Terminalia ivorensis and human health: the impact of Terminalia ivorensis on biomarkers of cyto- and genotoxicity in human cells in vitro.

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TERMINALIA IVORENSIS AND HUMAN HEALTH: THE IMPACT OF *TERMINALIA IVORENSIS* ON BIOMARKERS OF CYTO- AND GENOTOXICITY IN HUMAN CELLS *IN VITRO.*

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Ph.D. **2021**

Terminalia ivorensis and human health: the impact of *Terminalia ivorensis* on biomarkers of cyto- and genotoxicity in human cells *in vitro.*

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ABSTRACT

Terminalia ivorensis (TI) is a tree found in the tropical and sub-tropical zones of the world and is used in ethnomedicine for the treatment of diuresis, body pains, malaria and wound healing. There are no data investigating the impact of TI on human liver cells and cancer. Cancer is a global health problem causing an estimated 10 million deaths worldwide in 2020. Chemotherapy and radiotherapy are the commonly used methods for the management of cancer but are associated with severe side effects such as anxiety, bleeding, depression, heart problems, perforated gut and secondary cancers.

The aim of this study was to determine the influence of solvent, season and storage time on the phytochemical profile of TI and to search for potential safer treatment options for cancer by determining the impact of TI on biomarkers of cyto- and genotoxicity in human colon and liver cells *in vitro*.

Fresh TI samples were collected in the dry and rainy seasons from Asakakra Kwahu, Eastern region of Ghana, and a TI sample that had been stored at room temperature for 4 years was also used. The phytochemicals were isolated either by sequential Soxhlet extraction using petroleum ether, chloroform, ethyl acetate and ethanol, or by water extraction and were identified by liquid chromatography/mass spectroscopy mass spectroscopy. The study also measured the effect of TI extracts on human colon and liver cells. This was carried out using assays for drug metabolising enzymes (DME) such as cytochrome P450, catalase, glutathione peroxidase (GPx), glutathione reductase (GSR) and an essential tripeptide for drug metabolism, reduced glutathione (GSH). Cell viability was measured by the methyl thiazol tetrazolium bromide assay, cell proliferation by cell counting, DNA damage and DNA repair by single cell gel electrophoresis, and cell migration using the radial migration assay.

It was found that the chemical extraction procedure was consistent and robust for carrying out the study as it yielded comparable and consistent phytochemicals from different batches of extractions. Moreover, the phytochemicals isolated and identified from TI were significantly dependent on the solvent, season and storage. It was also found that different extracts of TI showed differential activity on hepatic

DME activities in human liver cells *in vitro*. Chloroform extract significantly ($p<0.05$) inhibiting the activities of cytochrome P450, GSH, GPx and GSR. In contrast, ethanol extract significantly ($p<0.05$) induced the activities of cytochrome P450, catalase, GSH, GPx and GSR. TI was more toxic in human colon (normal or cancerous) cells than in liver cells and showed potential anticancer effects by affecting some hallmarks of cancer including cell viability and proliferation, DNA damage, DNA repair and cell migration. Ethyl acetate and ethanol extracts were selectively more toxic in human colon cancer cells than in non-malignant colon cells. The impact of TI on human liver cells and anticancer properties shown by TI extracts were possibly due to the different phytochemicals present in the extracts. Hence, further work is required to isolate the active compounds and test for the potential anticancer effects.

Keywords: CaCo-2 cells, cell migration, DNA damage and repair capacity, cytotoxicity, Hep G2 cells, NCM460 cells, Phase I and Phase II drug metabolising enzymes, phytochemicals, Soxhlet extraction and *Terminalia ivorensis.*

DEDICATION

This work is dedicated to Maridia K. Adam, Mariam and James Williams.

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ANOVA – analysis of variance.

AU – arbitrary units.

BCA - Bicinchoninic acid.

BER – Base excision repair.

BSA - bovine serum albumin.

CaCo-2 – colon cancer cells.

CRC – colorectal cancer.

CV – coefficient of variation.

CYP – cytochrome P450.

DAPI - 4,6-diamidine-2-phenylindol dihydrochloride.

DME – drug metabolising enzymes.

DMEM – Dulbecco's Modification of Eagle's Medium.

DMSO – dimethyl sulfoxide.

DPPH – 1,1-diphenyl-2-picrylhydrazyl.

ECACC - European Collection of Cell Cultures.

EDTA - ethylenediaminetetraacetic acid.

FBS – fetal bovine serum.

Fe^{3+}- ferric ion.

Fe^{2+}- ferrous ion.

FRAP – ferric reducing antioxidant power.

GSH – reduced glutathione.

GPx – glutathione peroxidase.

GSR – glutathione reductase.

GSSG – glutathione disulphide.

H_2O_2 – hydrogen peroxide.

Hep G2 - human liver cell line.

IC_{50} – concentration for 50 % of effect.

KNUST – Kwame Nkrumah University of Science and Technology, Ghana.

LC_{50} – lowest concentration of TI that caused a 50 % increase in DNA SSB.

LC MS/MS – liquid chromatography mass spectroscopy / mass spectroscopy.

LDH – lactate dehydrogenase.

MS - mass spectrometry.

MTT – methyl thiazol tetrazolium bromide.

NaPi - sodium phosphate buffer.

NEAAs – non-essential amino acids.

PBS – phosphate buffered saline.

PCA - Principal component analysis.

PLS-DA - partial least squares discriminant analysis.

RINH – Rowett Institute of Nutrition and Health.

ROS – Reactive Oxygen Species

SBR – Strand break repair.

SCGE – single cell gel electrophoresis.

SEM – standard error of mean.

SOD – Superoxide Dismutase.

SSB – single strand breakage.

T25 cm^2 – 25 cm^2 tissue culture flask.

T75 cm^2 – 75 cm^2 tissue culture flask.

TB – trypan blue.

TE – Trolox equivalent.

TI – Terminalia ivorensis.

TPTZ - 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Plant-based products and phytochemicals derived from plants have made an important contribution to drug discovery (Du & Tang, 2014). Natural products (NPs); for example, from animals, microorganisms and plants have been used as a source of drugs in traditional medicine and to produce synthetic drugs in conventional medicine (Newman et al., 2003). For instance, in traditional medicine, melon juice is used as a laxative, *Atropa belladonna* in anaesthetics, olive oil to enhance wound healing and *Papaver somniferum* for parasitic infections and inflammation (Cragg & Newman, 2013; Ji et al., 2009). Olive oil has been adopted in conventional medicine for the removal of earwax and digitalis for oedema and cardiac problems (Du & Tang, 2014). Additionally, salicylic acid is a plant hormone that has been used to synthesise acetylsalicylic acid, referred to commonly as aspirin in conventional medicine (Vlot et al., 2009). The properties of these plant-based products have been associated with antioxidant activity, cell cycle inhibition, regulation of angiogenesis and induction of apoptosis (Du & Tang, 2014; Nichenametla et al., 2006). Analysis of The United States Food and Drug Administration (FDA) list of approved drugs for cancer over the period of 1981-2014 showed that more than 60 % of synthetic anticancer drugs were derived from NPs; and from which bioactive compounds derived from plants constitute approximately 75 % of all drugs approved for the management of tumours (Basmadjian et al., 2014; Newman & Cragg, 2016). The understanding of NP usage for medicinal purposes comes from the human search for alternative treatment for diseases (Dias et al., 2012). NPs have been shown from several studies to exhibit various levels of efficacy and have been used successfully as antidepressants and anxiolytics (e.g. *Onosma bracteatum*), antimalarials and antibacterials (e.g. *Terminalia ivorensis*), cosmetics (e.g. *Theobroma cacao*), and food additives (e.g. saffron) (Alavizadeh & Hosseinzadeh, 2014; Asif et al., 2019; Johnny et al., 2014).

Chemotherapy and radiotherapy are effective methods for the management of cancer. However, they can have severe side effects such as bleeding, headache, loss of appetite and nausea (Du & Tang, 2014). Therefore, the search for NPs with good efficacy, but with lesser toxic effects remains a priority for cancer therapy (Du

& Tang, 2014). Thus, *Terminalia ivorensis* (TI), a commonly used medicinal plant is assessed in this *in vitro* study for potential use as an anticancer agent in human colon cancer.

1.2 TERMINALIA IVORENSIS

1.2.1 Description and geological distribution

Terminalia ivorensis (TI, Ivory Coast almond) of the family *Combretaceae,* is found in tropical and sub-tropical zones of the world (Hawthorne, 1995; Orwa et al., 2009). It is a large forest tree that grows up to 15 – 50 m in height, and is branchless for up to 30 meters (Figure 1.1) (Burkill, 1985). TI trees serve as a supply of solid timber for the building and construction industries and the wood is also used for firewood and the production of charcoal (Burkill, 1985; Orwa et al., 2009; Smith, 1971). Besides its economic usage, TI also serves as a good source of phytochemicals for ethno-medicinal purposes (Annan et al., 2012). TI is commonly known in several languages as Framire in French, Terminalia in Spanish, Black Afara in English and Mwalambe in Swahilli (Orwa et al., 2009).

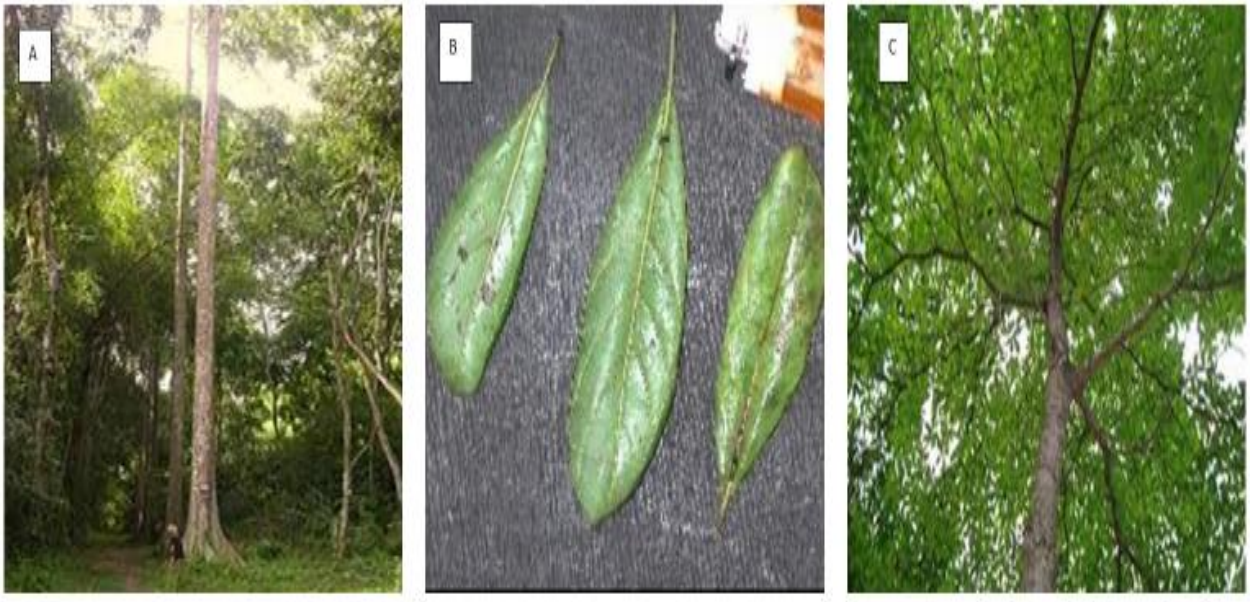


Figure 1.1: TI; (A) stem, (B) leaves and (C) stem with branches and leaves. Taken from Lamb and Ntima (1971).

1.2.2 Taxonomical classification of TI

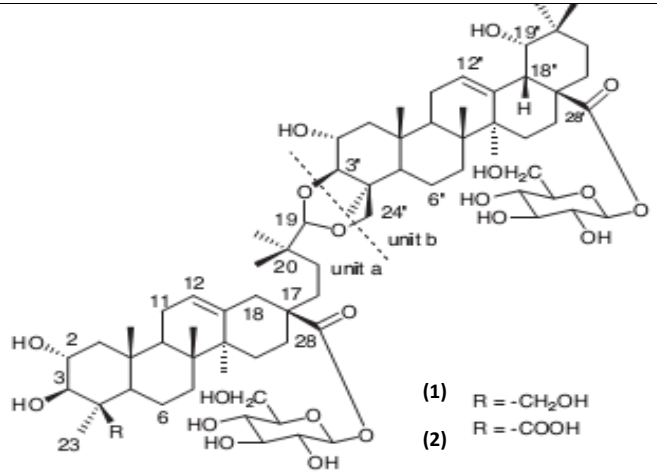
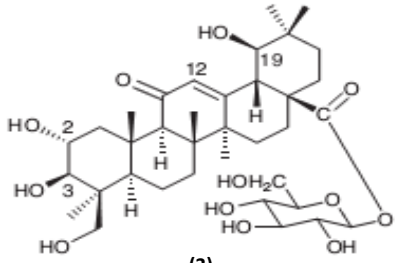
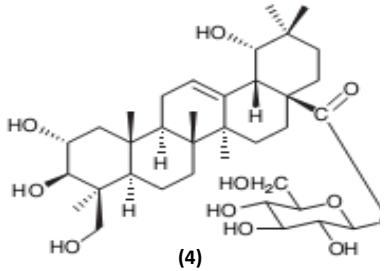
TI is classified in the eukaryotae domain, Kingdom Plantae, Phylum Angiosperm, Class Eudicots, Order Myrtales, Family *Combretaceae*, Genus Terminalia and Species ivorensis. Its specific epithet is *ivorensis (combretaceae)* A. Chev and botanically termed as Terminalia ivorensis A. Chev. (Corbineau & Côme, 1993; Lamb & Ntima, 1971).

1.2.3 Phytochemicals identified from TI.

In vitro analysis of the powdered stem bark of TI shows the presence of alkaloids, flavonoids, saponins and tannins (Annan et al., 2012). Samuel et al. (2014) extracted TI stem bark with acetone and used electron spectroscopy and NMR to identify 2-(3,5-dihydroxyphenyl)-benzofuran-5,6-diol as the bioactive compound and showed *in vitro* antibacterial effect against *Bacillus substilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Samuel et al., 2014). According to Coulibaly et al. (2014), *in vitro* analysis of aqueous and ethanolic stem bark extracts of TI identified the presence of polyphenols, saponins, tannins and terpenes. Tadem MS-MS spectrometric analysis of n-butanolic and methanolic stem bark extracts of bioactive saponins in TI identified the presence of ivorenoside A, B, C and sericoside and these compounds are associated with *in vitro* antioxidant activity and cell anti-proliferating effects against human breast (MDA-MB-231) and colon (HCT116) cancer cells (Ponou et al., 2010). Ponou et al. (2011) also studied the structures of triterpenes of TI stem bark using mass spectrometry which was successively extracted with methanol, ethyl acetate and butanol and showed the presence of arjungenin, arjunic acid, betulinic acid, ivorengenin A, ivorengenin B, oleanolic acid and sericic acid. Adiko et al. (2013) extracted TI stem bark with methanol, methylene chloride and ethyl acetate and electron spray ionization tandem mass spectrometry analysis of phenolic compounds from TI showed the presence of 3,3'-di-O-methylellagic acid; 3,7,8-tri-O-methylellagic acid; 3,3',4-O-trimethyl-4'-O-β-D-glucopyranosylellagic acid; progallin A; and punicalagin. Catteau et al. (2018) and Zhang et al. (2009) also reported the antioxidant and antibacterial activities against *Staphylococcus aureus*. Moreover, the leaves of TI were serially

extracted with chloroform, ethyl acetate and methanol and *in silico* analysis of the final extract showed the presence of alkaloids, bound quinones, flavonoids, leucoanthocyanins, polyphenols, saponins and tannins (Claudine et al., 2017). Additionally, essential oils were isolated from the flowers of TI through hydrodistillation and gas chromatography and electron mass spectroscopy analysis showed the presence of oils such as 1,8-cineole; α-humulene; α-pinene; β-caryophyllene; δ-3-carene; cedrol; myrcene and terpinolene (Ogunwande et al., 2019). Structures of some major compounds identified from TI and their *in vitro* biological activities are presented below (Table 1.1).

Table 1.1: Structures of bioactive compounds identified from extracts of TI using different solvents and their biological activities.

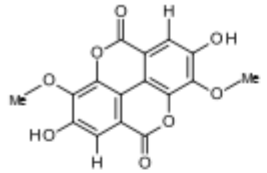
Bioactive compound	Structure	*In vitro* biological activity	Reference
(1) Ivorenoside A (0 – 200 µM) extracted serially with methanol, ethyl acetate and n-butanol from TI stem bark. (2) Ivorenoside B (0 – 200 µM) extracted serially with methanol, ethyl acetate and n-butanol from TI stem bark. (3) Ivorenoside C (0 – 200 µM) extracted serially with methanol, ethyl acetate and n-butanol from TI stem bark. (4) Sericoside (0 – 200 µM) extracted serially with methanol, ethyl acetate and n-butanol from TI stem bark.	 (1) R = -CH_2OH (2) R = -COOH  (3)  (4)	Antioxidant activities and anti-proliferative effects in human brain (T98G), breast (MDA-MB-231), colon (HCT116) and prostate (PC3) cancer cells lines.	Ponou et al. (2010). Ponou et al. (2011).

(5) 3,3'-di-O-methylellagic acid (0 – 400 µM) extracted from TI stem bark with methanol and washed with cyclohexane, chloroform and ethyl acetate.

(6) 2-hydroxy-3,7,8-trimethoxychromeno[5,4,3-cde]chromene-5,10-dione (0 – 400 µM) extracted from TI stem bark with methanol and washed with cyclohexane, chloroform and ethyl acetate.

(7) progallin A (0 – 400 µM) extracted from TI stem bark with methanol and washed with cyclohexane, chloroform and ethyl acetate.

(8) 3,3',4-O-Trimethyl-4'-O-β-D-glucopyranosylellagic acid (0 – 400 µM) extracted



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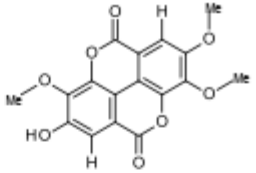

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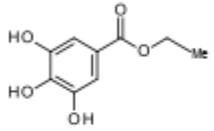

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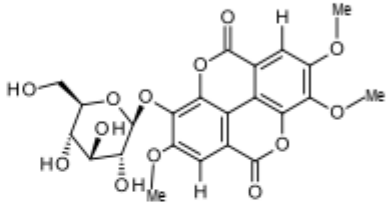

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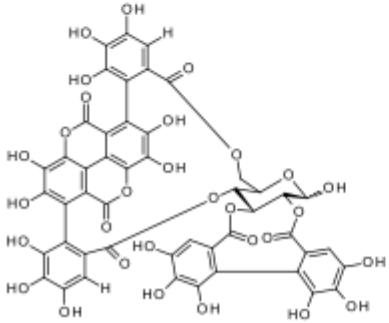
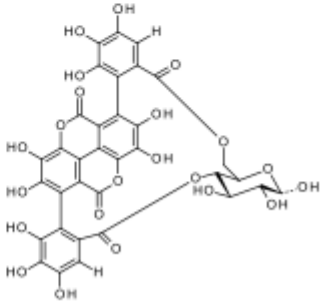


(9)



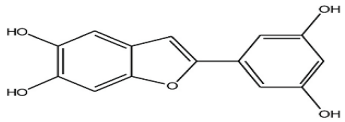
(10)

Antioxidant activity and antibacterial activity against *Staphylococcus aureus*.

