemist Ge Hong (葛洪), that revealed the original method to use sweet wormwood (*Artemesia annua* L., *Asteraceae*) for malaria was to take "a handful of *Qinghao* [sweet wormwood] immersed in two litres of water, wring out the juice and drink it all," which led to a lowering in temperature for the extraction, successful discovery of antiplasmodial activity through laboratory analysis and isolation of the active ingredient (Tu, 2016). A similar process, searching a 10th century Anglo-saxon herbal volume, has prompted scientific investigation into potential new antibiotics for drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Fuchs et al., 2018).

Following this approach, certain hypotheses can be generated that can be experimentally tested through mitochondrial functional analysis. These can include complete reproductions of experiments found in the literature, variations of experiments found in the literature, and novel experiments designed to test traditional indications that could reveal their mechanisms and lead to the publication of new research.

B. Reproduction of the Results

Any significant results that could be used for quality evaluation of the herb under analysis should be reproduced using verified samples to ensure they are consistent and reliable. This is important because reproducibility in scientific literature can be low (Baker, 2016; Errington et al., 2020) and adulteration in herbal supplies can be high (Booker A., Frommenwiler et al., 2016; Srirama et al.,

2017). This could extend to the materials used in the scientific literature, so it is essential to ensure that any research used to develop a standard is obtained using a validated test on a verified sample to ensure the reproducibility of the result.

C. Investigation into Functional Adulteration

The next area of research would include common forms of adulteration, especially functional adulteration, as described in section 2.6.1, which may be detected through mitochondrial analysis and evade other methods. This information is not always easily available and may require research techniques beyond a literature review, including qualitative techniques such as surveys and interviews with experts involved in quality evaluation of the specific herb or formulas under investigation, farmers, market traders, industry specialists, professional groups and end users such as herbalists that may have experience of batch variability of their supplies. Besides researching the methods of adulteration that have been found or suspected, this fieldwork can also serve as a means to acquire real world samples for the later stages of the investigation.

D. Comparison of Significant Results with Adulterated Samples

To determine whether mitochondrial analysis can be used to differentiate the adulterated samples, it will be necessary to compare the results from the authenticated samples with those of an adulterated sample. If possible, these should be based on real-world concerns, using methods that have been discovered in stage C. These should be artificially created in the laboratory in order to determine the sensitivity of the test and, wherever possible, related to the kinds of functional adulteration that is the strength of this method to detect.

E. Publication of the Results on a Database

All results need to be made available on a database that can be accessed by quality control organisations. This is similar to the HPTLC Atlas (HPTLC

Association, 2020), which publishes chromatographic profiles of herbs and the exact protocols to get optimal results, against which test samples can be compared. Herbal quality control organisations could contribute to the data, adding new tests, modifying existing ones for greater optimisation and challenging results which they cannot reproduce. Since the content of this database would be assays that have been replicated with enough frequency to be used as industry standards, it could become a benchmark for researchers to aspire towards since inclusion on the database would show their findings were consistent, similar to the adoption of specific protocols for controls in biological assays, and help mitigate the tendency of journals to only publish novel results (Sharma & Verma, 2019).

24.4. Mitochondrial Profiling of Other Herbs

The future herbs and formulas to be tested will be driven by the needs of industry to have standards for their particular products. Since the donor of the original material was Pukka Herbs, the future herbs that were planned were mainly dictated by the products they manufacture, starting with single herb teas such as liquorice, and gradually increasing in complexity to include two and three herb blends which could be compared against individual herb profiles, and then complex mixtures. These were cross referenced with the likelihood of them having a detectable effect on mitochondria that would enable their profiling based on table 7, section 20.2).

24.4.1. Cinnamon

Cinnamon (*C. verum* seu *cassia, Lauraceae*) has a history as long and rich as liquorice, being especially valued in CHM where the formula *Gui Zhi Tang* (桂枝湯, “Cinnamon Twig Decoction”) is said to be the “crown … of the Chinese herbal medicine” (Scheid et al., 2009, p. 17) and the major leader of all other formulae, around which the entire tradition of formula creation that characterises CHM has evolved (European TCM Association, 2019). Cinnamon usually refers to two main species: *C. verum* J.Presl (syn. *C. zeylanicum* Blume), known as Ceylon cinnamon or true cinnamon; and *C. cassia* (L.) J.Presl (syn. *C. aromaticum* Nees et

Neolitsea cassia (L.) Kosterm.), commonly referred to as Chinese cinnamon or cassia bark. Although Chinese cinnamon retains its traditional uses in acute infectious diseases (Bensky et al., 2004, pp.8-11; Chen & Chen, 2000, pp.40-42), much modern research has focused on the potential therapeutic properties of cinnamon in chronic, non-communicable diseases prevalent today, including the management of diabetes mellitus, glucose intolerance, dyslipidemia, obesity, arteriosclerosis, Alzheimer's and arthritis (Medagama, 2015; Hariri & Ghiasvand, 2016; Jiang, 2019). It also has a potential toxicity profile despite being a commonly consumed spice. The cassia species contains high levels of coumarin which has demonstrated hepatotoxic and carcinogenic properties (Abraham et al., 2010; Wang et al., 2013).

The mitochondrial modulating functions of cinnamon have already been discussed in section 3.2.5 but to briefly summarise, the metabolic nature of these disorders and involvement of oxidative stress suggest a mitochondrial mechanism. The research picture reflects this with 38 papers connecting cinnamon or one of its principal components with mitochondrial function. Only two of these attempted to profile its activity using the SeaHorse Flux Analyzer and only one looked at the herb itself (Li, X. et al., 2021). The other investigated e-cigarette liquids flavoured with cinnamaldehyde (Clapp et al., 2019). Li, X. et al. (2021) found that it induced uncoupling of the protons and electrons in the ETC leading to thermogenesis while Kwan et al. (2017) reported that it induced mitochondrial biogenesis, resulting in the browning of adipose tissue. These suggest a hormetic mechanism whereby uncoupling reduces the ATP being generated in favour of heat, resulting in activation of mitochondrial biogenesis to restore ATP levels and a net increase in mitochondrial ATP generation. Cinnamon also helped to restore insulin sensitivity and reduce apoptosis by reducing the sensitivity of the mPTP in the brains of mice fed a high fructose diet (Couturier et al., 2016). It also helped to stabilise serum glucose, insulin and cholesterol levels, reducing overall body weight while increasing mitochondrial biogenesis in skeletal muscle in another mouse model given a high fat diet (Song et al., 2017), potentially assisting with obesity that is also a causative factor in the development of diabetes mellitus.

These suggest several mitochondrial functional experiments that can be reproduced in order to generate a profile of cinnamon types. The use of antioxidant profiling has already been suggested to differentiate four species of cinnamon (Rana & Sheu, 2023) but their profiling only used simple antioxidant assays (DPPH and ABTS assays) rather looking at the functional effects cinnamon has on ROS in a complex biological system. Different cinnamon species could equally be evaluated using a DCFDA or MitoSOX assay, and the proposed uncoupling effects could be measured using a TMRE assay with its uncoupling potential compared against FCCP as a positive control. This would provide a more complete picture to enable comparison of the species. Uncoupling is a physiological process used to reduce the production of ROS (Hass & Barnstable, 2021), so it could be predicted that both of these assays could be used and the results compared to determine the uncoupling capacity of cinnamon species. Finally, Li, X. et al. (2021) used the Seahorse assay to measure uncoupling through an inhibited respiratory rate and raised proton leak meaning that this assay could also be utilised as a third reference point.