Adiko et al. (2013).

Catteau et al. (2018).

Zhang et al. (2009).

from TI stem bark with methanol and washed with cyclohexane, chloroform and ethyl acetate. (9) punicalagin (0 – 400 µM) extracted from TI stem bark with methanol and washed with cyclohexane, chloroform and ethyl acetate. (10) punicalin (0 – 400 µM) extracted from TI stem bark with methanol and washed with cyclohexane, chloroform and ethyl acetate.			
(11) 2-(3,5-dihydroxyphenyl)benzofuran-5,6-diol (0 – 25 mg/m l) extracted from TI stem bark with acetone.	 **(11)**	Antibacterial activity against *Bacillus substilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.*	Samuel et al. (2014).

As shown from earlier studies (section 1.2.3), the choice of solvent is a strong determinant of the type of secondary metabolite isolated from TI and hence, the medicinal efficacy. While previous studies have identified different secondary metabolites from various parts of TI using different chemical solvents, none of the studies investigated the impact of water versus chemical solvents on the phytochemical profile of TI. Meanwhile in traditional medicine, TI is routinely extracted with water. Therefore, the phytochemicals extracted using the chemical solvents may not be directly comparable with the drug interaction for extracts used in ethnomedicine. Therefore, the present study investigated the impact of chemical extraction versus water (traditional extraction) on the phytochemical profile of TI.

Moreover, season and length of storage of plants have also been shown to affect the secondary metabolites present in plant products. For example, storage of *Cosmos caudatus* for 12 h resulted in depletion of the levels of α-tocopherol, benzoic acid, catechin, cyclohexen-1-carboxylic acid, lycopene, myoinositol and stigmasterol when compared to the fresh plant sample (Javadi et al., 2015). Additionally, different amounts of alkaloids, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids were measured from Aloe vera collected from different locations of India with different rainfall patterns and temperatures (Kumar et al., 2017). However, there are no data comparing the effect of season or storage on the phytochemical profile of TI. Therefore, this study further investigated the impact of season and storage on the phytochemical profile of TI.

1.2.4 TI and human health

TI has been documented as an antioxidant in *in in vitro* studies. For example, TI ethanol or methanol extracts (200 µM) have been shown to possess strong antioxidant activity against 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Kouassi et al., 2019; Ponou et al., 2010; Ponou et al., 2011).

Previous studies have also reported the antimicrobial effects of TI. For instance, treatment of *Staphylococcus aureus, Staphylococcus coaggulase negative* and *Staphylococcus epidermidis* with 0.156 mg/ml of 70 % ethanolic stem bark extract of TI for 24 h showed significant ($p<0.05$) inhibition up to 100 % in all bacteria strains when compared to the antibiotic control (oxacillin, 5 µg/ml) (Coulibaly et al., 2014). Samuel et al. (2014) also demonstrated the antibacterial effect of TI by administering 125 mg/ml of TI acetone stem bark extract for 24 h to *Bacillus substilis, Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus* and showed that TI extract significantly ($p<0.05$) inhibited the growth of the four bacteria strains by approximately 55, 100, 70, and 40 % respectively. According to Claudine et al. (2017), incubation of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium tuberculosis spp* with 20 – 50 µg/ml of TI methanolic extract for 8 weeks inhibited the growth of *Mycobacterium tuberculosis* H37Rv by 100 % and moderately inhibited (<25 %) the growth of *Mycobacterium tuberculosis spp*.

Furthermore, Sitapha et al. (2013) reported the antifungal effect of TI by treating *Aspergillus fumigatus and Candida albicans* with 100 – 390 µg/ml of TI aqueous and hydroalcoholic extracts for 48 h and showed that both extracts reduced the growth of both fungi by 100 % and showed higher activity as compared to the antifungal control (ketoconazole, 390 µg/ml). A study by Kouassi et al. (2019) also reported that culturing Fusarium species (*Fusarium oxysporum sp* and *Fusarium oxysporum sp tulipae*) with 12.5 – 50 µg/ml of TI ethanolic extract for 6 days inhibited the growth of the fungi by up to 100 % in both fungi as compared to *Terminalia mantaly* ethanolic extract. Similar antifungal research showed that treating *Fusarium oxysporum lycopersici* and *Sclerotium rolfsii* with 25 mg/ml of 70 % ethanolic extract of TI for 48 h inhibited the radial growth up to 70 and 100 % for *Fusarium oxysporum lycopersici* and

Sclerotium rolfsii respectively as compared to the untreated control (Coulibaly et al., 2020).

An *in vitro* anti-plasmodial effect of TI was reported by Annan et al. (2012) who treated *Plasmodium falciparum* parasites with 25 µg/ml of TI ethanolic stem bark extract for 72 h and reported 83 % inhibition in growth of the parasites. Earlier studies also reported that administering 200 µM of saponins (Ivorenosides A, B and C or sericoside) or triterpenes (ivorengenin A and ivorengenin B) from TI to brain (T98G), colon (HCT116), human breast (MDA-MB-231) and prostate (PC3) cancer cells for 24 h were shown to inhibit cell proliferation of the cell lines by 50 – 80 % compared to the respective controls (Ponou et al., 2010; Ponou et al., 2011).

In animal models, Ben-Azu et al. (2016) investigated the anti-psychotic effect of TI by treating Swiss albino mice with 125 – 1000 mg/kg of TI ethanol extract and found that the TI extract significantly ($p<0.05$) reduced ketamine-induced cognitive dysfunction and hyperactivity up to 100 % after 1 h of treatment as compared to 20 mg/kg ketamine control. A similar study demonstrated the neuroprotective effects of TI extract by treating Swiss albino mice with 125 – 1000 mg/kg of TI ethanol extract for 14 days and showed significant ($p<0.05$) reduction up to 100 % against ketamine-induced schizophrenia-like behaviours as compared to the control (20 mg/kg ketamine) (Ben-Azu et al. 2016). Administering 100 – 400 mg/kg of TI methanol extract to Wistar rats also showed significant ($p<0.001$) anti-nociceptive and anti-inflammatory effects by 50 – 80 % after 30 min and 1 h of treatment respectively when compared to the untreated controls (Avoseh et al., 2018). Ansah et al. (2016) also exposed Sprague-Dawley rats to 300 – 1000 mg/kg of TI ethanolic stem bark extract for 14 days and observed that TI extract protected the kidney and liver architecture from damage by gentamicin. Seven days treatment of Sprague-Dawley rats with 300 – 1000 mg/kg of TI ethanolic stem bark extract was also shown to protect kidney structures against potassium dichromate-induced damage in rats (Moomin et al., 2020).

In traditional medicine, TI stem bark extract has been reported to be used for the treatment of diuresis, general body pains, haemorrhoids, malaria, ulcers, wounds and yellow fever (Akinyemi et al., 2006; Etukudo, 2003; Sitapha et al., 2013).

Several studies have documented the medicinal properties of TI but only a few explored the impact of TI phytochemicals on cancer cells and to the best of my knowledge, no study has investigated the impact of different TI extract on human liver or colon cells. Therefore, the present study examined the impact of TI extract on cell survival and the effect on drug metabolising enzymes in human liver cells *in vitro*.

1.3 LIVER AND DRUG METABOLISING ENZYMES

The liver functions in the absorption, distribution and storage of endogenous substances in the body (Liu et al., 2008; Luis et al., 2003). For instance, when there is excess glucose in the blood, the liver controls blood glucose levels by facilitating the storage of glucose as glycogen and ultimately the conversion of glycogen back to glucose during hypoglycaemic or stressful conditions (Scanlon & Sanders, 2007). It also serves as a storage organ for various vitamins and minerals such as copper, iron and vitamins A, B12, D, E and K (Saladin, 2003).

Additionally, the liver is the site for the synthesis of amino acids, clotting factors (for instance prothrombin), plasma proteins (for instance albumin), bile and cholesterol (Scanlon & Sanders, 2007). Bile produced from the liver is used for emulsification of fats and lipoproteins and transports the fats from the blood into other tissues with elimination of excess cholesterol into the faeces (Luis et al., 2003; Saladin, 2003). The liver also has Kupffer cells which phagocytose and destroy old red blood cells and bacteria which are excreted into the bile for elimination (Liu et al., 2008; Luis et al., 2003; Scanlon & Sanders, 2007).

Moreover, the liver is the principal site for the biotransformation of endogenous and exogenous substances in the body, for instance drugs (Scanlon & Sanders, 2007). While other sites like the gut, kidney, lung and plasma are involved in xenobiotic or drug metabolism, the liver contains higher concentrations of most of the major metabolic enzymes (for example catalase, cytochrome P450, glutathione peroxidase and glutathione reductase) and the tripeptide GSH compared to the other organs in the body (Almazroo et al., 2017). The liver expresses enzymes that are used in the catalysis of Phase I and Phase II metabolic processes (Meyer, 1996). These processes help to increase the overall polarity of drugs or xenobiotics for excretion (Scanlon & Sanders, 2007).

1.3.1 Phase I metabolism

Most pharmacologically active substances are lipid-soluble and at physiological pH they are only partially ionised or remain un-ionised (Meyer, 1996). Most lipophilic endogenous compounds or drugs are enzymatically biotransformed into water-soluble forms for excretion, or in some cases, become more active or toxic products of the parent compound (Phang-Lyn & Llerena, 2020; Scanlon & Sanders, 2007). Phase I metabolism is the first stage in the biotransformation pathway and involves the conversion of a lipid-soluble compound to a water-soluble metabolite which may still be active and used as a substrate for subsequent Phase II reactions (Phang-Lyn & Llerena, 2020; Zöllner et al., 2010). Phase I metabolism enzymatically alters the structure of the lipophilic compound rendering it unable to interact with lipid target sites in the cell (Berenbaum & Johnson, 2015). In Phase I reactions, there is the addition of groups such as an amino, carboxyl or hydroxyl group through hydrolysis, oxidation or reduction reactions to increase the polarity of the parent compound (Danielson, 2002). This involves the use of metabolic enzymes including alcohol and aldehyde dehydrogenases, amide and ester hydrolases, cytochrome P450, flavine monooxygenases and epoxide hydrolase (Meyer, 1996; Zöllner et al., 2010).

1.3.1.1 Cytochrome P450

Cytochrome P450 enzymes are a group of haemoproteins that catalyse the oxidation, peroxidation, and reduction of xenobiotics and several endogenous substances (Venkatakrishnan et al., 2000). Cytochrome P450s are the most important of the Phase I enzymes that catalyse the biotransformation of drugs (Zöllner et al., 2010). Cytochrome P450s are capable of bioactivating xenobiotics to their toxic products as well as deactivating xenobiotics for excretion (Cao et al., 2008; Venkatakrishnan et al., 2000).

Pharmacological studies investigating cytochrome P450 enzymes have been used to address both the side effects of drugs and the interindividual differences in human drug metabolism (Danielson, 2002). For instance, the cytotoxic and genotoxic potentials of bromobenzene, 2-aminofluorene, aflatoxin B and benzo(a)pyrene were determined by studying cytochrome P450 activity in human liver cells (Cao et al., 2008; Doostdar et al., 1988; Plazar et al., 2007). In

addition, an individual's ability to genetically express cytochrome P450 enzymes influences the ability to metabolise drugs and the differences in metabolism between individuals may be due to genetic polymorphism (Chen et al., 2011; Hodges & Minich, 2015). Various drugs and food may alter the activity of cytochrome P450 enzymes pathways by inducing, inhibiting the specific enzyme activity, or serving as substrate of the enzyme, and hence, interfering with the metabolism of other drugs (Chen et al., 2011; Phang-Lyn & Llerena, 2020). Typical inducers of cytochrome P450 enzymes include alcohol (chronic use), ginseng, phenobarbital, phenytoin, and tobacco (smoking) and inhibitors include alcohol (acute use), amiodarone and valproate acids (Phang-Lyn & Llerena, 2020).

1.3.2 Phase II drug metabolism

Phase II drug metabolism involves conjugation of metabolites produced during Phase I drug metabolism with endogenous hydrophilic groups to increase their solubility for excretion or transport (Berenbaum & Johnson, 2015). This conjugation process usually involves groups such as acetyl, glucuronyl, methyl and sulphyl groups and results in the elimination of the conjugated products in the faeces or urine (Phang-Lyn & Llerena, 2020; Timbrell, 1989). Major Phase II metabolic enzymes include aminotransferases, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferases, methyltransferases, N-acetyl transferases, superoxide dismutase and UDP-glucuronosyl transferases. Additionally, reduced glutathione (GSH) is not an enzyme but is an important tripeptide involved in drug metabolism (Berenbaum & Johnson, 2015; Hodges & Minich, 2015). While there are other relevant Phase II enzymes, this study focused on enzymes and GSH involved in the glutathione redox cycle which include catalase, glutathione peroxidase, glutathione reductase and GSH (Figure 1.2).

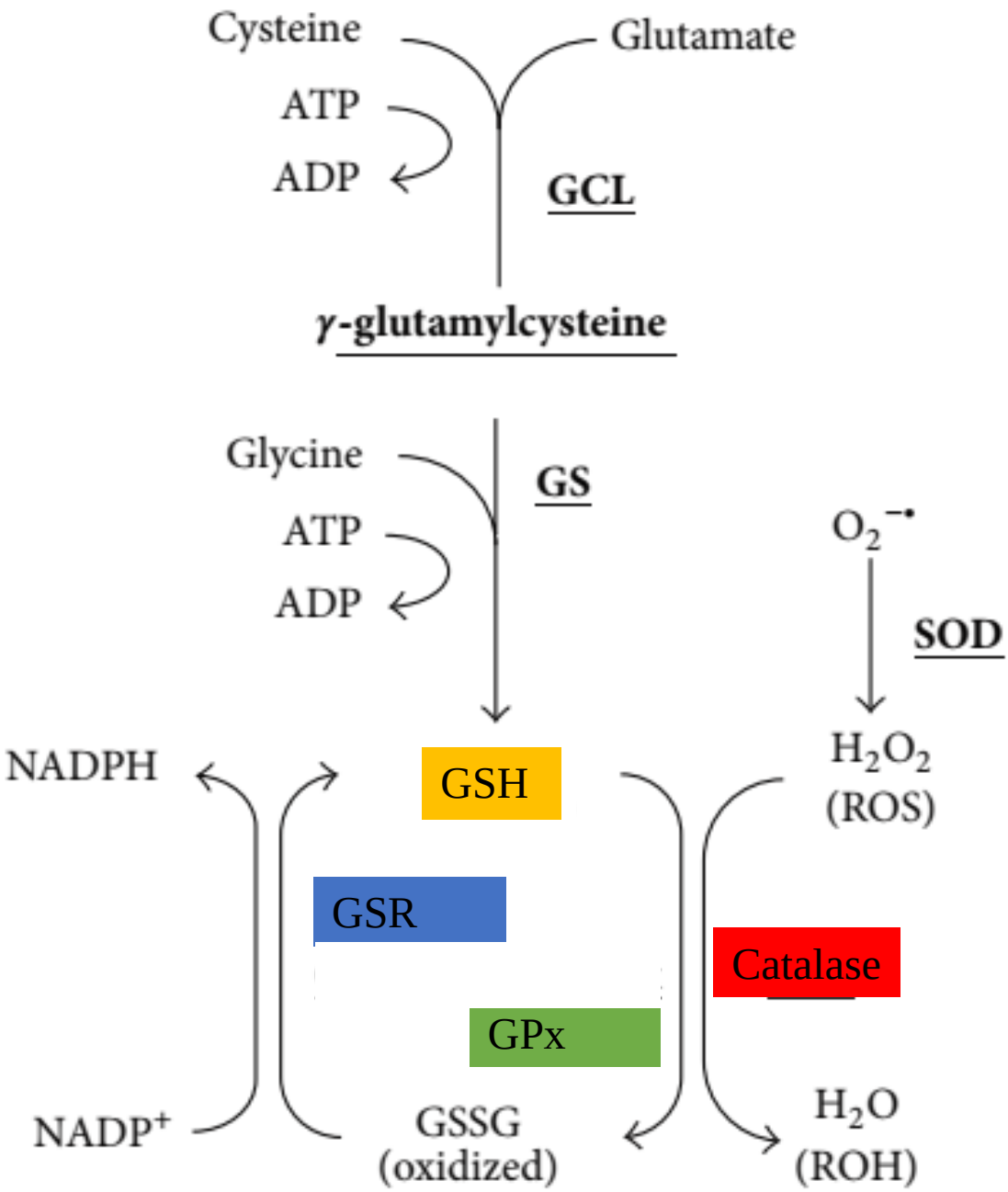


Figure 1.2: Glutathione redox cycle showing the synthesis of GSH from cysteine, glutamine and glycine, and the detoxification of ROS to water.

GSH (reduced glutathione) and DME of interest are highlighted. Glutamate cysteine ligase (GCL), GPx (glutathione peroxidase), GSR (glutathione reductase), glutathione synthetase (GS), hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide phosphate (NADPH), superoxide anion ($O_2^{-\cdot}$), SOD (superoxide dismutase) and oxidized glutathione (GSSG). Taken from Simpson et al. (2015).

1.3.2.1 Catalase

Catalase is a haem-containing enzyme that is present in almost all aerobic organisms (Prakash et al., 2009). In mammals, catalase is highly concentrated in the liver, erythrocytes and kidney (Kodydková et al., 2014; Liu et al., 2008). In human cells, it is mainly found in the peroxisome where it is involved in the catalysis of hydrogen peroxide (H_2O_2) to water and oxygen (Scibior & Czeczot, 2006; Sivanandham, 2011). In the cell, H_2O_2 is normally generated by intracellular oxidative reaction (Agarwal et al., 2008). H_2O_2 however, causes damage to proteins, lipids and DNA of cells and therefore must be detoxified from the body (Sivanandham, 2011; Weydert & Cullen, 2010). At high concentrations of H_2O_2, catalase acts catalytically to remove it, whereas at low concentration of H_2O_2, catalase utilises hydrogen from a donor (like ethanol, phenol or methanol) to remove H_2O_2 (Scibior & Czeczot, 2006). Low levels of catalase and a high concentration of H_2O_2 are implicated in many pathological conditions including asthma, diabetes, dyslipidaemia, hepatotoxicity, inflammation and nephrotoxicity in mice and rat models (Ansah et al., 2016; Aslam et al., 2013; Kodydková et al., 2014).

1.3.2.2 Reduced glutathione

Reduced glutathione (GSH) is a water-soluble cellular tripeptide consisting of glutamine, cysteine and glycine (Townsend et al., 2003). GSH is synthesised from cysteine, glutamine and glycine in a two-step pathway that requires energy in the form of ATP and is catalysed by glutamate cysteine ligase and glutathione synthetase (Figure 1.2) (Bajic et al., 2019; Simpson et al., 2015). GSH is essential for cellular survival and maintenance by functioning as a non-enzymatic antioxidant, a redox buffer, a co–factor for signal transduction and electrophile defence (Johnson et al., 2012; Townsend et al., 2003). The thiol group on GSH is a potent reducing agent in mammalian tissues (Townsend et al., 2003). GSH serves as a co-factor for detoxification of various compounds in reactions with glutathione reductases and glutathione peroxidases (Johnson et al., 2012; Townsend et al., 2003). Low levels of GSH within a cell predispose it to damage by oxidative stress. Insufficient amounts of GSH in tissues has been linked with various human disease conditions such as cancer, liver damage, renal injury,

neurodegenerative diseases and pancreatitis (Johnson et al., 2012; Townsend et al., 2003).

1.3.2.3 Glutathione reductase

Glutathione reductase (GSR) uses NADPH as a co-factor to convert oxidised glutathione (glutathione disulphide; GSSG) to its reduced form (GSH) (Johnson et al., 2012). After GSH is utilized for detoxification of ROS and other processes, GSR is responsible for recycling GSSG to GSH (Alam et al., 2013). The ratio of GSH to GSSG in a cell or tissue is used to assess the toxicity within the cell or tissue (Townsend et al., 2003).

1.3.2.4 Glutathione peroxidase

Human glutathione peroxidase (GPx) is a seleno-enzyme that uses GSH to neutralise hydroperoxides in the system to produce more soluble glutathione disulfides (GSSG) and hydroperoxide products (Alam et al., 2013). Low activity of GPx is an early indication of disturbance in antioxidant balance in the system and can consequently result in oxidative stress (Alam et al., 2013; Liu et al., 2008). Different isozymes of GPx are expressed in different tissues for instance gastrointestinal GPx, cellular GPx, extracellular GPx and phospholipid hydroxide GPx (Alam et al., 2013).

1.4 CANCER AND ITS GLOBAL BURDEN

Cancer is a condition characterised by cellular mutation, uncontrolled proliferation, and aberrant cell growth (Rang et al., 2014). Cancer is caused by factors including infectious agents (for example human papilloma virus), chemicals (for instance benz[a]anthracene) and radiation such as UV light (Liu et al., 2008; Rang et al., 2014). It is estimated that 70–90% of all cancers in human are caused by behavioural, dietary, hereditary or environmental factors such as smoking, radiation, lack of physical activity and environmental pollution (Finkel et al., 2009; Liu et al., 2008; Rajesh et al., 2015).

Cancer is developed through a series of processes which involve initiation, promotion and progression (Liu et al., 2008). The initiation stage which is the

first stage of the carcinogenesis process involves stable and heritable changes in DNA such as mutations (Brigelius-Flohé & Kipp, 2009). Examples of initiators include nitrosamines, X-rays and fungi such as aflatoxin (Liu et al., 2008; Rang et al., 2014). The promotion stage of carcinogenesis involves the selective clonal expression of initiated cells that have been phenotypically and genetically altered to exhibit a higher rate of uncontrolled proliferation (Brigelius-Flohé & Kipp, 2009; Liu et al., 2008). Moreover, progression is the final stage in carcinogenesis and involves the irreversible conversion of benign lesions to cancer (Liu et al., 2008). In this stage, there is increase DNA synthesis and cell proliferation (Liu et al., 2008; Rang et al., 2014).

Globally, an estimated 18.1 and 19.3 million total newly diagnosed cases of cancer were recorded in 2018 and 2020 respectively while 9.6 and 10 million cancer related deaths were recorded in these same years (Bray et al., 2018; World Health Organization, 2020). In 2020, female breast cancer was the most diagnosed cancer worldwide annually with 2.3 million total new cases, followed by lung and colorectal cancers with 2.2 and 1.93 million new cases respectively. Conversely, lung cancer caused the highest number of deaths of 1.8 million people followed by colorectal and breast cancers with 940 and 690 thousand deaths respectively (Sung et al., 2021; World Health Organization, 2020).

1.4.1 Colorectal cancer

Colorectal cancer (CRC) refers to cancer of the appendix, colon and rectum (Sameer, 2013). Globally, CRC is the second leading cause of cancer related deaths, and its incidence is steadily increasing in both developed and developing countries (Sung et al., 2021). Developed countries have a higher rate of CRC cases than developing countries (Figure 1.3) (Rawla et al., 2019). Historically, a lower incidence of CRC was found in Asia, sub-Saharan Africa and South America compared to Australia, North America and Western Europe (Center et al., 2009). However, due to the increasing adoption of western dietary habits and lifestyles, the cases of CRC are increasing in countries that showed previously low incidence such as Asia and sub-Saharan Africa (Vainio & Miller, 2003).

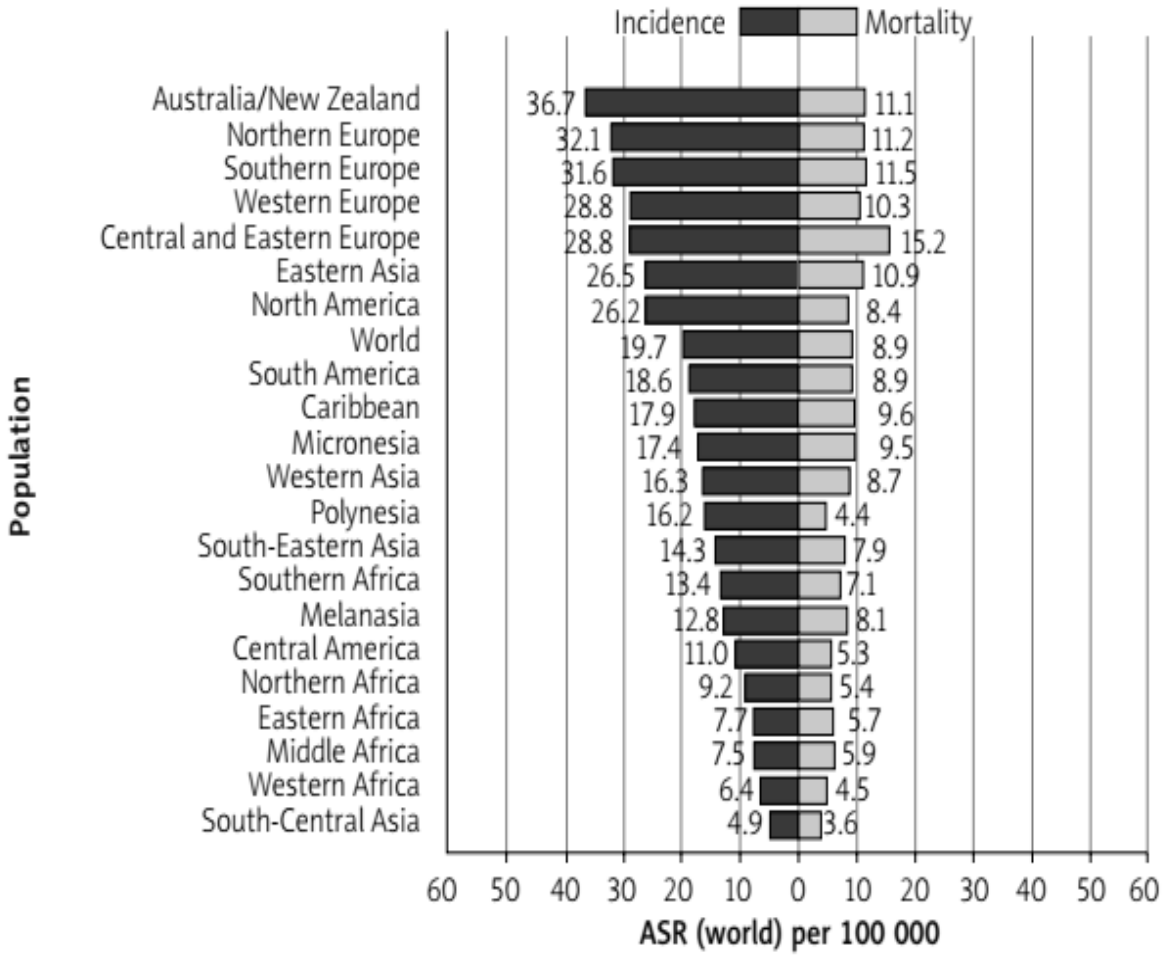


Figure 1.3: Global incidence and mortality rates of CRC per 100,000 population for the different continental regions. ASR means age-standardised incidence rates. Taken from Rawla et al. (2019).

In 2018, Hungary recorded the highest number of CRC cases in men globally, with 70.6/100,000 population while Norway recorded the highest number of cases in females (29.3/100,000 population) (Figure 1.4). Additionally, CRC is the most diagnosed cancer in men in countries such as Bahrain, Kuwait, Oman, Qatar, Saudi Arabia, Slovakia, South Korea, United Arab Emirates, United Kingdom and Yemen while Africa and South Asian countries recorded the lowest incidence rates among both males and females (Rawla et al., 2019).

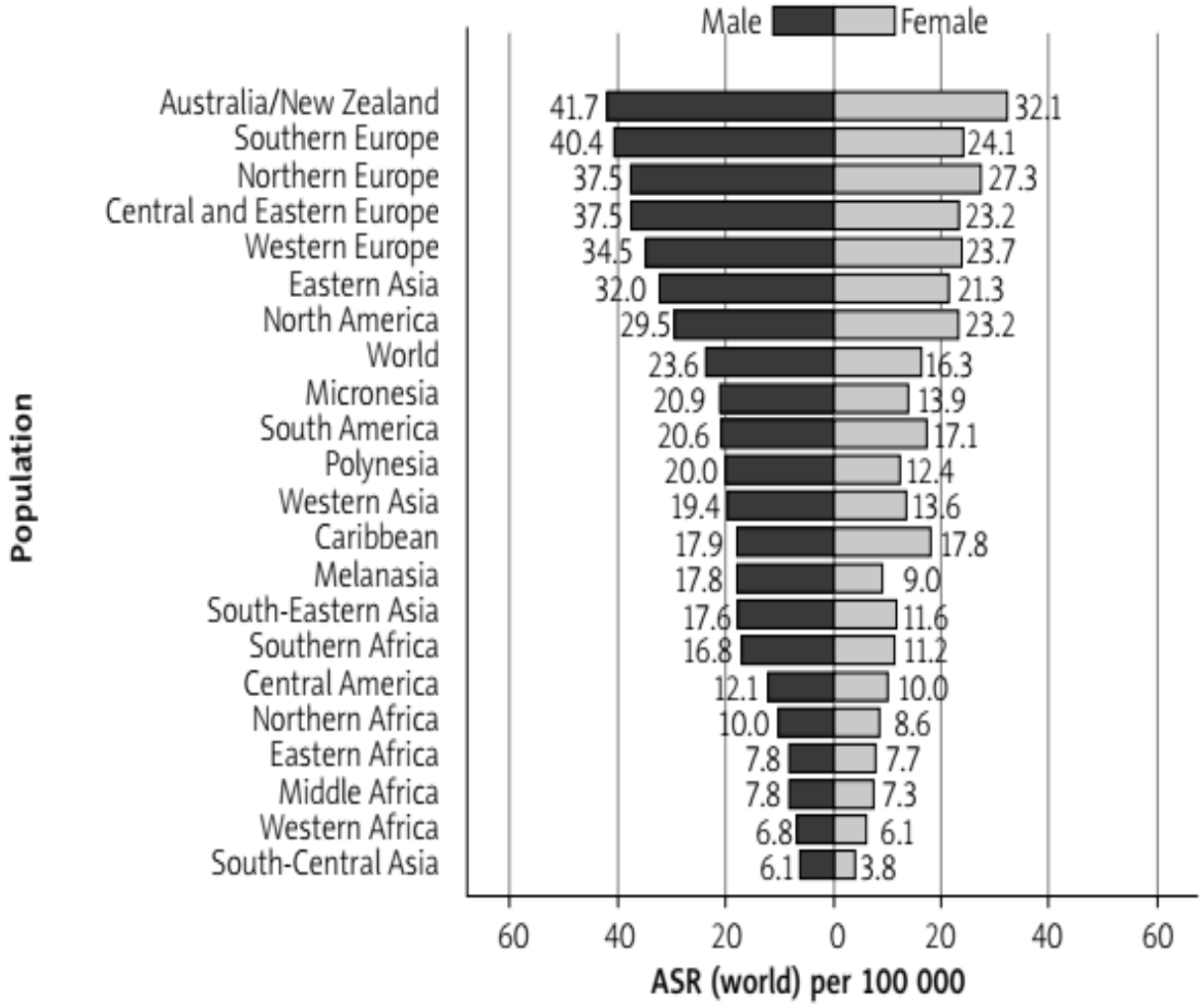


Figure 1.4: Incidence rates of CRC among males and females per 100,000 population for the different continental regions of the world. ASR means age-standardised incidence rates. Taken from Rawla et al. (2019).

CRC is classified as inherited or sporadic based on the aetiology and genetic predisposition (Nguyen & Duong, 2018). Sporadic CRC may result from aging, dietary or environmental factors such as viruses, bacteria and smoking, and constitutes over 70 % of CRC cases (Nguyen & Duong, 2018; Sameer, 2013). The remaining CRC cases are inherited, thus, have a family history of CRC (Sameer, 2013).

According to Fearon and Vogelstein's genetic model of sporadic CRC, the multifactorial development of CRC from normal colonic tissues involves genetic and epigenetic changes that lead to the selective proliferation of modified cells or tissues, consequently resulting in invasion and development of tumours (Fearon & Vogelstein, 1990; Hanahan & Weinberg, 2000). CRC occurs principally through

three mechanisms namely: chromosomal instability, microsatellite instability and CpG island methylator phenotype (CIMP) (Nguyen & Duong, 2018). Chromosomal instability is characterised by mutations in one or both alleles of a chromosome in normal human cells and occurs in approximately $2x10^{-8}$ mutations/nucleotide/generation (Nachman & Crowell, 2000; Sameer, 2013). Chromosomal instability is the most common form of genomic instability and accounts for almost 85 % of all CRC cases (Sameer, 2013). The Microsatellite instability pathway involves the inactivation of DNA nucleotide mismatch repair genes which lead to the accumulation of frameshift mutations contributing to tumour development and progression (Mudassar et al., 2014). Microsatellite instability is found in 12 – 15 % of CRC cases (Sameer, 2013). Lastly, CIMP pathway results in the altering of tumour suppressor genes through hypermethylation and hypomethylation of DNA at promoter CpG islands of the genes and accounts for approximately 17 % of CRC (Mudassar et al., 2014; Nguyen & Duong, 2018). Some genes associated with these mechanisms include APC, CDC4, DCC, KRAS, hMLH1 and p53 (Figure 1.5) (Nguyen & Duong, 2018)

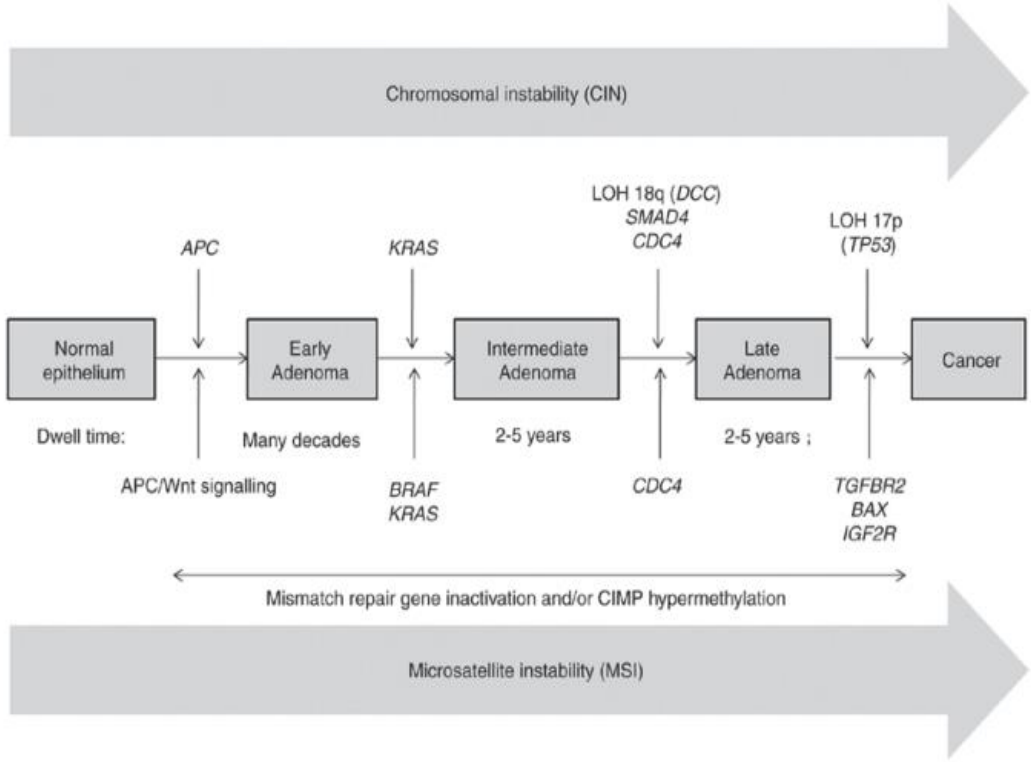


Figure 1.5: Genetic alterations and inactivation of tumour suppressor genes and tumour proteins that occur during the development of CRC from normal tissues.

CpG island methylator phenotype (CIMP); adenomatous polyposis (APC); KRAS proto-oncogene GTPase (KRAS); B-Raf proto-oncogene serine/threonine kinase (BRAF); tumour protein 53 (TP53);

loss of heterozygosity (LOH); hereditary non-polyposis colorectal cancer (HNPPC); mutL homolog 1 (MLH1); mutS homolog 2 (MSH2); deleted in colorectal cancers gene (DCC); transforming growth factor-β receptor (TGFBR); BCL2 associated X apoptosis regulator (BAX); insulin like growth factor 2 receptor (IGF2R); cell division control protein 4 (CDC4). Taken from Nguyen and Duong (2018).

It is well known that certain cellular and molecular alterations are indicative of the onset, progression and metastasis of cancer. These are known as the hallmarks of cancer (Hanahan & Weinberg, 2000; Nguyen & Duong, 2018). Detection of these molecular prognostic biomarkers helps to identify people at higher risk of developing cancer and aids in the selection of appropriate treatment (Nguyen & Duong, 2018).