24.4.2. Ginger

Ginger (*Z. officinalis*) has enjoyed enormous popularity as both a culinary spice and medicinal herb throughout the world and over time (Spence, 2023). After liquorice, ginger is perhaps one of the most frequently used herbs in CHM, estimated to be in around half of all formulas (Shahrajabian, Sun & Cheng, 2019). It forms two distinct herbs in the Chinese pharmacopoeia, depending on whether it is fresh or dried (Bensky et al., 2004, p. ???), a division supported by modern chemometric analysis which reveals that heat sensitive gingerols are converted to shogaols as it dehydrates (Jolad et al., 2005).

One of the most common uses for ginger is in the alleviation of gastrointestinal disorders. Clinical trials showing a reduction in vomiting among children with acute gastroenteritis (Nocerino, 2021) have led to speculation that it could ameliorate the burden of child deaths from gastroenteritis related dehydration in parts of the world where pharmaceutical interventions are

unavailable (Matthews-King, 2018). It has also been researched for immunomodulatory activity, having an anti-inflammatory effect through Akt inhibition and NF-KB activation, while reducing oxidative stress via the Nrf2 signalling pathway activation (Ayustaningwarno et al., 2024).

As well as its potential in reducing infant mortality from infectious diseases, ginger has been researched into its role in alleviating the burden of non-communicable diseases such as metabolic disorders and cancer that affect industrialised nations. Seo, Kang & Fang (2021) found that ginger supplementation attenuated the effects of a high fructose diet which is known to induce mitochondrial dysfunction and oxidative stress. Oh et al. (2017) connected this to increased mitochondrial biogenesis in rats fed a high fat diet, with Alhamoud et al. (2023) finding that 6-gingerol may increase uncoupling protein 1 expression and thermogenesis leading to mitochondrial biogenesis and browning of adipose tissue, similar to cinnamon. In regards to cancer, ginger is suspected to act on several mechanisms, including cell cycle arrest, induction of apoptosis, disrupting redox balance, and inhibition of proliferation, angiogenesis, migration and dissemination (Zadorozhna & Mangieri, 2021). Mitochondria are central to apoptosis and redox balance in particular, with many of the other mechanisms having reliance on ATP that mitochondria produce. In one instance, Nedungadi et al. (2021) found that ginger extract induces apoptosis in MDA-MB-231 (triple negative breast cancer) and A549 (non-small cell lung cancer) cell lines by inducing ER stress and drastic reduction in $\Delta\Psi m$ and ATP, along with excessive ROS production. This resulted in translocation of apoptosis inducing factor to the nucleus and DNA fragmentation.

The importance of ginger as a common culinary spice and medicinal herb, used in lay and professional herbalism for communicable and non-communicable diseases, makes it important to understand. Furthermore, its activity on mitochondria may be behind many of these uses making it amenable to mitochondrial analysis but to date no Seahorse analysis has been conducted on ginger.

24.4.3. Tea

Tea (*C. sinensis* (L.) Kuntze, *Theaceae*) is an important herb to understand since the global industry is the largest in the world, worth an estimated US$22.7 billion market by 2025 (Market Research Future, 2019), more than 5x the entire herbal tea market combined. It also has an ancient history of use in China with traces of tea being found in Western Han dynasty tombs (206 BCE - 9 BC) (Lu et al., 2016) and is still consumed for medicinal purposes as varied as anti-inflammatory, anti-carcinogenic, anti-mutagenic, antioxidative, antimicrobial, hypolipidemic, antidiabetic, angiogenesis prevention, memory enhancement, bone health improvement and skin protective effects when consumed internally (Saeed et al., 2017) and used as an active ingredient in cosmetics (Koch et al., 2019).

Some of these actions are likely to involve mitochondria, especially as some of its constituents are known to have mitochondrial modulatory effects. Caffeine has been demonstrated to induce mitochondrial biogenesis through activation of PGC-1α which may mimic or improve exercise adaptations (Yamada et al., 2022) and increase muscular endurance by acting as a Ca^{2+} ionophore, AMPK activator and triggering myokine release (Takada, Fumoto & Kinugawa, 2022). However, its actions on mitochondria can also induce cardiac toxicity in high enough doses (Sardão, Oliveira & Moreno, 2002; Wink et al., 1999). Epigallocatechin 3-gallate also has both therapeutic and toxic effects if consumed as a concentrated extract (Dekant et al., 2017; Hu et al., 2018), which are mitochondria mediated (Oliveira et al., 2016; James, Kennett & Lambert, 2018).