1.4.2 Hallmarks of Cancer

The Hallmarks of Cancer are biological changes that develop during the growth of human tumours (Hanahan & Weinberg, 2000). These Hallmarks provide the basis for understanding the biology of cancer and are used for assessing cancer development. They include DNA damage and inhibition of DNA repair; sustaining cell proliferation and resisting cell death; evading cell growth suppression; sustained angiogenesis; and activating cell invasion and metastasis (Foulds, 1954; Nowell, 1976). Conceptual progress in the last decade has added two emerging hallmarks and these are reprogramming of energy metabolism and evading immune destruction (Hanahan & Folkman, 1996b).

1.4.2.1 Increased ability to inherit DNA damage and elevated repair.

DNA replication is error-prone and DNA damage can result from DNA polymerases adding a wrong base during replication (Liu et al., 2008). DNA damage may also be caused by genotoxins from endogenous or exogenous sources, for instance acrylamide, hydrogen peroxide, hydroxyl radicals, mercury, superoxide ions and X-rays (Bertin & Averbeck, 2006; Liu et al., 2008). Within the nucleus, the ends of chromosomes are protected from damage or fusing with other chromosomes by a cap of nucleotides called the telomere. With every cycle of cell replication, approximately 50-100 telomeric nucleotides are lost. This progressive loss of telomeres activates the DNA damage response, for instance tumour suppressor genes (such as TP53), leading to cell growth arrest

(senescence) or cell death (Shay & Wright, 2000). However, cancerous cells circumvent this proliferative barrier and maintain telomeric DNA at lengths sufficient to avoid senescence or apoptosis leading to unlimited proliferation of cells that proceed to form tumours (Blasco, 2005; Shay & Wright, 2000). Cancerous cells also have the ability to increase DNA repair capacity to extend the telomere length through upregulation of telomerase (a DNA polymerase that adds telomere segments to the ends of telomeric DNA) or by an alternative recombination-based telomere maintenance mechanism (Ince et al., 2007; Passos et al., 2007). Additionally, toxicity to the DNA (genotoxicity) may include mutations, mismatched bases, strand breakages (single and double strand breakages) which causes genomic instability and consequently leads to the accumulation of frameshift mutations and selective proliferation of modified cells or tissues contributing to tumour development and progression (Fearon & Vogelstein, 1990; Hanahan & Weinberg, 2011; Liu et al., 2008).

1.4.2.2 Sustaining cell proliferation and resisting cell death.

The most fundamental trait of cancer cells involves their ability to sustain chronic proliferation (Hynes & MacDonald, 2009). Normal tissues homeostasis maintains tissue architecture and function by carefully controlling the production and release of growth signals that initiate entry into, and progression through, cell growth and division (Lemmon & Schlessinger, 2010; Wertz & Dixit, 2010). However, growth factors are deregulated in cancer cells and act in an autocrine manner to synthesise and secrete their own. The cancer cells additionally send signals to stimulate normal surrounding cells which in response produce growth factors such as transforming growth factor-β (TGF-β) and vascular epithelial growth factor (VEGF) for continuous proliferation (Cabrita & Christofori, 2008; Wertz & Dixit, 2010). Cancer cells can also increase the levels of receptor proteins for growth factors and become hyper-responsive to otherwise limited growth ligands in order to continue proliferation (Bhowmick et al., 2004).

Moreover, normal cells are programmed to eliminate defective or unnecessary cells from the body during development or after cellular exposure to stress such as oxidative stress (Fernald & Kurokawa, 2013). This programmed cell death, also known as apoptosis, serves as a barrier to continuous cell growth and eventual development of cancer (Adams & Cory, 2007; Fernald & Kurokawa,

2013). Apoptosis is controlled by external apoptotic signals (extrinsic pathway) and internal apoptotic signals (intrinsic pathway) (Adams & Cory, 2007). These pathways regulate cellular processes that lead to the activation of a family of cysteine-dependent aspartate-specific proteases (CASPASE) that initiate a cascade of proteolysis for the execution of apoptosis (Adams & Cory, 2007; Lowe et al., 2004).

1.4.2.3 Evading growth suppression

In addition to the ability to induce and sustain positive acting growth-stimulatory signals, cancer cells become insensitive to growth suppressor proteins such as tumour protein 53 (P53) and retinoblastoma-associated (RB) protein (Burkhart & Sage, 2008). P53 and RB are key cellular regulatory proteins that modulate the ability of cells to proliferate or activate senescence and apoptosis (Deshpande et al., 2005). RB receives extracellular signals and P53 integrate intracellular signals and these proteins control whether a cell should progress through its growth-and-division cycle (Sherr & McCormick, 2002). During the development of cancer, the activity of these proteins is inhibited for the cells to continue growing (Burkhart & Sage, 2008; Sherr & McCormick, 2002).

1.4.2.4 Sustained angiogenesis

Like normal tissues, tumour cells develop new blood vessels from existing blood vessels in a process called angiogenesis. This facilitates the supply of oxygen and nutrients, and the removal of metabolic waste such as carbon dioxide and nitrogenous compounds (Hanahan & Folkman, 1996). Angiogenesis in non-cancerous cells is counter regulated by positive and negative mechanisms that induce or suppress angiogenesis (Baeriswyl & Christofori, 2009). Vascular endothelial growth factor-A (VEGF-A) protein induces angiogenesis to produce new blood vessels during embryonic and postnatal development and in the homeostasis of endothelial cells, as well as in pathological conditions in the adult (Ferrara, 2009; Stefanini et al., 2008). The negative mechanism of anti-angiogenesis is controlled by thrombospondin-1 (TSP-1) which binds to transmembrane receptors on endothelial cells and suppresses signals that counteract proangiogenic stimuli (Kazerounian et al., 2008). However, in cancerous cells the TSP-1 pathway is inhibited and the angiogenic pathway

(VEGF-A pathway) remains activated to continually sprout new vessels that help sustain expanding neoplastic growth (Baeriswyl & Christofori, 2009; Bergers & Benjamin, 2003).

1.4.2.5 Activating cell migration and metastasis.

Cancerous cells develop the ability to modify their shape and detach from their original site of formation and then attach to other cells and to the extracellular matrix to form new colonies. This process is termed metastasis (Berx & van Roy, 2009; Cavallaro & Christofori, 2004). In normal cells, cell-cell and cell-extracellular matrix adhesion molecules (e. g. E-cadherin) help maintain the quiescence of cells (Berx & van Roy, 2009). However, these adhesion molecules are downregulated in cancer cells (Berx & van Roy, 2009; Hanahan & Weinberg, 2011). In contrast, adhesion molecules that aid cell migration during embryogenesis and inflammation (e. g. N-cadherin) are often upregulated in many invasive carcinoma cells (Berx & van Roy, 2009; Cavallaro & Christofori, 2004).

1.5 HYPOTHESES

1) The phytochemical profile of TI is significantly affected by seasonal changes and storage of TI sample.

2) Traditional method of extraction is more efficient in extracting more phytochemical compounds than chemical extraction.

3) TI does not have major effects on human liver DME activity, and on liver cell growth and viability.

4) TI selectively inhibit certain hallmarks of cancer in human colon cancer cells but not in their normal counterparts.

5) TI is rich in metabolites that may have anticancer properties.

1.6 MAIN AIMS

1) To develop optimum solvent extraction processes and identify the major phytochemical metabolites present in TI.

2) To investigate the effects of traditional and chemical extraction methods, storage and seasonal variation on phytochemical profile of TI.

3) To investigate the effect of TI extract on human liver cell viability, proliferation and DME in culture.

4) To investigate the therapeutic effects of TI on certain Hallmarks of Cancer in both human colon cancer and non-malignant colon cells in culture.

1.7 STRUCTURAL LAYOUT OF THESIS

The thesis is composed of 6 chapters. Chapter 1 gives a general overview and literature review of the research. Chapter 2 establishes and develops the extraction method for isolating TI metabolites reproducibly and robustly from TI extracts for use in subsequent chapters. Chapter 3 investigates the impact of TI extracts on human liver cell viability, proliferation and DME in culture. Chapter 4 investigates the therapeutic effect of TI in colon cancer and non-malignant colon cells in culture. Chapter 5 shows the influence of traditional and chemical extraction methods, storage and season on phytochemical profile of TI. Chapter 6 presents the overall discussion, conclusions and recommendations for further research.

CHAPTER TWO

ASSESSMENT OF THE EFFICIENCY AND CONSISTENCY OF THE EXTRACTION PROCEDURE

2.1 INTRODUCTION

The purpose of extraction is to separate the soluble components of a substance from the insoluble residue (Azwanida, 2015). The quality and quantity of phytochemicals isolated from a substance depends on the type of starting material, origin of the plant, moisture content, particle size and degree of pre-extraction processing (Ncube et al., 2008; Tiwari et al., 2011).

Both fresh and dried natural product (NP) samples are used as starting material in the extraction processes, but dried samples are more commonly preferred for extraction (Ncube et al., 2008; Tiwari et al., 2011). Due to the unequal distribution of water in many plant tissues, air-drying is used to obtain a constant weight of plant material before extraction (Tiwari et al., 2011). Other commonly used forms of drying NP samples include freeze-drying, microwave drying, oven drying and sun-drying (Tiwari et al., 2011).

Particle size also influences the amount of phytochemicals extracted from plant material (Azwanida, 2015). Grinding helps to reduce sample size leading to an increase in surface area for efficient extraction to occur (Azwanida, 2015). Mills, electric blenders, mortar and pestle are routinely used to reduce particle size (Borhan et al., 2013). A particle size smaller than 0.5 mm is considered ideal for efficient extraction (Sulaiman et al., 2011).

The composition and yield of phytochemicals obtained by extraction are also dependent on the temperature of extraction, extraction time, and of the nature, concentration and polarity of solvent (Ncube et al., 2008; Tiwari et al., 2011).

Successful isolation of biologically active compounds from a plant material is principally dependent on the type of solvent used in the extraction procedure (Eloff, 1998). A good solvent for extraction should yield high quantities of target compounds and should not allow the compounds to dissociate easily or complex in solution (Eloff, 1998). Since the final crude extract may contain traces of the residual solvent, the solvent should be non-toxic and should not affect any subsequent bioassays to be carried out using the extract (Das et al., 2010; Ncube et al., 2008). To ensure that as wide a profile of compounds as possible is extracted, sequential extractions are carried out with solvents of increasing polarity, from non-polar to more polar solvents (Das et al., 2010).

Several extraction procedures are used for extracting various phytochemicals from plant material and some of the procedures include maceration, decoction, Soxhlet extraction, sonication, percolation, microwave-assisted extraction, superficial fluid extraction and accelerated solvent extraction (Azwanida, 2015; Tiwari et al., 2011). Maceration involves soaking a material (whole or powdered) in a solvent in a stoppered container for a defined time with frequent agitation at room temperature (Handa et al., 2008; Ncube et al., 2008). This method is best suited for extracting thermolabile compounds (Ncube et al., 2008). Decoction involves boiling plant material in a specified volume of solvent for a specific time (Handa et al., 2008). Decoction is suitable for extracting thermostable compounds, hard plant material (for instance roots and barks) and more oil-soluble compounds (Handa et al., 2008; Ncube et al., 2008). With Soxhlet extraction, finely ground sample is extracted through a porous bag or "thimble" in a heated solvent. The solvent vaporises the sample in the thimble and then condenses it (Handa et al., 2008). This method requires only a small amount of solvent compared to maceration, and is suitable for extracting compounds with limited solubility, and thermostable and oil-soluble compounds (Handa et al., 2008; Nikhal et al., 2010). The conventional Soxhlet extraction method involves the use of a Franz von Soxhlet extractor but has the disadvantage of exposing the operator to potentially toxic emissions and hazardous and flammable organic solvents (Naude et al., 1998). Automated Soxhlet extraction technology is usually employed to reduce these hazards associated with the temperature and pressure in the traditional Soxhlet extraction (Rahmalia et al., 2015).

Therefore, sequential automated Soxhlet extraction was considered the most suitable choice of extraction procedure for this research as it enables the safe extraction of a wide range of phytochemicals with different polarity in different solvents. Moreover, an automated Soxhlet extraction technology (Soxtherm machine, see Figure 2.2) was used for the extraction in order to control the temperature, pressure and limit exposure to solvents.

In this study, three samples of TI bark (from the same batch of sample) were sequentially extracted using Soxhlet extraction and the extracts were then used to determine how efficient the Soxhlet extraction procedure is in yielding representative samples which could be used as single sample for subsequent extractions or for use in further experiments. It was important to ascertain

consistency of specific biomarkers that are used to assess the impact of TI on human liver cells (viability, growth and DME), and hallmarks of cancer in subsequent chapters.

2.1.1 Aim:

In order to develop and validate methods for this study, the aim was to assess the efficiency and consistency of the Soxhlet extraction procedure, with regards to metabolite extraction and effect on biomarkers.

2.1.2 Specific objectives:

- To determine the efficiency of Soxhlet extraction in producing consistent amounts of yield between sequential extractions from TI.
- To determine the consistency in phytochemical profile between different batches of TI extractions.
- To determine the consistency in antioxidant capacity for extracts from the different batches of TI extractions.
- To determine the consistency in effect of extracts on cell viability and proliferation for the different batches of TI extractions.

2.2 METHODS

2.2.1 Collection, preparation and isolation of phytochemicals from TI

2.2.1.1 Collection of plant material

Bark samples of TI were collected from Asakraka Kwahu in the Eastern region of Ghana in September 2014. Samples were collected from trunks of five TI trees assessed by a certified herbalist to be of approximately the same size and age. The samples were collected by a professional herbalist in an ethical, and sustainable manner (see appendix 1 for sample authenticity certification). The total amount of sample collected was 6.3 kg (at the time of collection). The samples were washed thoroughly with running tap water and air dried at room temperature for 2 weeks. The dried samples were transported to the Rowett Institute of Nutrition and Health (RINH), University of Aberdeen, UK for subsequent preparation.

2.2.1.2 Preparation of plant material

The TI sample was prepared at the RINH. To ensure a homogeneous and representative sample, all of the sample (6.5 kg) was placed in a clean container, mixed thoroughly and then spread into 6 grids (Figure 2.1). A 32 g sample was weighed from each grid using a Mettler Toledo balance (MH-124, Thomson Scientific, UK) into 33 trays (120x170x35 mm) to increase the surface area for further drying. Each tray was covered with cotton wool held in place with a rubber band and packed into 3 shelves of a freezer and freeze-dried at -20 °C for 4 days. The freeze-dried sample was then placed in an ultra-low temperature freezer (Innova U725, New Brunswick Scientific, UK) to decrease the temperature further (-80 °C) so that any increase in temperature due to subsequent processing would not degrade the phytochemicals within the sample. The sample was taken from -80 °C and broken down further into smaller pieces using a 5-second pulse with a domestic food processor (FDP301S, Kenwood, Gouwut). After preparing 19 trays (out of 33), the processor was left to cool for 10 min to prevent heating of the sample and consequent degradation of the phytochemicals. This processing step was carried out to ensure that a greater

volume of sample could be placed into a freezer mill (SPEX sample prep 6870, Fisher Scientific, Loughborough, UK) tube during the freeze-milling step.

The processed sample (1.57 kg each) was divided into four plastic bags and 200 g of the sample was sub-sampled from each bag and freeze-milled to obtain a total of 800 g freeze-milled TI sample. The remaining sample in the four bags was vacuum-sealed and stored at -80 °C to serve as a reserve if required. The 800 g freeze-milled sample was then mixed and divided into two plastic bags each containing 400 g, vacuum-sealed and stored at -80 °C until required for isolation of phytochemicals.

Figure 2.1: Composite ground-up samples from five TI trees divided into grids for homogeneity and selection of a representative sample for extraction.

2.2.1.3 Isolation of phytochemicals from TI sample

To assess the efficiency and consistency of the extraction procedure, three subsamples of TI bark were weighed and labelled A, B or C (36 g per extraction). A sequential Soxhlet extraction (Gerhardt Soxtherm, SX PC 1.40, Gerhardt, Germany, Figure 2.2) was employed using six solvents to obtain different phytochemical profiles from the TI samples. Solvents were used in order of increasing polarity: petroleum ether (40-60 °C, extra dry, Fischer Scientific); chloroform (99.8+%, stabilized with ethanol, Fischer Scientific); dichloromethane (99.99%, HPLC grade, Fischer Scientific); ethyl acetate (99.98%, HPLC grade, Fischer Scientific); acetone (99.5%, Fischer Scientific) and ethanol (99.8%, absolute, Fischer Scientific).

The Soxhlet extraction was carried out essentially as described previously (Liu et al., 2012). Finely ground TI sample (6.0 g) was weighed into each of 6 cellulose thimbles and extracted with 140 ml petroleum ether. A 118 min extraction was carried out at 150 °C. The remaining solvent was allowed to cool to room temperature and each of the extracts poured into a 25 ml round bottom flask which was pre-labelled (1-6) and pre-weighed. Each replicate was evaporated at 40 °C using a Buchi rotavapor (R-200, Sigma-Aldrich, UK) to complete dryness to obtain a powdered petroleum ether extract of TI. The dried extracts were weighed separately before being pooled to a pre-weighed bottle which was also weighed, wrapped with tin foil to prevent light degradation and stored at -80 °C for further analysis. The TI residue left in the thimbles after the petroleum ether extraction was vacuum dried for 18 h at room temperature using a Heraeus vacutherm (VT 6025, Kendro, Germany) and reweighed. The drying was carried out in a vacuum to prevent the TI from oxidising (Liu et al., 2012). Using the same procedure as for petroleum ether extraction, the residue was sequentially extracted with chloroform (125 min extraction) with rotary evaporation at 62 °C; dichloromethane (105 min extraction) with rotary evaporation at 39 °C; ethyl acetate (110 min extraction) with rotary evaporation at 77 °C; acetone (118 min) with rotary evaporation at 56 °C and ethanol (115 min extraction) with rotary evaporation at 78 °C (Figure 2.2).

The percentage yield for each thimble (replicate) and solvent system was calculated using the formulae below:

a) Yield (g) =weight of sample and bottle (g) – weight of bottle alone (g).
b) Yield (%) = [yield (g) / sample before extraction (g)] x 100.

Figure 2.2: Isolation of phytochemicals from TI using different solvent extractions. (A) Ground TI; (B) Automated Gerhardt Soxtherm and (C) Sequential extraction of TI.

2.2.1.4 Identification of phytochemicals isolated from TI samples.

The method used for the identification of phytochemicals was as described previously (Russell et al., 2011). Solvent extracts from samples A – C (10 mg/ml) were prepared in methanol and 20 µl of each suspension was added to 40 µl of an internal standard and 40 µl of methanol. The internal positive standard was 2-amino-3,4,7,8-tetramethylimidazol [4,5-f] quinoxaline (100 ng/µL) and the negative standard was ^{13}C benzoic acid (400 ng/µL). The samples were centrifuged for 3 min at 10,000 x g at 4 °C, and the supernatant was subjected to liquid chromatography mass spectroscopy / mass spectroscopy (LC MS/MS) analysis. Phytochemicals were separated by liquid chromatography using an Agilent 1100 HPLC system with Zorbax Eclipse 5-µm, 150x4 mm column (Agilent Technologies, Wokingham, UK).