Despite the popularity of this drink and the mitochondrial modulation activity it possesses, its characterisation is still incomplete with no analysis using the Seahorse Flux Analyser and much disagreement about its exact mechanisms. Furthermore, there are few current reviews of its mitochondrial modulating activity, except some regarding the effect of caffeine of skeletal muscle (Yamada et al., 2022; Takada, Fumoto & Kinugawa, 2022) and the effects of epigallocatechin 3-gallate on the liver (Oliveira et al., 2016) making a thorough review of the current

literature and some additional characterisation to fill the current knowledge gaps overdue.

24.4.4. Ginseng

Asian ginseng (*P. ginseng*) is another herb with a history dating back to the earliest classics of CHM and remains popular today. It has been trialled for improvements in psychomotor performance, physical performance, circulation, glucose metabolism, the respiratory system, erectile dysfunction, immunomodulation, mood regulation, antioxidant effects, cancer, menopausal symptoms and dry mouth, with the most promising results in the areas of glucose metabolism and moderating the immune response, suggesting potential implications for several diseases, including type 2 diabetes and chronic respiratory conditions (Shergis et al., 2013). This suggests that mitochondria may be the main target of ginsenosides, the active compounds found in ginseng. Current evidence suggests that ginsenosides may enhance mitochondrial respiration while suppressing glycolysis, encouraging greater reliance on OXPHOS, protect against Ca^{2+} induced apoptosis, and both stimulate mitochondrial biogenesis and fusion, as well as initiate fission and mitophagy of damaged mitochondria (Wang & Roh, 2020). Ginseng also has a favourable toxicology profile (Mancuso & Santangelo, 2017). While this herb has been tested using a full array of mitochondrial assays including the Seahorse Flux Analyzer (Huang et al., 2019), most methods favour alcohol extracts over water extracts making them poor representations of brewing a commercial tea blend, and many CHM formulas, which also favour water decoctions.

24.4.5. Ginkgo

Ginkgo (*G. biloba*) is uncommon to find in any commercial teas, but it is a very popular herbal supplement and received the most results on a search for herbs in relation to mitochondria (table 7, section 20.2). It is also one of the most adulterated herbs in the marketplace with as many as 94% of products being adulterated (Booker, A., Frommenwiler et al., 2016). Industry regulatory standards

demand a flavonoid content of 22-27% quercetin, kaempferol and isorhamnetin and 5-7% terpene lactones (ginkgolides A, B, C at 2.8 - 3.4% and bilobalide at 2.6 - 3.2%) and less than 5 ppm of toxic ginkgolic acids (EMA, 2014). These can all be detected using HPLC-MS techniques (Ding et al., 2006) but supplements have been found containing rutin and quercetin from much cheaper buckwheat sources, which remain difficult to differentiate from the same ginkgo derived flavonoids without using advanced equipment such as NMR spectroscopy (Booker, A., Frommenwiler et al., 2016).

Ginkgo has also attracted attention for its effects on mitochondria long before the current scientific trend with the majority of studies on its mitochondrial modulating activities being published from 1999 to 2012. These were mostly assessing its ability to prevent Alzheimer’s disease through selective effects on enzymes that assemble the ETC resulting in a reduction in oxidative stress and an increase in ATP generation which, in turn, reduced β-amyloid plaques and extended lifespan in mouse and *C. elegans* models (Eckert, 2012). The toxic ginkgolic acid component also operates through a mitochondrial mechanism, inhibiting mitochondrial biogenesis and promoting excessive mitophagy (Wang et al,. 2019). Indirect effects that may affect mitochondrial function include an inhibitory effect of platelet aggregating factors and vasodilatory action that may improve blood circulation, which will increase oxygenation of ischaemic tissues (Tao et al., 2020). Clinical trials have had mixed results with greater efficacy in relieving symptoms of cognitive impairment than in prevention (Lejri et al., 2019).