A 3-gradient elution method was used with the mobile phase solvents as water and acetonitrile containing 0.1 % acetic acid. An injection volume of 5 µL was used with a flow rate of 300 µL/min. The liquid chromatography eluent was directed into an ABI 3200 triple quadruple mass spectrometer (Applied Biosystems, Warrington, UK) fitted with a turbo ion-spray source. The mass spectrometer was run in a negative-ion mode for the analysis of indoles and phenolics with the settings: ion-spray voltage of -4500; source temperature of 400 °C; gases 1, 2 and curtain gas were set at 15, 40 and 10 respectively. For the analysis of heterocyclic amines, the mass spectrometer was run in positive-ion mode with the settings: ion-spray voltage of 5500 V; source temperature of 400 °C; gases 1, 2 and curtain gas were set at 14, 40 and 10 respectively. All metabolites were quantified by multiple-reaction monitoring and ion transition for each of the analytes determined based on their molecular ion and a strong-fragment ion. Differing elution times were used to overcome similarities in molecular ions and fragment ions for the different categories of compounds. Declustering potential, voltage variables, collision energy, collision cell entrance potential and collision cell exit potential were individually optimized for each analyte (Russell et al., 2011).

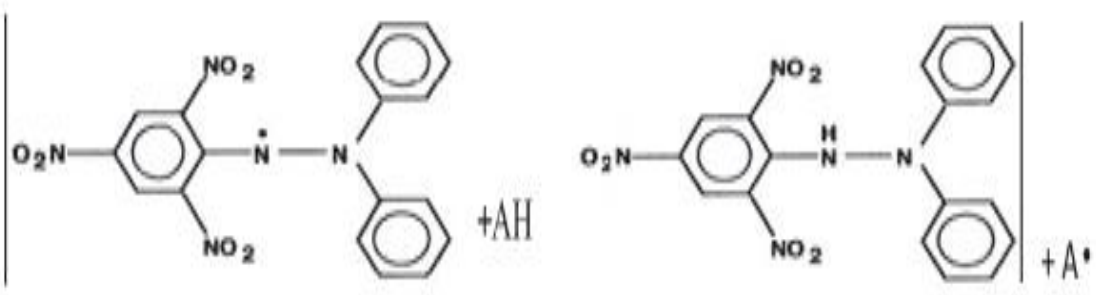
2.2.1.5 Analysis of phytochemical profile in TI sample

Consistency in the main phytochemical components of samples A – C in the various solvents was compared using Principal Component Analysis (PCA) and the amount of phytochemicals in each sample A – C was analysed using analysis of variance (ANOVA). The mean amount of phytochemicals for samples (A – C) in the different solvents was presented as heat maps.

2.2.2 Consistency in antioxidant activity for TI samples.

2.2.2.1 1,1-diphenyl-2-picrylhydrazyl scavenging activity for TI sample.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) molecule has a delocalised electron that gives the molecule a characteristic purple colour. When the electron reacts with a hydrogen donated by a suitable substrate (test compound) (Figure 2.3), there is a decrease in the intensity of the purple coloration (Alam et al., 2013). To examine the antioxidant potential of a substance, its DPPH radical scavenging activity is measured spectrophotometrically as its ability to bind to the unpaired electrons and produce a decrease in optical density at 517 nm (Alam et al., 2013; Sivakrishnan & Muthu, 2013).



DPPH (free radical) (antioxidant) DPPH (non-radical)

Figure 2.3: The reaction between DPPH and an antioxidant. Taken from Alam et al. (2013).

The DPPH method was as described previously (Sagdic et al., 2011). The amount of sample for each solvent extract was prepared such that a plot of absorbance (%) against concentration produced a plateau curve. DPPH (Fisher Scientific, Loughborough, UK) solution was prepared at a concentration of 0.1 mM. Chloroform, dichloromethane or petroleum ether extracts were prepared at a concentration of 1 mg/ml in methanol with serial dilutions of 3.9 – 1000 µg/ml. For acetone, ethanol or ethyl acetate extracts, 200 µg/ml was prepared in

methanol with serial dilutions of 0.78 – 200 µg/ml. Different stock solutions were prepared for the different solvent extracts because while optimising the DPPH protocol, extracts from acetone, ethanol or ethyl acetate showed higher activity than those from chloroform, dichloromethane or petroleum ether extracts.

Methanol (50 µl) was used as control, and 50 µl of extracts from samples A, B and C were added to the appropriate wells on a 96-well plate. DPPH solution (100 µl) was added, making a final concentration of 1.3 – 333.3 µg/ml for chloroform, dichloromethane or petroleum ether extracts and 0.26 – 66.7 µg/ml for acetone, ethanol or ethyl acetate extracts. The plates were incubated in the dark for 30 min at room temperature and the absorbance read spectroscopically at 517 nm (Sagdic et al., 2011). The percent absorbance was calculated using the equation:

Absorbance (%) = (A_{sample} / $A_{control}$) x 100, where A is absorbance.

Absorbance (%) was plotted against concentration (ug/ml). From the linear part of the graph, the derived regression equation was used to calculate the concentration of extract (IC_{50}) at which 50 % of the DPPH radicals were scavenged (Sagdic et al., 2011). The consistency in DPPH scavenging activity between samples A, B and C for each solvent extraction was analysed by One-Way Analysis of Variance (ANOVA) and $p<0.05$ was considered as significant.

2.2.2.2 Ferric reducing antioxidant power for TI sample.

Ferric reducing antioxidant power (FRAP) is based on the reduction of ferric ion to an intense blue ferrous ion at low pH, which absorbs maximally at 593 nm (Arya et al., 2013; Benzie & Strain, 1996). The results of the FRAP assay are presented with reference to the reaction kinetics of bilirubin, ascorbic acid, Trolox or uric acid (Benzie & Strain, 1996).

The FRAP assay was performed as described previously by Benzie and Strain (1996) with slight modification. The FRAP reagent was prepared by adding 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ; 2.5 ml, 10 mM in 40 mM HCl), ferric chloride ($FeCl_3$; 2.5 ml, 20 mM in distilled water) and acetate buffer (25 ml, 300 mM, pH 3.6) and stored at 37 °C until further use. Serial dilutions of Trolox (standard) were prepared in water:ethanol (4:10) with concentrations ranging from 31.2 – 312.5 µg/ml. Each TI extract was also prepared in water:ethanol (4:10) with

concentrations ranging from 31.2 – 1000 µg/ml. Extract, blank or standard (10 µl) were mixed with 190 µl of FRAP reagent to yield a final concentration of 1.6 – 15.6 µg/ml for standard, and 1.6 – 50 µg/ml for extract in each well. The mixture was incubated in the dark at 37 °C for 30 min and the absorbance read at 593 nm (BioTek µQuant). A graph was plotted with extract concentration against absorbance, and a Trolox calibration curve was also plotted. Using the regression equation derived from the Trolox calibration curve, absorbance from the linear part of the extract graph was used to calculate the FRAP activity. The results were expressed as Trolox equivalents (TE) and given as TE/ µg dry extract (Arya et al., 2013; Benzie & Strain, 1996). The formula used was:

TE/ g dry extract = C (g/ml)*[(V (ml)/ m (g)],

where C is the concentration obtained from the Trolox calibration graph, V is the volume of extract and m is the mass of extract (Arya et al., 2013). The consistency in FRAP activity between samples A, B and C were analysed using One-Way Analysis of Variance (ANOVA) and $p<0.05$ was considered as significant.

2.2.3 Consistency in effect of TI samples on cell viability and proliferation of human liver cells *in vitro*

2.2.3.1 Recovery of Hep G2 cells

The human liver cell line, Hep G2, (European Collection of Cell Cultures, ECACC No. 15L026, passage 100) was preserved in liquid nitrogen (vapour phase). Recovery of Hep G2 cells from liquid nitrogen was carried out as described previously (Patlolla et al., 2009). Prior to use, a cryovial of cells was thawed by gentle agitation for 2 min at 37 °C in a water bath. The cells were centrifuged at 1500 g for 5 min and the supernatant was discarded to remove any dimethyl sulfoxide (DMSO). The cells were then transferred to a 75 cm^2 tissue culture (T75 cm^2) flask containing 10 ml medium composed of high glucose Dulbecco's Modification of Eagle's Medium (DMEM, Fisher Scientific, Loughborough, UK) supplemented with 10 % (v/v) fetal bovine serum (FBS, lot number 015M3344, Sigma-Aldrick, Gillingham, UK), 1 % (v/v) penicillin (100 U/ml, Fisher Scientific, Loughborough, UK): streptomycin (100 µg/ml, Fisher Scientific, Loughborough, UK) and 1 % (v/v) non-essential amino acids (NEAAs, Fisher Scientific,

Loughborough, UK). The cells were incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 / 95 % air to allow the cells to attach and form a monolayer (Duthie & Collins, 1997; Patlolla et al., 2009).

2.2.3.2 Routine culture of Hep G2 cells

The routine culture of Hep G2 cells was as described previously (Duthie & Collins, 1997). 24 h after recovery, the cells were washed twice with sterile 5 ml phosphate buffered saline (PBS) (Oxoid Dulbecco A tablets, Fisher Scientific, Loughborough, UK) and maintained in fresh medium. After 7 days of growth, the cells were washed twice with 5 ml sterile PBS and harvested with 5 ml of 0.25 % trypsin (v)/(v) 0.03 % ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, Loughborough, UK) for 10 min at 37 °C. The cells were resuspended in 5 ml of medium, transferred into 15 ml centrifuge tubes and centrifuged at 1500 g for 5 min. The supernatant was discarded, and the pellet resuspended in 5 ml medium. The cells were seeded at a density of 1.5 x 10^6 cells/flask and routinely passaged 1:3 every 7 days with the medium changed every 4 days (Duthie & Collins, 1997).

2.2.3.3 Preservation of Hep G2 cell stock

After 3 passages, cells in T75 cm^2 flasks were washed twice with 5 ml sterile PBS and harvested with 5 ml of 0.25 % trypsin (v)/(v) 0.03 % EDTA. The cells were resuspended in 5 ml of medium (DMEM, 10 % FBS, 1% penicillin/streptomycin and 1% NEAAs), transferred into 15 ml centrifuge tubes and centrifuged at 1500 g for 5 min. The supernant was discarded and the cell pellet resuspended in 5 ml of cryopreserving medium (composed of DMEM supplemented with 10 % DMSO and 25 % FBS). The cell suspension (1 ml) was transferred into a 1.5 ml cryovial and put in a commercially available receptacle called a Mr. Frosty (containing isopropanol) to ensure slow freezing before being stored at -80 °C for 24 h. The vials were then transferred into liquid nitrogen (vapour phase) for long-term storage.

2.2.3.4 Optimising the solubility of TI samples in different solvents.

In order to assess the efficiency of TI samples in subsequent cell-based assays, the solubility of TI extracts was determined to provide the most appropriate solvent to solubilise extracts for administration to cells. Each solvent extract (1 mg/ml) was added to ethanol, methanol, water or DMSO (Sigma-Aldrick, Gillingham, UK), vortexed for 5 min and the solubility observed visually. The TI extracts in the different solvents were then sonicated for 15 min and incubated at room temperature for 30 min. The efficiency of extracts either to dissolve in solution, or precipitate out of solution, was observed visually after 30 min and observations recorded.

2.2.3.5 Optimising DMSO concentration on Hep G2 cell viability.

The MTT assay measures the metabolic activity of the mitochondria. In this study, the MTT assay was adopted as a measure of cell viability. The cell viability assay was carried out as described previously (Patlolla et al., 2009). Hep G2 cells were seeded at a density of 1.5 x 10^6 cells/flask and allowed to grow to 80-90% confluency. The cells were then washed twice with 5ml PBS, trypsinised with 3 mL of 0.25 % trypsin (v)/(v) 0.03 % EDTA and centrifuged at 1500 g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 5 ml fresh medium. Cells were counted with a haemocytometer and seeded at 7.5 x 10^3 cells/well in 96-well microtiter tissue culture plates with 100 µl of complete culture medium and incubated at 37 °C for 24 h in 95% air /5% CO_2. After 24 h, DMSO (0, 0.5, 1.0, 2.5 or 5.0 % final concentration in the medium) was added to the plate and medium was added to a final volume of 200 µl. The cells were incubated at 37 °C for 24 h. The medium was then removed from each well and 100 µl of 1 mg/ml methyl thiazol tetrazolium bromide (MTT, Fisher Scientific, Loughborough, UK) solution was added to each well. The plate was wrapped in tin foil and incubated at 37 °C for 4 h. The MTT was removed and 200 µl DMSO (100 %) was added to all wells. The plate was wrapped in tin foil, shaken on a plate shaker (Cole-Parmer Stuart, SSL5, Fisher Scientific, UK) for 20 min and the absorbance read at 560 nm with a spectrophotometer. The viability of cells was observed as their ability to convert yellow MTT to purple formazan, with the deeper a purple colour intensity, the higher the cell viability. Cell viability in the

various concentrations of DMSO was compared using ANOVA and $p<0.05$ was considered as significant. Cell viability was calculated using the formula below:
Cell viability (%) = (Abs / MAbc) x 100,

where Abs is absorbance of sample and MAbc is the mean absorbance of control (0 % DMSO) (Patlolla et al., 2009).

2.2.3.6 Optimising DMSO concentration on Hep G2 cell growth.

For assessing the effect of DMSO on cell growth, the method was as described previously (Duthie & Collins, 1997). Hep G2 cells were seeded at a density of 1.5 x 10^6 cells/flask and allowed to grow to 80-90% confluency. The cells were washed twice with 5ml PBS, harvested with 3 mL of 0.25 % trypsin (v)/(v) 0.03 % EDTA, and centrifuged at 1500 g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 5 ml fresh medium. Cells were counted with a haemocytometer and seeded at 7.5 x 10^4 cells/well in 12-well microtiter tissue culture plates with 1 ml of medium and incubated at 37 °C for 24 h in 95% air / 5% CO_2 (Duthie & Collins, 1997). DMSO (0, 0.5, 1.0, 2.5 or 5.0 % final concentration in the medium) was added to the plates and medium was added to a final volume of 2 ml. The cells were incubated at 37 °C for 72 h. The medium was removed, and cells were washed twice with 1 ml sterile PBS and incubated with 200 µl of 0.25 % trypsin (v)/(v) 0.03 % EDTA at 37 °C for 5 min. Complete medium (500 µl) was added to each well, cells were uniformly suspended in solution by gently pipetting the solution up and down several times, and transferred to Eppendorf tubes. Cells were counted with a haemocytometer and the percentage growth was calculated as indicated below:

Cell growth (%) = (NCs/MNCc) x 100,

where NCs is the number of cells in the sample and MNCc is the mean number of cells in controls (0 % DMSO) (Duthie & Collins, 1997).

2.2.3.7 Consistency of effect of TI samples on cell viability and cell growth.

The consistency between TI samples (A – C) for each extract was determined as previously described (sections 2.2.3.5 and 2.2.3.6) for cell viability and cell growth respectively, with sample concentrations (0 – 5.0 mg/ml final concentrations in the medium). Hep G2 cells were seeded at 7.5 x 10^3 cells/well in 96-well plate for cell viability, and at 7.5 x 10^4 cells/well in 12-well plates for cell growth and incubated with TI extracts for 24 and 72 h for cell viability and growth respectively.

2.3 RESULTS

2.3.1 Efficiency of extraction and consistency in yields of TI samples (A – C) with different solvents.

2.3.1.1 Efficiency of the extraction process as yields from solvent extraction.

From 108 g (3x36 g) TI sample used in the extraction, a combined total yield of 42.71 % was recovered using all six solvents. Sample A showed the highest mean yield compared with samples B and C (Figure 2.4). Conversely, lower yield efficiency was seen for sample A extracted with ethyl acetate compared with samples B or C. Sample B also showed higher efficiency in mean yields than sample C in petroleum ether, chloroform, dichloromethane or ethyl acetate extracts. However, mean yields in acetone and ethanol extracts were higher for sample C compared with sample B.
Acetone was the most efficient solvent for the extraction of TI with the highest mean yield across all three samples while dichloromethane was the least efficient solvent.

Sample A showed the greatest inconsistency between replicates, while samples B and C showed more consistency between their replicate yields (Figure 2.4).

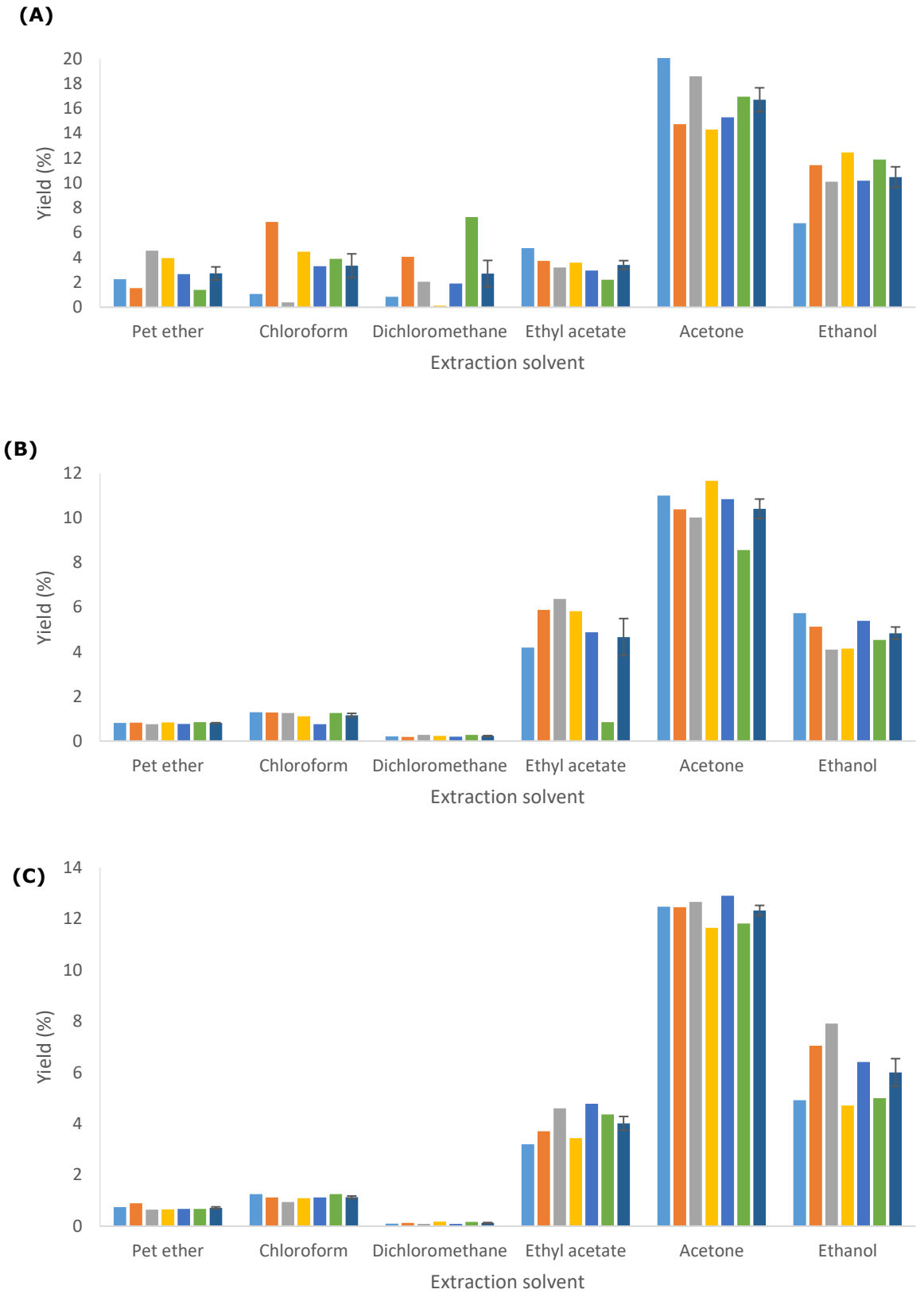


Figure 2.4: Efficiency of yield obtained from sequential Soxhlet extraction of TI samples using different solvents (n=6). (A) subsample A; (B) subsample B and (C) subsample C. The different colours indicate the six replicates for each solvent extraction and the dark blue bar is the mean of the six replicates in each solvent extraction, presented as mean ± standard error of mean (SEM).

2.3.1.2 Consistency in yield between TI samples (A – C).

Samples extracted with petroleum ether, chloroform, dichloromethane, acetone or ethanol showed a significantly ($p<0.05$) lower yield for samples B and C as compared to sample A. The mean yield of the three samples extracted with petroleum ether, acetone or ethanol was also significantly ($p<0.05$) lower as compared to yield of sample A. However, no statistically significant difference in yields was recorded between samples B and C extracted with the six solvents (Figure 2.5).

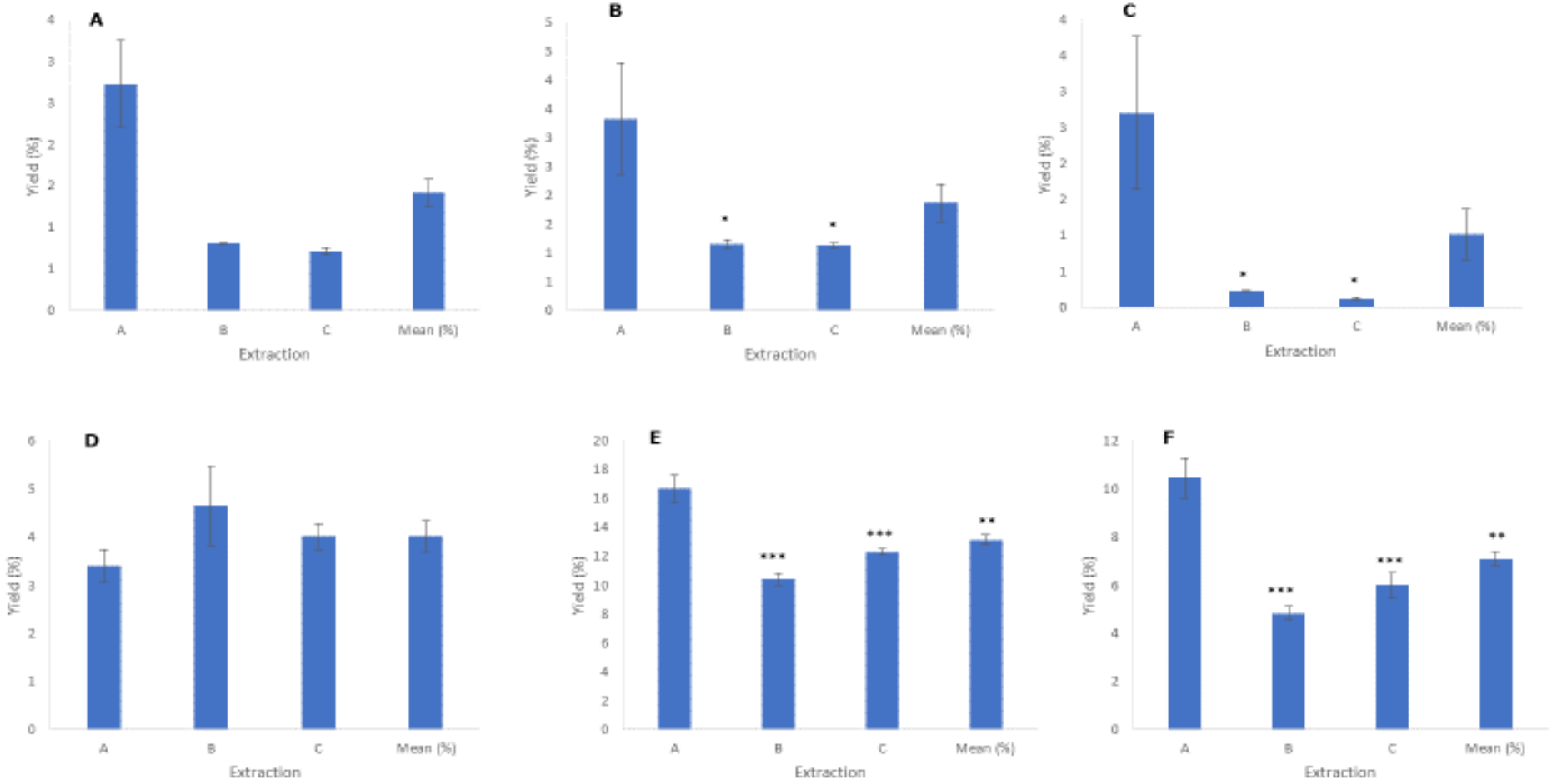


Figure 2.5: Comparison of extraction yield from the six solvents: (A) petroleum ether extract; (B) chloroform extract; (C) dichloromethane extract; (D) ethyl acetate extract; (E) acetone extract and (F) ethanol extract. Data are presented as mean ± SEM; n=6; *p<0.05; **p<0.01 and ***p<0.001 as compared to sample A by ANOVA followed by Bonferroni's multiple comparison test.

2.3.2 Consistency in phytochemical components isolated from TI samples (A – C) in the different solvents.

2.3.2.1 Consistency in phytochemicals identified from petroleum ether extracts of TI samples (A – C).

29 phytochemical metabolites were identified from the 62 phytochemical metabolites analysed from petroleum ether extracted samples (A – C). No significant difference was observed between the amount of the 29 phytochemical compounds present in samples A, B and C, which showed 100 % similarity between petroleum ether extracts (Table 2.1). The most abundant phytochemical metabolites isolated from the petroleum ether extracts of TI were 4-hydroxyphenylpyruvic acid (127.9 pg/mg), benzoic acid (19.4 pg/mg) and vanillin (17.9 pg/mg).

Table 2.1: Phytochemical metabolites identified from petroleum ether extracts of TI samples A – C.

Phytochemical class	Phytochemical metabolites	A	B	C	Mean ABC	P-value
Benzoic acids	Benzoic acid	18.4	21.7	18.2	19.4	ns
	Salicylic acid	-	356.3	0.1	-	-
	Vanillic acid	-	6.8	-	-	-
Benzaldehydes	Vanillin	22.4	15.0	16.4	17.9	ns
	Syringin	1.8	2.6	3.4	2.6	ns
	3,4-dimethoxybenzaldehyde	0.5	0.5	-	-	-
	3,4,5-trimethoxyacetophenone	0.2	0.2	0.2	0.2	ns
Phenylpyruvic acids	Phenylpyruvic acid	0.7	0.6	0.7	0.7	ns
	4-hydroxyphenylpyruvic acid	55.6	118.9	149.4	108.0	ns
Phenyllactic acids	Phenyllactic acid	0.8	0.9	0.6	0.8	ns
Phenols	Anthranilic acid	-	0.2	0.2	-	-
	Quinadilic acid	-	0.1	0.1	-	-
	Ethylferulate	16.8	-	-	-	-
	4-ethylphenol	-	-	1.9	1.9	-

	4-methylcatechol	0.4	0.5	0.7	0.5	ns
Phenolic dimers	Ferulic dimer (5-5 linked)	0.7	0.5	0.5	0.6	ns
	Ferulic dimer (5-5 hydrogenated)	1.0	1.0	-	1.0	-
Indoles	Indole-3-carbinol	1.3	1.2	2.2	1.6	ns
Flavonoids	8-methylpsoralen	0.2	0.2	0.2	0.2	ns
	Bergapten	0.3	0.3	0.3	0.3	ns
	Tangeretin	1.0	1.0	1.1	1.0	ns
	Imperatorin	0.2	0.3	0.4	0.3	ns
	Luteolinidin	2.3	1.8	1.4	1.8	ns
	Glycitein	0.2	0.2	0.2	0.2	ns
Lignans	Secoisolariciresinol	0.9	1.1	0.9	1.0	ns
	Matairesinol	0.7	0.7	0.7	0.7	ns
	Enterodiol	0.8	0.8	0.7	0.8	ns
	Enterolactone	1.2	1.3	1.0	1.2	ns
	Indole-3-carboxaldehyde	0.2	0.3	0.3	0.3	ns

Data are presented as pg/mg amount for A, B and C and mean of ABC; ns indicates not significant ($p>0.05$) by ANOVA followed by Bonferroni's post hoc test; - indicates below detectable limit and no statistics were carried out for any of these analyses.

2.3.2.2 Consistency in phytochemicals identified from chloroform extracts of TI samples (A – C).

Of the 62 phytochemicals analysed from samples A, B and C, 50 were identified from chloroform extracts. No significant difference was observed in the amounts of phytochemicals identified from samples A, B and C which showed a similarity index of 100 % (Table 2.2). This shows that specific phytochemical compounds are consistently extracted from the samples using chloroform and going forward can be regarded as representative of each other. Table 2.2 shows that the most abundant phytochemicals isolated with chloroform were 4-hydroxyphenylpyruvic acid (54.8 pg/mg), benzoic acid (116.9 pg/mg), ferulic acid (23.7 pg/mg), naringenin (19.1 pg/mg), secoisolariciresinol (57.2 pg/mg), syringic acid (16.1 pg/mg), syringin (25.3 pg/mg), vanillic acid (90.6 pg/mg) and vanillin (93.1 pg/mg).

Table 2.2: Phytochemical metabolites identified from chloroform extracts of TI samples A – C.

Phytochemical class	Phytochemical metabolites	A	B	C	Mean ABC	P-value
Benzoic acids	Benzoic acid	102.9	132.6	115.2	116.9	ns
	Salicylic acid	13.7	11.7	13.8	13.1	ns
	P-hydroxybenzoic acid	8.3	9.6	9.5	9.2	ns
	Anthranilic acid	0.3	0.3	0.3	0.3	ns
	Protocatechuic acid	8.9	-	14.5	-	-
	Gallic acid	3.5	-	4.8	-	-
	Vanillic acid	90.8	86.8	94.2	90.6	ns
	Syringic acid	15.9	13.2	19.3	16.1	ns
Benzaldehydes	P-hydroxybenzaldehyde	5.7	6.5	5.9	6.0	ns
	Protocatachaldehyde	15.6	18.6	21.4	18.5	ns
	Vanillin	92.3	88.7	98.1	93.1	ns
	Syringin	25.2	20.4	30.3	25.3	ns
	3,4-dimethoxybenzaldehyde	0.7	0.9	0.7	0.8	ns
Cinnamic acids	P-coumaric acid	4.6	3.0	4.9	4.2	ns

	Ferulic acid	23.2	20.6	27.3	23.7	ns
Phenylpropionic acids	4-hydroxyphenylpropionic acid	-	98.6	118.7	-	-
Acetophenones	4-hydroxyacetophenone	9.4	8.4	8.9	8.9	ns
	4-hydroxy-3-methoxyacetophenone	2.1	1.4	2.6	2.1	ns
	3,4-dimethoxyacetophenone	0.5	-	0.5	-	-
	3,4,5-trimethoxyacetophenone	0.2	0.2	0.1	0.1	ns
Phenylpyruvic acids	Phenylpyruvic acid	0.6	0.6	-	-	-
	4-hydroxyphenylpyruvic acid	59.4	53.2	51.8	54.8	ns
Phenyllactic acids	Phenyllactic acid	1.0	0.7	1.0	0.9	ns
Phenols	Quinadilic acid	0.2	-	0.1	-	-
	4-ethylphenol	-	1.6	-	-	-
	4-methylcatechol	0.7	0.7	0.7	0.7	ns
Phenolic dimers	Ferulic dimer (5-5 linked)	0.7	0.5	0.6	0.6	ns
Indoles	Indole	1.3	1.4	1.5	1.4	ns
	Indole-3-carbinol	1.6	1.6	1.6	1.6	ns
Flavonoids	Bergapten	0.2	0.2	0.2	0.2	ns
	Tangeretin	1.3	1.1	0.9	1.1	ns

	Phloretin	1.2	0.2	1.1	0.8	ns
	Imperatorin	0.3	0.4	0.2	0.3	ns
	Naringenin	21.6	15.9	19.8	19.1	ns
	Hesperitin	1.1	1.0	1.0	1.0	ns
	Quercetin	1.7	-	1.7	-	-
	Taxifolin	2.2	-	1.9	-	-
	Scopoletin	0.2	0.4	0.1	0.2	ns
	Luteolin	1.3	-	1.2	-	-
	Luteolinidin	1.3	1.4	1.5	1.4	ns
	Isorhamnetin	3.2	3.2	3.2	3.2	ns
	Apigenin	1.8	1.3	1.8	1.6	ns
	Glycitein	0.2	0.2	0.2	0.2	ns
Lignans	Secoisolariciresinol	63.8	48.1	59.6	57.2	ns
	Matairesinol	0.7	0.6	0.5	0.6	ns
	Enterodiol	0.6	0.5	0.4	0.5	ns
	Enterolactone	1.1	1.0	1.0	1.0	ns
	Syringaresinol	47.8	-	53.2	-	-

Pinoresinol	9.4	3.8	8.0	7.1	ns
Indole-3-carboxaldehyde	1.6	1.5	1.6	1.6	ns

Data are presented as pg/mg amount for A, B and C and mean of ABC; ns indicates not significant ($p>0.05$) by ANOVA followed by Bonferroni's post hoc test; - indicates below detectable limit and no statistics were carried out for any of these analyses.

2.3.2.3 Consistency in phytochemicals identified from dichloromethane extracts of TI samples (A – C).

48 phytochemicals were identified from dichloromethane extracted samples A, B and C. From the 48 phytochemicals, 9 (18.8 %) were significantly different and 39 (81.2 %) showed no statistically significant difference between the samples (A – C). Sample C showed significantly ($p<0.05$) higher amounts of benzoic acid, ferulic acid, p-hydroxybenzoic acid, syringin and vanillic acid as compared to samples A or B. Sample C also showed significantly ($p<0.05$) higher amounts of syringic acid, p-hydroxybenzaldehyde, protocatachaldehyde and 4-hydroacetophenone as compared to sample A, but were not significantly different to sample B. The most abundant phytochemicals isolated by dichloromethane extraction of TI were 4-hydroxyphenylpyruvic acid (110.1 pg/mg), benzoic acid (64.4 pg/mg), ferulic acid (25.3 pg/mg), naringenin (28.7 pg/mg), secoisolariciresinol (57.8 pg/mg), syringaresinol (43.3 pg/mg), syringic acid (33.2 pg/mg), syringin (58.2 pg/mg), vanillic acid (149.7 pg/mg) and vanillin (192.8 pg/mg) (Table 2.3).

Table 2.3: Phytochemical metabolites identified from dichloromethane extract of TI samples.

Phytochemical class	Phytochemical metabolites	A	B	C	Mean ABC	P-value
Benzoic acids	Benzoic acid	33.4	49.0	110.7***	64.4	0.0013
	Salicylic acid	0.7	1.3	3.8	1.9	ns
	P-hydroxybenzoic acid	4.2	4.3	18.9*	9.1	0.0216
	Protocatechuic acid	-	-	7.1	-	-
	Vanillic acid	70.7	89.8	288.5**	149.7	0.0120
	Syringic acid	17.9	25.8	55.8*	33.2	0.0305
Benzaldehydes	P-hydroxybenzaldehyde	5.0	7.5	19.0*	10.5	0.0266
	Protocatachaldehyde	7.1	10.3	32.1*		0.0290
	Vanillin	116.8	133.0	128.6	126.1	ns
	Syringin	33.6	35.3	105.7***	58.2	0.0011
	3,4-dimethoxybenzaldehyde	1.4	1.4	1.7	1.5	ns
Cinnamic acids	Cinnamic acid	-	1.0	1.6	-	-
	P-coumaric acid	-	1.5	8.8	-	-
	Ferulic acid	16.3	18.7	41.0*	25.3	0.0441

Phenylpropionic acids	4-hydroxyphenylpropionic acid	-	-	236.7	-	-
Acetophenones	4-hydroxyacetophenone	11.3	12.9	29.3*	17.8	0.0328
	4-hydroxy-3-methoxyacetophenone	2.4	3.5	4.1	3.3	ns
	3,4-dimethoxyacetophenone	0.8	1.0	1.2	1.0	ns
	3,4,5-trimethoxyacetophenone	0.3	0.3	0.4	0.3	ns
Phenylacetic acids	Phenylacetic acid	2.8	2.2	3.9	2.9	ns
Phenylpyruvic acids	4-hydroxyphenylpyruvic acid	85.3	87.4	157.6	110.1	ns
Phenyllactic acids	Phenyllactic acid	0.8	1.0	1.2	1.0	ns
Phenols	Anthranilic acid	-	-	0.4	-	-
	Quinadilic acid	0.1	-	0.2	-	-
	4-methylcatechol	0.8	0.9	1.2	1.0	ns
Phenolic dimers	Ferulic dimer (5-5 linked)	0.7	0.7	0.7	0.7	ns
Indoles	Indole	1.6	1.9	3.8	2.4	ns
	Indole-3-carbinol	1.8	1.7	1.2	1.6	ns
	Indole-3-carboxylic acid	-	-	1.0	-	-
	Indole-3-pyruvic acid	-	-	17.3	-	-
Flavonoids/Coumarins	Coumarin	-	0.3	0.6	-	-

	Bergapten	0.2	0.2	0.2	0.2	ns
	Tangeretin	0.7	0.6	0.8	0.7	ns
	Phloretin	0.1	0.2	1.1	0.5	ns
	Imperatorin	0.2	0.2	0.2	0.2	ns
	Naringenin	24.2	26.0	36.1	28.7	ns
	Hesperitin	0.5	1.2	1.9	1.2	ns
	Luteolinidin	1.0	1.0	1.8	1.1	ns
	Isorhamnetin	-	-	4.1	-	-
	Apigenin	0.9	1.0	1.8	1.2	ns
	Glycitein	0.2	-	-	-	-
Lignans	Secoisolariciresinol	46.8	61.77	65.1	57.8	ns
	Matairesinol	0.8	0.8	1.2	0.9	ns
	Enterodiol	0.5	0.5	0.4	0.5	ns
	Enterolactone	0.9	0.9	0.9	0.9	ns
	Syringaresinol	35.0	38.2	56.8	43.3	ns
	Pinoresinol	12.0	11.3	16.9	13.4	ns
	Indole-3-carboxaldehyde	1.8	2.1	4.1	2.7	ns

Data are presented as pg/mg amount for A, B and C and mean of ABC; ns indicates not significant ($p>0.05$); *$p< 0.05$, **$p< 0.01$ and ***$p< 0.001$ as compared to sample A or C by ANOVA followed by Bonferroni's post hoc test; - indicates below detectable limit and no statistics were carried out for any of these analyses.