This combination of adulteration practices being common, using substances that closely imitate the genuine product making chemometric testing complex, and its proposed mechanism involving modulation of mitochondrial function, make ginkgo supplements a prime candidate for mitochondrial functional testing. One important finding that may emerge from this research is whether the more cheaply derived flavonoids also affect the mitochondrial modulating function of the supplements similarly to authentic ginkgo. If adulterated ginkgo supplements perform comparably in the mitochondrial functional testing, then there may be an argument to evaluate these cheaper flavonoids on their own and start producing products which openly declare a mixture of the two extractions, helping to protect

this high demand, endangered plant (Sun, 1998) from overharvesting, while providing a cheaper product for consumers.

24.5. Mitochondrial Profiling of Blends and Formulas

Once some significant individual herbs have been tested, the next stage would be to examine some blends and formulas. An important aspect of formula creation is the principle of synergy between the herbs, creating a combined effect that is greater than the sum of its parts (Zhou et al., 2016). When testing a formula for its mitochondrial modulations in relation to a specific clinical effect, this can be compared against its individual herbs to find out if the effect is equal to the individual herb, greater, or less. For example, in relation to this study, if a formula is being examined for its potential antivenom effects, as Galen's Theriac formula was said to do (Karaberopoulos, Karamanou & Androutsos, 2012), it can be compared against the equivalent dosage of liquorice alone, both in equal dose and compared to the amount of liquorice that is in the formula. This will determine if any effect is solely due to the liquorice alone, or if simply taking an equivalent dose of liquorice may be sufficient. It is also possible that an equivalent dose of liquorice will have the same effect, but would exceed recommended toxicity profiles, suggesting that the formula may be effective at producing the detoxification result without triggering the effects of pseudoaldosteronism, hypokalemia and hypertension that liquorice alone can cause (Nazari, Rameshrad & Hosseinzadeh, 2017). It could also be observed if the detoxifying effect of liquorice enables other toxic herbs to retain their therapeutic effect with fewer side effects when included in a formula, as practised in Chinese herbal medicine (Bensky et al., 2004, p,734).

The aim of starting testing with liquorice was due to the ubiquity of its use in herbal formulas and in commercial medicinal herbal tea blends in the UK such as Pukka and Yogi Tea (Rolfe, 2023; Yogi Tea, 2023). It would be advisable to start examining the synergistic effects of liquorice and other herbs though gradually increasing complexity, starting with herb pairs that use liquorice to modify their actions. Fortunately there are several classical CHM formulas that utilise liquorice with another herb to modify its effect which include:

24.5.1. Cinnamon and Liquorice

Cinnamon and Liquorice decoction is a classic CHM formula from the *Discussion of Cold Damage* (c. 220 C.E.) that was originally described for heart palpitations after a patient suffering from an externally contracted illness had been encourage to sweat excessively (Zhang & Liu, 2016, p. 211-213). The same combination is sold in commercial blends of tea today (Pukka Herbs, 2020b) which would make it a particularly useful combination to understand, being of interest to CHM practitioners, industry and consumers alike.

24.5.2. Liquorice and Ginger

Liquorice and dry ginger is another combination that is derived from the *Discussion of Cold Damage* (c. 220 C.E.), originally described for when a patient suffering from an externally contracted illness experiences cold hands and feet, dry throat, vexation and agitation, and vomiting after being mistakenly given the wrong formula for their condition (Zhang & Liu, 2016, p. 110). This combination has also received some modern research into their interaction, whereby liquorice was observed to increase the extraction of 8-gingerol and 6-shogaol while reducing the “acrid and drying” 6-gingerol component (Jiang et al., 2020). This shift in composition through the addition of liquorice could have important implications for its therapeutic potential when compared against ginger alone.