2.3.2.4 Consistency in phytochemicals identified from ethyl acetate extracts of TI samples (A – C).

A total of 45 phytochemicals were identified from samples A, B and C extracted using ethyl acetate (Table 2.4). No significant difference was observed in the phytochemical profile of samples A, B or C which showed 100 % similarity index. This shows that specific phytochemical compounds are consistently extracted from the samples using ethyl acetate and going forward can be regarded as representative of each other. The predominant phytochemical metabolites in these extracts were 4-hydroxyphenylpyruvic acid (23.2 pg/mg), benzoic acid (47.7 pg/mg), catechin (712.1 pg/mg), gallic acid (626.4 pg/mg), protocatechuic acid (96.0 pg/mg), secoisolariciresinol (34.0 pg/mg), syringaresinol (26.5 pg/mg), vanillic acid (30.0 pg/mg) and vanillin (23.3 pg/mg).

Table 2.4: Phytochemical metabolites identified from ethyl acetate extracts of TI samples.

Phytochemical class	Phytochemical metabolites	A	B	C	Mean ABC	P-value
Benzoic acids	Benzoic acid	40.7	70.8	31.5	47.7	ns
	Salicylic acid	0.3	0.2	0.2	0.2	ns
	P-hydroxybenzoic acid	8.2	7.9	7.2	7.8	ns
	Protocatechuic acid	96.6	98.3	93.0	96.0	ns
	Gallic acid	613.5	688.3	577.3	626.4	ns
	Vanillic acid	29.7	30.5	29.8	30.0	ns
	Syringic acid	4.4	5.2	3.8	4.5	ns
Benzaldehydes	P-hydroxybenzaldehyde	-	1.4	-	-	-
	Protocatachaldehyde	11.2	13.6	12.7	12.5	ns
	Vanillin	24.0	21.4	24.5	23.3	ns
	Syringin	8.5	7.1	7.7	7.8	ns
	3,4-dimethoxybenzaldehyde	1.6	1.4	1.8	1.6	ns
Cinnamic acids	P-coumaric acid	5.5	5.2	5.2	5.31	ns
	Ferulic acid	7.9	6.2	8.1	7.4	ns

Acetophenones	4-hydroxyacetophenone	4.1	3.4	4.9	4.2	ns
	3,4,5-trimethoxyacetophenone	0.3	0.3	0.3	0.3	ns
Phenylpyruvic acids	4-hydroxyphenylpyruvic acid	25.3	20.2	24.2	23.2	ns
Phenyllactic acids	Phenyllactic acid	0.6	0.9	0.6	0.7	ns
Phenols	Quinadilic acid	-	0.3	-	-	-
	4-methylcatechol	0.5	0.6	0.6	0.6	ns
Phenolic dimers	Ferulic dimer (5-5 linked)	-	0.5	-	-	-
Indoles	Indole-3-carbinol	0.9	0.9	0.8	0.8	ns
	Indole-3-pyruvic acid	10.7	13.5	14.3	12.9	ns
Flavonoids	Bergapten	0.3	0.3	0.3	0.3	ns
	Tangeretin	1.5	1.7	1.6	1.6	ns
	Catechin	744.0	619.5	773.0	712.1	ns
	Gallocatechin	10.0	9.2	11.0	10.1	ns
	Phloretin	5.0	4.7	4.8	4.8	ns
	Imperatorin	0.5	0.6	0.6	0.6	ns
	Naringenin	15.9	17.1	17.1	16.7	ns
	Hesperitin	1.2	1.1	1.3	1.2	ns

	Quercetin	7.8	7.2	7.1	7.4	ns
	Taxifolin	16.4	14.3	14.8	15.2	ns
	Phloridzin	14.3	11.6	13.8	13.2	ns
	Luteolin	2.6	2.5	2.3	2.4	ns
	Luteolinidin	5.0	3.4	3.1	3.8	ns
	Apigenin	1.7	1.7	1.6	1.7	ns
	Glycitein	-	0.4	-	-	-
Lignans	Secoisolariciresinol	33.9	33.5	33.5	34.0	ns
	Matairesinol	0.5	0.6	0.6	0.6	ns
	Enterodiol	0.2	0.4	0.2	0.3	ns
	Enterolactone	0.2	0.9	0.5	0.6	ns
	Syringaresinol	28.9	22.2	28.3	26.5	ns
	Pinoresinol	3.3	3.4	4.0	3.6	ns
	Indole-3-carboxaldehyde	1.2	1.2	1.3	1.2	ns

Data are presented as pg/mg amount for A, B and C and mean of ABC; ns indicates not significant ($p>0.05$) by ANOVA followed by Bonferroni's post hoc test; - indicates below detectable limit and no statistics were carried out for any of these analyses.

2.3.2.5 Consistency in phytochemical metabolites in acetone extracts of TI samples (A – C).

41 phytochemicals were identified from 62 phytochemicals analysed from acetone extracts of samples (A – C). No significant difference was observed between the samples. This shows that specific phytochemical compounds are consistently extracted from the samples using acetone and going forward can be regarded as representative of each other. The predominant phytochemical metabolites in acetone extracts were benzoic acid (24.9 pg/mg), catechin (237.8 pg/mg), gallic acid (410.9 pg/mg), protocatechuic acid (48.6 pg/mg), secoisolariciresinol (26.0 pg/mg), syringaresinol (17.1 pg/mg), vanillic acid (12.7 pg/mg) and vanillin (10.0 pg/mg).

Table 2.5: Phytochemical metabolites identified from acetone extracts of TI samples.

Phytochemical class	Phytochemical metabolites	A	B	C	Mean ABC	P-value
Benzoic acids	Benzoic acid	24.4	28.1	22.2	24.9	ns
	Salicylic acid	-	0.4	0.1	-	-
	P-hydroxybenzoic acid	3.2	2.2	3.0	2.8	ns
	Protocatechuic acid	53.7	37.4	54.6	48.6	ns
	Gallic acid	451.1	347.9	433.7	410.9	ns
	Vanillic acid	14.0	11.6	12.4	12.7	ns
Benzaldehydes	Protocatachaldehyde	7.3	4.4	8.4	6.7	ns
	Vanillin	10.8	8.9	10.2	10.0	ns
	Syringin	3.1	2.0	2.7	2.6	ns
Cinnamic acids	P-coumaric acid	1.7	1.6	2.5	1.9	ns
	Ferulic acid	2.6	1.5	2.7	2.3	ns
Acetophenones	4-hydroxyacetophenone	1.8	-	-	-	-
	3,4,5-trimethoxyacetophenone	0.4	0.3	0.3	0.3	ns
Phenyllactic acids	Phenyllactic acid	0.9	0.6	0.7	0.7	ns

Phenolics others	Quinadilic acid	-	0.3	-	-	-
Phenols	4-methylcatechol	0.6	0.6	0.6	0.6	ns
Phenolic dimers	Ferulic dimer (5-5 linked)	0.5	0.4	0.6	0.5	ns
Indoles	Indole-3-carbinol	1.0	0.7	0.8	0.8	ns
	Indole-3-pyruvic acid	13.2	-	8.8	-	-
Flavonoids	8-methylpsoralen	0.2	-	-	-	-
	Bergapten	0.4	0.4	0.4	0.4	ns
	Tangeretin	1.6	1.7	1.6	1.6	ns
	Catechin	241.1	223.2	249.3	237.8	ns
	Gallocatechin	3.0	3.7	3.0	3.2	ns
	Phloretin	1.3	1.3	1.2	1.3	ns
	Imperatorin	0.6	0.5	0.5	0.5	ns
	Naringenin	7.9	5.8	8.2	7.3	ns
	Quercetin	4.7	4.7	4.5	4.6	ns
	Taxifolin	6.0	5.6	5.9	5.8	ns
	Phloridzin	2.3	2.8	2.0	2.4	ns
	Luteolin	1.8	1.8	1.8	1.8	ns

	Luteolinidin	4.2	3.5	3.8	3.8	ns
	Apigenin	1.4	1.3	1.5	1.4	ns
	Glycitein	0.5	0.4	0.3	0.4	ns
Lignans	Secoisolariciresinol	27.0	23.3	27.6	26.0	ns
	Matairesinol	0.6	0.5	0.5	0.6	ns
	Enterodiol	0.4	0.4	0.2	0.3	ns
	Enterolactone	0.7	0.6	0.5	0.6	ns
	Syringaresinol	18.9	15.5	16.8	17.1	ns
	Pinoresinol	3.9	3.3	3.5	3.6	ns
	Indole-3-carboxaldehyde	1.0	1.0	0.9	1.0	ns

Data are presented as pg/mg amount for A, B and C and mean of ABC; ns indicates not significant ($p>0.05$) by ANOVA followed by Bonferroni's post hoc test; - indicates below detectable limit and no statistics were carried out for any of these analyses.

2.3.2.6 Consistency in phytochemical metabolites in ethanol extracts of TI samples (A – C).

A total of 45 phytochemicals were identified from 62 phytochemicals analysed from the ethanol extracted samples (A, B and C). No significant difference was observed in 44 (97.8 %) out of 45 phytochemicals. Catechin amounts were shown to be significantly ($p<0.01$) lower in sample B as compared to sample A or C. The samples showed overall variability index of 2.2 % and 97.8 % similarity index, hence, were regarded as representative of each other. The predominant phytochemical metabolites in the ethanol extracts were 4-hydroxyphenylpyruvic acid (38.2 pg/mg), benzoic acid (40.9 pg/mg), catechin (248.7 pg/mg), gallic acid (853.2 pg/mg), indole-3-pyruvic acid (23.9 pg/mg), protocatechuic acid (112.7 pg/mg), secoisolariciresinol (35.7 pg/mg), syringaresinol (45.6 pg/mg), vanillic acid (32.8 pg/mg) and vanillin (25.6 pg/mg).

Table 2.6: Phytochemical metabolites identified from ethanol extracts of TI samples.

Phytochemical class	Phytochemical metabolites	A	B	C	Mean ABC	P-value
Benzoic acids	Benzoic acid	40.7	46.7	35.3	40.9	ns
	Salicylic acid	0.1	0.3	0.3	0.2	ns
	P-hydroxybenzoic acid	7.3	7.7	7.4	7.5	ns
	Anthranilic acid	0.9	0.9	0.9	0.9	ns
	Protocatechuic acid	107.0	104.5	126.7	112.7	ns
	Gallic acid	796.0	834.2	929.4	853.2	ns
	Vanillic acid	33.9	30.7	33.7	32.8	ns
	Syringic acid	7.9	9.4	5.8	7.7	ns
Benzaldehydes	P-hydroxybenzaldehyde	-	-	1.5	-	-
	Protocatachaldehyde	12.9	11.9	17.3	14.0	ns
	Vanillin	28.7	19.2	29.0	25.6	ns
	Syringin	7.2	5.7	7.3	6.7	ns
Cinnamic acids	P-coumaric acid	6.3	6.3	7.3	6.6	ns
	Ferulic acid	6.8	5.9	7.3	6.7	ns

Acetophenones	4-hydroxyacetophenone	2.5	-	2.2	-	-
	3,4,5-trimethoxyacetophenone	0.3	0.3	0.3	0.3	ns
Phenylpyruvic acids	4-hydroxyphenylpyruvic acid	37.3	36.8	40.6	38.2	ns
Phenyllactic acids	Phenyllactic acid	0.7	0.7	0.7	0.7	ns
Phenols	4-methylcatechol	0.5	0.8	0.8	0.7	ns
Phenolic dimers	Ferulic dimer (5-5 linked)	1.0	0.8	0.9	0.9	ns
Indoles	Indole-3-carbinol	0.8	1.1	0.9	0.9	ns
	Indole-3-pyruvic acid	26.2	20.1	25.3	23.9	ns
Amines	Tyromine	0.7	0.7	0.7	0.7	ns
Flavonoids	Bergapten	0.3	0.3	0.3	0.3	ns
	Tangeretin	1.3	1.1	1.2	1.2	ns
	Catechin	351.2*	49.1	345.9*	248.8	0.001
	Gallocatechin	4.3	-	3.4	-	-
	Phloretin	1.9	1.1	1.8	1.6	ns
	Imperatorin	0.4	0.4	0.3	0.4	ns
	Naringenin	12.5	10.0	11.1	11.2	ns
	Hesperitin	0.6	0.4	0.5	0.5	ns

	Quercetin	5.0	5.5	4.7	5.1	ns
	Taxifolin	11.8	11.8	13.1	12.3	ns
	Phloridzin	1.2	0.9	0.8	1.0	ns
	Luteolin	2.4	2.1	2.1	2.2	ns
	Luteolinidin	3.8	4.2	4.2	4.1	ns
	Apigenin	1.6	1.5	1.6	1.5	ns
	Glycitein	0.7	-	-	-	-
Lignans	Secoisolariciresinol	35.3	34.4	37.3	35.7	ns
	Matairesinol	0.8	0.9	0.7	0.8	ns
	Enterodiol	0.3	0.2	0.3	0.3	ns
	Enterolactone	0.5	0.5	0.4	0.5	ns
	Syringaresinol	36.1	51.0	49.7	45.6	ns
	Pinoresinol	8.9	7.6	8.6	8.3	ns
	Indole-3-carboxaldehyde	1.6	0.9	1.4	1.3	ns

Data are presented as pg/mg amount for A, B and C and mean of ABC; ns indicates not significant ($p>0.05$); *$p< 0.05$ as compared to sample A or C by ANOVA followed by Bonferroni's post hoc test; - indicates below detectable limit and no statistics were carried out for any of these analyses.

2.3.2.7 Phytochemical distribution in the different solvent extracts.

To visualise the most abundant compounds extracted using each solvent, the mean amounts of phytochemicals extracted from samples (A – C) were presented as heat maps. Extraction of TI with the 6 solvents showed the presence of acetophenones, benzaldehydes, benzoic acids, cinnamic acids, flavonoids, indoles, lignans, phenolics, phenylacetic acids, phenyllactic acids and phenylpyruvic acids.

Petroleum ether extract showed the highest amount of phenylpyruvic acids (particularly 4-hydroxyphenylpyruvic acid). Similarly, chloroform and dichloromethane extracts showed the highest amounts of benzoic acids (particularly benzoic acid, salicylic acid, syringic acid and vanillic acid); benzaldehydes (particularly 3,4-dimethoxybenzaldehyde, P-hydroxybenzaldehyde, protocatachaldehyde, syringin and vanillin); cinnamic acids (particularly ferulic acid) and lignans (particularly secoisolariciresinol). On the other hand, benzoic acids like gallic acid and protocatechuic acid; and flavonoids like catechin, quercetin and taxifolin were detected only in acetone, ethanol or ethyl acetate extract. These findings confirmed the clustering of the principal components shown in the PCA plot (Figure 2.8).

The reproducibility of the extractions was also rigorously investigated on key biomarkers such as: antioxidant activity and effects on cell viability and cell growth.

Table 2.7: Amounts and distribution of phytochemical metabolites identified from different solvent extracts of TI.

Phytochemical class	Phytochemical metabolites	Petroleum ether	Chloroform	Dichloromethane	Ethyl acetate	Acetone	Ethanol
Benzoic acids	Benzoic acid	19.4	116.9	64.4	47.7	24.9	40.9
	P-hydroxybenzoic acid	-	9.2	9.1	7.8	2.8	7.5
	Protocatechuic acid	-	-	-	96.0	48.6	112.7
	Anthranilic acid	-	0.3	-	-	-	0.9
	Gallic acid	-	-	-	626.4	410.9	853.2
	Vanillic acid	-	90.6	149.7	30.0	12.7	32.8
	Salicylic acid	-	13.1	1.9	0.2	-	0.2
	Syringic acid	-	16.1	33.2	4.5	-	7.7
Benzaldehydes	P-hydroxybenzaldehyde	-	6.0	10.5	-	-	-
	Protocatachaldehyde	-	18.5	16.5	12.5	6.7	12.2
	Vanillin	17.9	93.1	192.8	23.3	10.0	25.6
	Syringin	2.6	25.3	58.2	7.8	2.6	6.7
	3,4-dimethoxybenzaldehyde	-	0.8	1.8	1.6	-	-
Cinnamic acids	P-coumaric acid	-	4.2	-	5.3	1.9	6.6

	Ferulic acid	-	23.7	25.3	7.4	2.3	6.7
Acetophenones	4-hydroxyacetophenone	-	8.9	17.8	4.2	-	-
	4-hydroxy-3-methoxyacetophenone	-	2.1	5.7	-	-	-
	3,4,5-trimethoxyacetophenone	0.2	0.1	0.3	0.3	0.3	0.3
	3,4-dimethoxyacetophenone	-	-	1.0	-	-	-
Phenylacetic acids	Phenylacetic acid	-	-	2.9	-	-	-
Phenylpyruvic acids	4-hydroxyphenylpyruvic acid	127.9	54.8	110.1	23.2	-	38.2
	Phenylpyruvic acid	0.7	-	-	-	-	-
Phenyllactic acids	Phenyllactic acid	0.8	0.9	1.0	0.7	0.7	0.7
Phenols	4-methylcatechol	0.5	0.7	1.0	0.6	0.6	0.7
Phenolic dimers	Ferulic dimer (5-5 linked)	0.6	0.6	0.7	-	0.5	0.9
Indoles	Indole	-	1.4	2.4	-	-	-
	Indole-3-carbinol	1.6	1.6	1.6	0.8	0.8	0.9
	Indole-3-carboxaldehyde	0.3	1.6	2.7	1.2	1.0	1.3
	Indole-3-pyruvic acid	-	-	-	12.9	-	23.9

	Tyromine	-	-	-	-	-	0.7
	coumarin	-	-	-	-	-	-
Flavonoids	8-methylpsoralen	0.2	-	-	-	-	-
	Bergapten	0.3	0.2	0.2	0.3	0.4	0.3
	Tangeretin	1.0	1.1	0.7	1.6	1.6	1.2
	Catechin	-	-	-	712.1	237.8	248.8
	Gallocatechin	-	-	-	10.1	3.2	-
	Phloretin	-	0.8	0.5	4.8	1.3	1.5
	Imperatorin	0.3	0.3	0.2	0.6	0.5	0.4
	Naringenin	-	19.1	28.7	16.7	7.3	11.2
	Hesperitin	-	1.0	1.5	1.2	-	0.5
	Quercetin	-	-	-	7.4	4.6	5.1
	Taxifolin	-	-	-	15.2	5.8	12.3
	Scopoletin	-	0.2	-	-	-	-
	Phloridzin	-	-	-	13.2	2.4	1.0
	Luteolin	-	0.8	-	2.4	1.8	2.2
	Luteolinidin	1.8	1.4	1.1	3.8	3.8	4.1

	Isorhamnetin	-	3.2	-	-	-	-
	Apigenin	-	1.6	1.2	1.7	1.4	1.5
	Glycitein	0.2	0.2	-	-	0.4	-
Lignans	Secoisolariciresinol	1.0	57.2	57.8	34.0	26.0	35.7
	Matairesinol	0.7	0.6	0.9	0.6	0.6	0.8
	Enterodiol	0.8	0.5	0.5	0.3	0.3	0.3
	Enterolactone	1.2	1.0	0.9	0.6	0.6	0.5
	Syringaresinol	-	-	43.3	26.5	17.1	45.6
	Pinoresinol	-	7.1	13.4	3.6	3.6	8.3

Data are presented as mean; n=3 and – indicates phytochemicals not detected. Mean amounts of phytochemicals ≥100 pg/mg was denoted as red; 50 – 99 pg/mg (orange); 20 – 49 pg/mg (yellow); 5 – 19 pg/mg (light green) and below 5 pg/mg (light blue).