24.5.3. Peony and Liquorice

Another classic CHM formula consisting of only a pair of herbs featuring liquorice that is of great importance in CHM is peony and liquorice decoction. This consists of peony (*P. lactiflora*) and liquorice, usually in equal measure and was originally used for cramps in the legs but has become widely used for a number of disorders that involve spasms, including abdominal pain (Guo, 2016, pp. 110 & 116-124). While there appears to be little research into peony and mitochondria, if this combination is expected to work on muscle cramps, then it is likely to be

having an effect on the mitochondria rich muscle cells. The precise causes of muscular spasms are unclear, but an ischaemic model has been proposed (Coletti, 2022) which would implicate mitochondrial dysfunction creating an energy crisis due to lack of oxygen inhibiting OXPHOS and therefore the ATP required for muscles to relax as well as contract. If this model is correct, then the findings of this study that liquorice helps maintain normal function on lower oxygen consumption, could be relevant to both the clinical application and theoretical development of this model.

24.5.4. More Complex Blends

One of the main advantages of using an end-point such as the effect on mitochondrial function to assess medicinal herbal teas is that a profile can be generated regardless of the complexity of the formula. Testing these particular herb pairs would have a particular advantage for scientific discovery though: All of the above herbs, cinnamon, peony, ginger and liquorice are combined together, with the addition of jujube berries (*Ziziphus jujuba* Mill., *Rhamnaceae*) in the classic CHM formula Cinnamon Twig Decoction (*Gui Zhi Tang*, 桂枝湯), discussed in section 24.4.1 above, around which the entire formula system of CHM has been devised. Therefore testing these individual herbs and pairs would then enable comparison to a small formula whose individual constituents and main combinations have already been profiled. The combined effect of each herb and pair can then be compared against the formula to determine the proportional influence of each herb, along with new effects arising from the entire formula not seen in any individual herb or combination to establish whether the whole formula has effects that cannot be attributed to any of its parts. Further small variations on this central formula, such as Cinnamon Twig Decoction with Extra Cinnamon (*Gui Zhi Jia Gui Tang*, 桂枝加桂湯), Cinnamon Twig Decoction minus Peony (*Gui Zhi Qu Shao Yao Tang*, 桂枝加芍藥湯) and Cinnamon Twig Decoction plus Peony (*Gui Zhi Jia Shao Yao Tang*, 桂枝加芍藥湯) which, as their name describes, have a higher amounts of cinnamon, or the peony removed, and Cinnamon Twig and Ginseng Decoction (*Gui Zhi Ren Shen Tang*, 桂枝人參湯) that utilises cinnamon, ginseng, liquorice, dried ginger and one other herb (*Atractylodes macrocephala*

Koidz, *Asteraceae*) (Zhang & Liu, 2016, pp. 362, 73, 687 & 482). These each contain very similar ingredients which will have all been profiled individually and in combination with each other, but have very different indications, so they could become potential future targets against which to assess the effect of combinations and complex formulas compared against each other.

Chapter XXV

25. Conclusions

For the first time mitochondrial functional analysis was used to detect functional adulteration with poor quality herbs of correct species in medicinal herbal teas, an issue that has concerned herbalists for millennia. This has remained largely unaddressed in the literature due to the technical challenges it presents using common existing methods that excel at identifying added drugs or incorrect species, but encounter difficulties when these two criteria are met. To date the main methods of evaluating functional quality were to use animal or human subjects which present ethical and financial challenges today, preventing routine use. Instead, only post-hoc evaluation of the efficacy of medicinal herbal teas is usually performed by herbalists or the consumers of medicinal herbal teas who evaluate their effects based on experience. Mitochondrial functional analysis presents an alternative that can provide objective measurements based on the mitochondrial modulations that are produced by herbal tea extractions in relation to their intended use. This can be used alongside existing methods of quality evaluation to ensure that herbs are both correct species, do not have added drugs and produce effects on cell populations that relate to their clinical use.

The strength of mitochondrial functional analysis is that mitochondria have been identified as central to many biological functions and are the likely target for many herbs, especially those in the class of the adaptogens. This means that the complex effects brought about by herbs can be evaluated through their effects on a complex biological system, avoiding the issues of having to use marker compounds as a proxy. This is especially problematic when active compounds are unknown, or in the case of blends and formulas, where the potential ingredients responsible for the effects may complex and naturally vary between harvests and batches, or may be substituted for legitimate reasons. Despite this variability in chemotype and genotype, the intended effect on the biological system should be equivalent and can be evaluated directly through mitochondrial functional analysis.

In this study several modulations in mitochondrial function were identified from exposure to a liquorice tea extract including modulations in ROS and respiration. In particular, the reduction in effects brought about by mitochondrial modulating drugs used in the Seahorse assay was correlated with the clinical use of liquorice in CHM and further assays identified the liquorice tea extract to have a cytoprotective effect in non-cancerous MCF10A cells treated with cisplatin that was not reproduced in either the cancerous MCF7 cell line, or when using the adulterated tea. This indicates a unique property to the unadulterated liquorice tea extract that could be used as a functional quality indicator. Furthermore, while HPTLC analysis was easily able to detect other common types of adulteration, such as incorrect species and different extraction methods, it was very challenging to detect any difference between the two extracts that produced significantly different mitochondrial functional profiles.

Other potential mitochondrial functional quality markers were also discovered including a drop in basal OCR in MCF10A cells despite no apparent decline in other mitochondrial functional markers. A potential link between this and the contemporary use of liquorice in CHM for cardiovascular disease was proposed and this was presented as another possible mitochondrial functional test that could be developed. As knowledge progresses, superior mitochondrial functional markers of the clinical effects of liquorice could be found and made into better standards. Other herbs and blends were presented for priority profiling and a pipeline process to streamline the procedure was presented. This pipeline process should look at traditional indications for the herbs as well as scientific literature in order to devise hypotheses which can be tested, adding to the knowledge base of these herbs and repeatable standards for comparison.

Over the course of this work, much has been learned that could improve the development of new quality assessment measures in future. A review of historical and contemporary literature illustrated the extent of the problem of adulteration of medicinal herbs. The developments in technology leading to modern methods were explored and the principle behind each was evaluated, finding their specific strengths and limitations. Recent discoveries regarding the role of mitochondria in

cellular adaptation and cell fate were reviewed, and the centrality of mitochondrial modulation to traditional medical techniques with non-specific targets was speculated. Techniques for evaluating mitochondrial function were examined and their mechanisms understood in order to develop a system for evaluating the effect of herbs on mitochondrial function. Practical skills were also developed in cell culture and experimental assays to achieve the results presented in this study. In discussing these results, the effect of liquorice on the drugs used in the Seahorse assay led to researching how cells detoxify harmful substances and reviewing studies suggesting that liquorice may encourage the expression of efflux transporters and maintain the mitochondrial ATP production necessary for them to function at maximal efficiency. Another unexpected result that implied that liquorice treated cells were consuming less oxygen than untreated cells led to further investigation on the antioxidant mechanisms of liquorice that proposed glycyrrhizin can capture electrons and act as a trap for short-lived radicals, simultaneously explaining the reduced oxidative stress in cancerous cell lines which deliberately produce radicals in order to maintain an optimal redox state, and the ability to maintain normal function while consuming less oxygen in liquorice treated non-cancerous cells. Further potential mitochondrial functional tests for liquorice and other popular herbs with mitochondrial modulating functions were also perused and discussed. These attest to the fact that by incorporating mitochondrial functional testing into existing methods of adulteration detection, a new dimension can be added to current approaches, addressing a deficiency in modern techniques that has been a concern to those using herbs for their medicinal benefits for thousands of years.

26. References

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