2.3.2.8 Principal components analysis of samples (A – C).

Principal component analysis (PCA) on a univariate scale was used to assess consistency of the main phytochemical metabolites isolated from samples A, B and C. Similarity in phytochemicals between samples was shown by how closely clustered they were to each other in the same quadrant of a plot. From the PCA plot (Figure 2.6), samples A, B and C were comparable for all solvent extracts except for sample C using dichloromethane extraction.

Looking across the 3 samples (A – C), metabolites from petroleum ether extract were completely different from metabolites identified from the other solvent extracts. Metabolites from chloroform and dichloromethane extracts were similar while ethyl acetate, acetone and ethanol extracts also showed similar metabolites. However, ethyl acetate showed the highest amount of catechin and could be considered as a different group for further extraction. Therefore, for further extractions to be used in this research (that is samples D, E and F), TI can be extracted with (1) petroleum ether, (2) chloroform or dichloromethane, (3) ethyl acetate and (4) acetone or ethanol to obtain 4 solvent extracts that will capture all the phytochemical compounds previously extracted with six solvents.

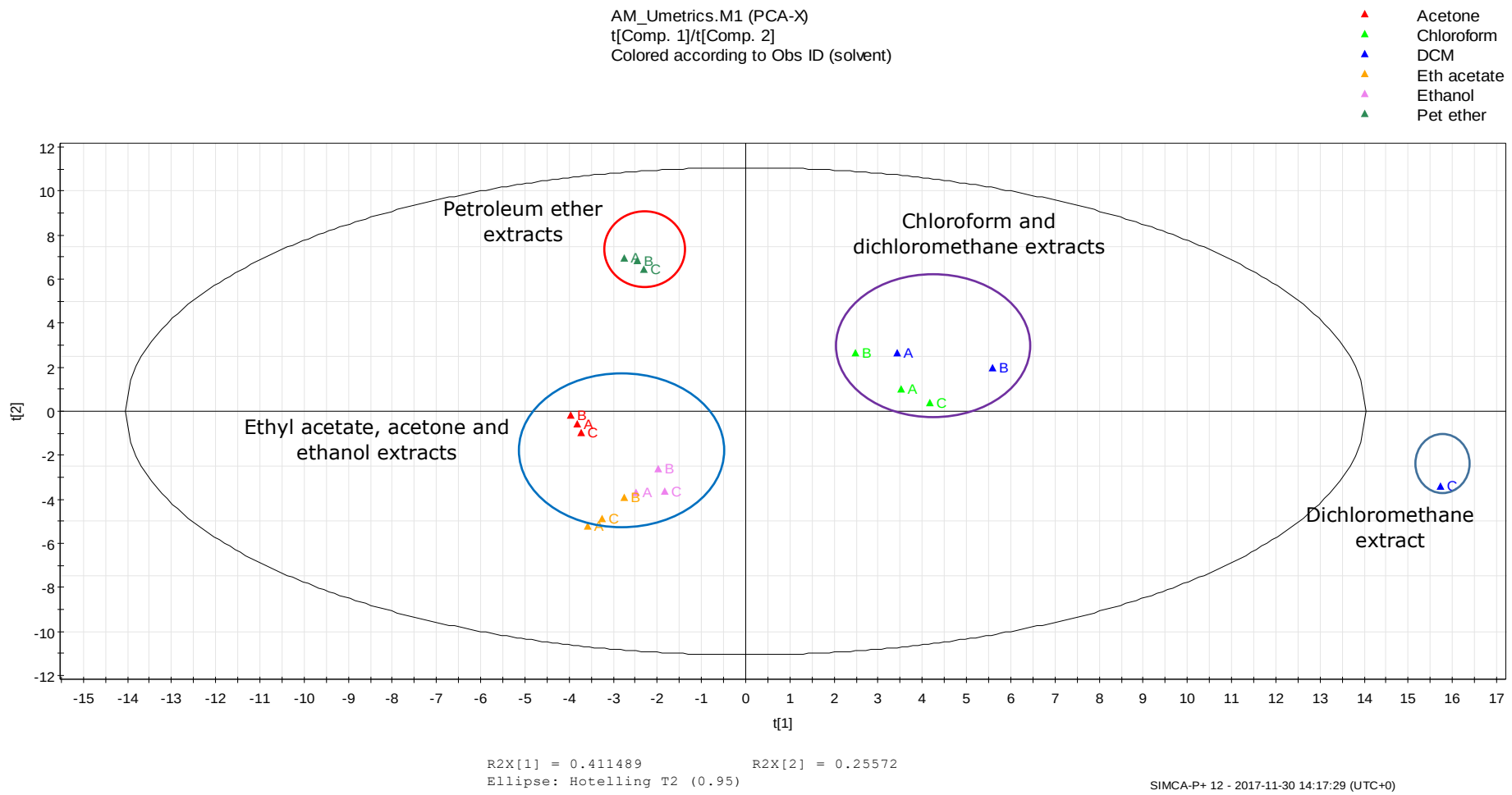


Figure 2.6: Principal component analysis on a univariate scale showing the similarity in phytochemical components between samples A, B and C extracted with the various solvents.

2.3.3 Consistency in antioxidant activity for TI samples.

2.3.3.1 DPPH radical scavenging activity of TI samples (A – C).

The concentration of TI at which half the concentration of DPPH radicals was scavenged (IC_{50}) was calculated and used to determine the consistency in antioxidant activity between the 3 samples. All samples (A – C) showed similar DPPH scavenging activity. Extraction of samples with chloroform showed the highest variation (coefficient of variation, CV=19.9 %) in DPPH scavenging activity, while extraction with petroleum ether showed the least variability (CV=0.9 %). The samples extracted with petroleum ether, dichloromethane, ethyl acetate or acetone showed consistent DPPH scavenging activity as shown by the IC_{50} values (CV<10 %) (Table 2.8).

For the individual extracts arranged in order of increasing DPPH scavenging activity: Chloroform extract < dichloromethane extract < petroleum ether extract < ethyl acetate extract < acetone extract < ethanol extract.

Table 2.8: DPPH scavenging activity (IC_{50}) for TI samples A – C.

Extract/ IC_{50} (µg/ml)	A	B	C	Mean ABC	CV (%)
Petroleum ether	1151.0	1170.0	1169.0	1163.3	0.9
Chloroform	1773.0	1832.0	1246.0	1617.0	19.9
Dichloromethane	1177.0	1227.0	1117.0	1173.7	4.6
Ethyl acetate	9.3	9.3	9.5	9.4	1.2
Acetone	8.7	8.4	8.0	8.4	4.2
Ethanol	6.4	8.5	8.9	7.9	16.9

Data is presented as IC_{50}, n=3, CV is the coefficient of variation.

2.3.3.2 Consistency in FRAP activity of TI samples (A – C)

There was no statistically significant difference in FRAP activity between TI samples A, B and C for any of the solvent extracts. For the individual extracts arranged in order of increasing FRAP activity: chloroform extract < petroleum ether extract < dichloromethane extract < ethanol extract < acetone extract < ethyl acetate extract (Figure 2.7).

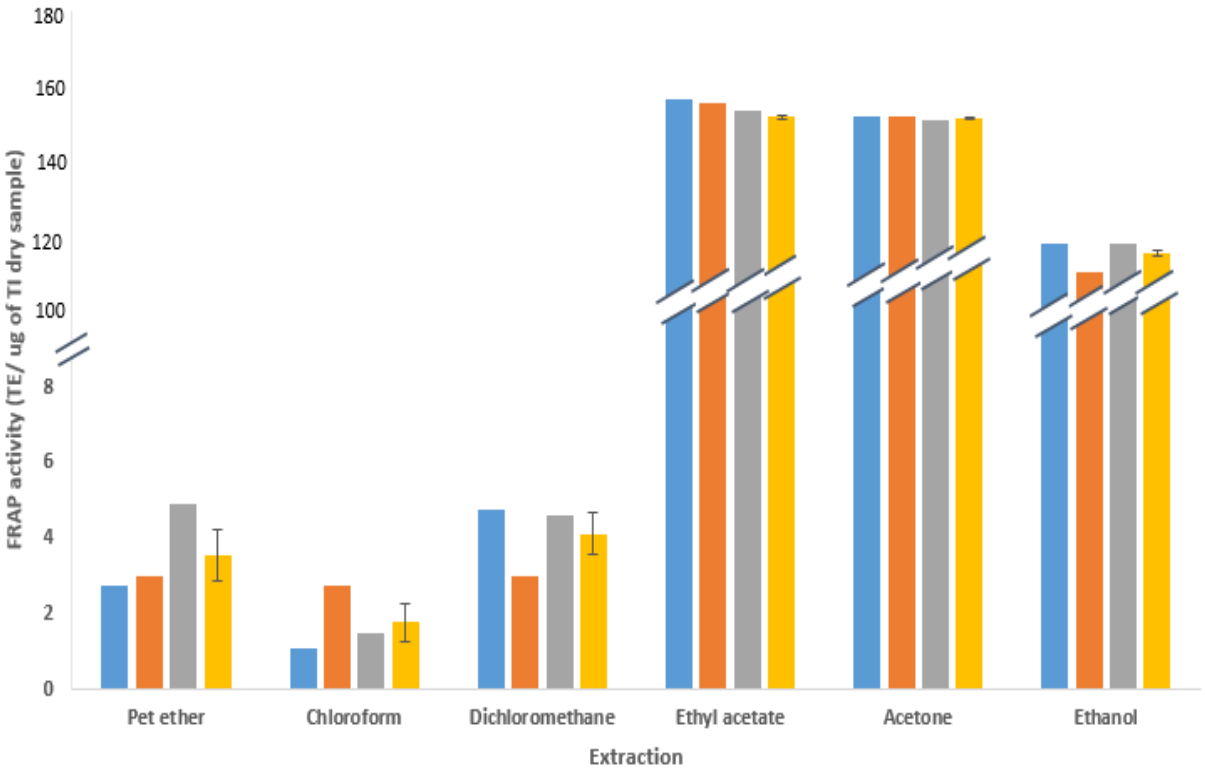


Figure 2.7: FRAP activity between samples A – C. Data are presented as FRAP activity ± SEM; blue bar is sample A; orange bar is sample B; grey bar is sample C and yellow bar is mean ABC; n=3. Consistency in FRAP activity between samples compared by ANOVA followed by Bonferroni post hoc test ($p>0.05$).

2.3.4 Consistency in effect of TI samples on cell viability and proliferation of human liver cells *in vitro.*

2.3.4.1 Optimising solubility of TI samples in different solvents.

Samples extracted with ethyl acetate, acetone or ethanol were visually observed to completely dissolve in water, ethanol, methanol or DMSO after 5 min of vortexing. Conversely, extracts from petroleum ether, chloroform or dichloromethane did not dissolve in water as visual inspection showed the presence of extract particles. These 3 extracts only partially dissolved in methanol, ethanol or DMSO. However, after 15 min of sonication, extracts were observed to completely dissolve in methanol, ethanol or DMSO whereas those in water did not dissolve, as shown by the presence of particles. After 30 min of incubation, extracts from petroleum ether, chloroform or dichloromethane precipitated out of solution from methanol or ethanol while those in DMSO were dissolved uniformly. Therefore, DMSO was taken as the solvent of choice to dissolve all extracts for further assessment of the effect of TI on cells *in vitro*. To this effect, DMSO was tested for its effects on Hep G2 cell viability and growth to determine the non-toxic dose of DMSO to administer to the cells when dissolving TI extract for further experiments.

2.3.4.2 Optimising DMSO concentration on Hep G2 cell viability and growth.

Cells were treated with varying concentrations (0 – 5 %) of DMSO for 24 h and cell viability and growth were determined by MTT assay and cell count respectively. As the concentration of DMSO increased, Hep G2 cell viability decreased. DMSO significantly ($p<0.001$) decreased Hep G2 cell viability at concentrations ≥2.5 % when compared with untreated control. However, there was no significant effect on cell viability at DMSO concentrations ≤1.0 % (Figure 2.8A).

Similarly, cell growth decreased with increasing DMSO. Treatment of Hep G2 cells with DMSO at concentrations ≥2.5 % significantly ($p<0.001$) decreased cell proliferation. No significant effect on cell growth was observed at concentrations ≤1.0 % when compared with untreated control (Figure 2.8B).

DMSO at concentrations ≤1.0 % showed no significant effect on cell growth and viability as determined above. Therefore, 0.5 % was used as the DMSO concentration to solubilise extracts and as the solvent control for all subsequent experiments.

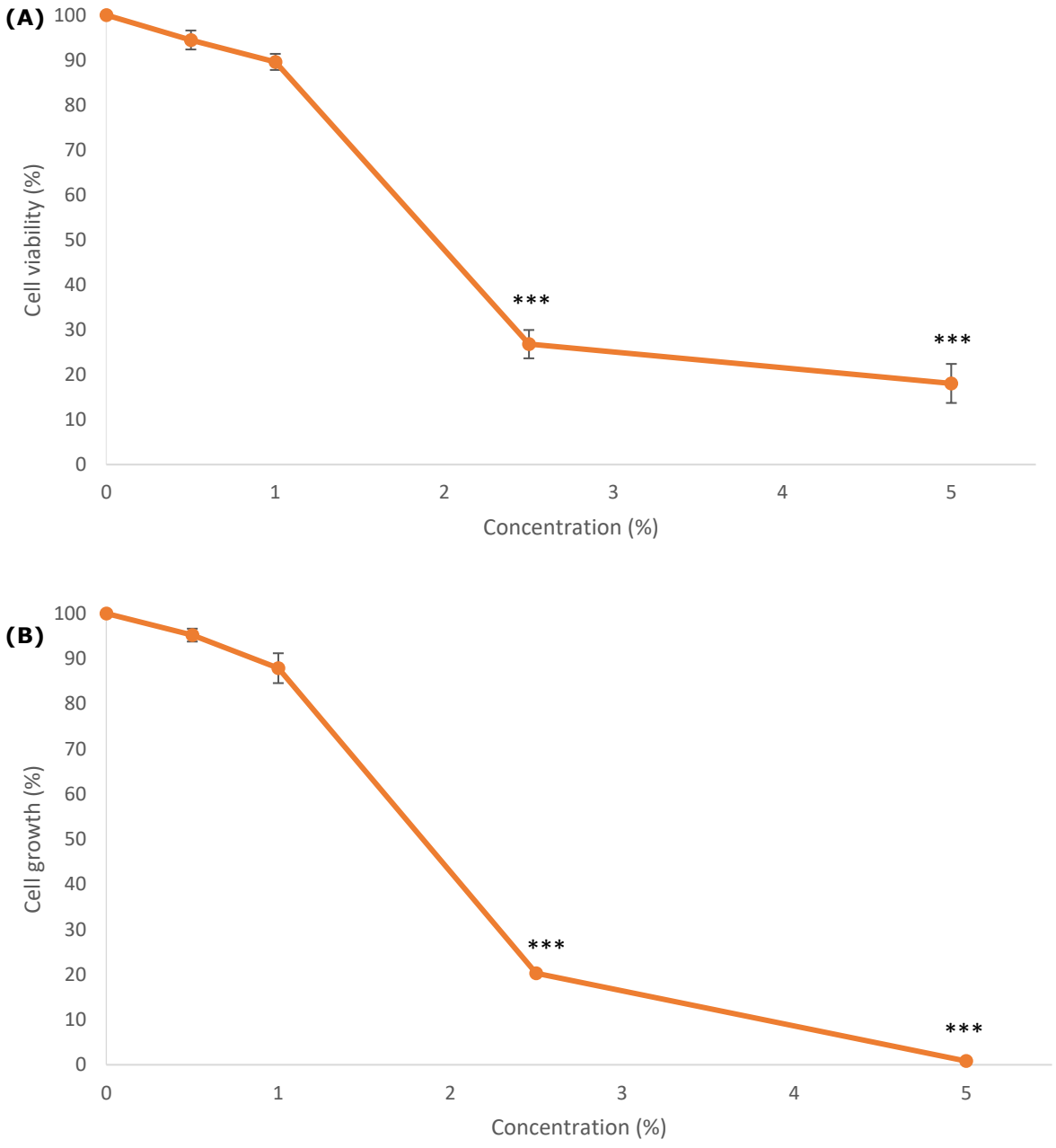


Figure 2.8: Effect of DMSO on (A) cell viability and (B) cell growth after 24 h treatment of Hep G2 cells. Data are presented as mean± SEM; n=3; ***p<0.001 when compared with untreated control by ANOVA followed by Bonferroni's post hoc test.

2.3.4.3 Consistency of effect of TI samples (A – C) on cell viability.

Cells were treated with varying concentrations (0 – 5 mg/ml) of TI for 24 h and cell viability was determined by MTT assay. As the concentration of TI extract increased, there was a decrease in the viability of Hep G2 cells. Extract concentrations ≥2.5 mg/ml significantly ($p<0.001$) decreased cell viability when compared to DMSO control. Similarly, cell viability was significantly ($p<0.05$) reduced after treatment with samples (1 mg/ml) extracted with all solvents. No significant effect in viability was observed at lower concentrations (<1.0 mg/ml) for the samples.

No significant difference on cell viability was observed between samples (A, B and C). This shows that samples A, B and C can be regarded as similar and representative of each other for future experiments (Figure 2.9).

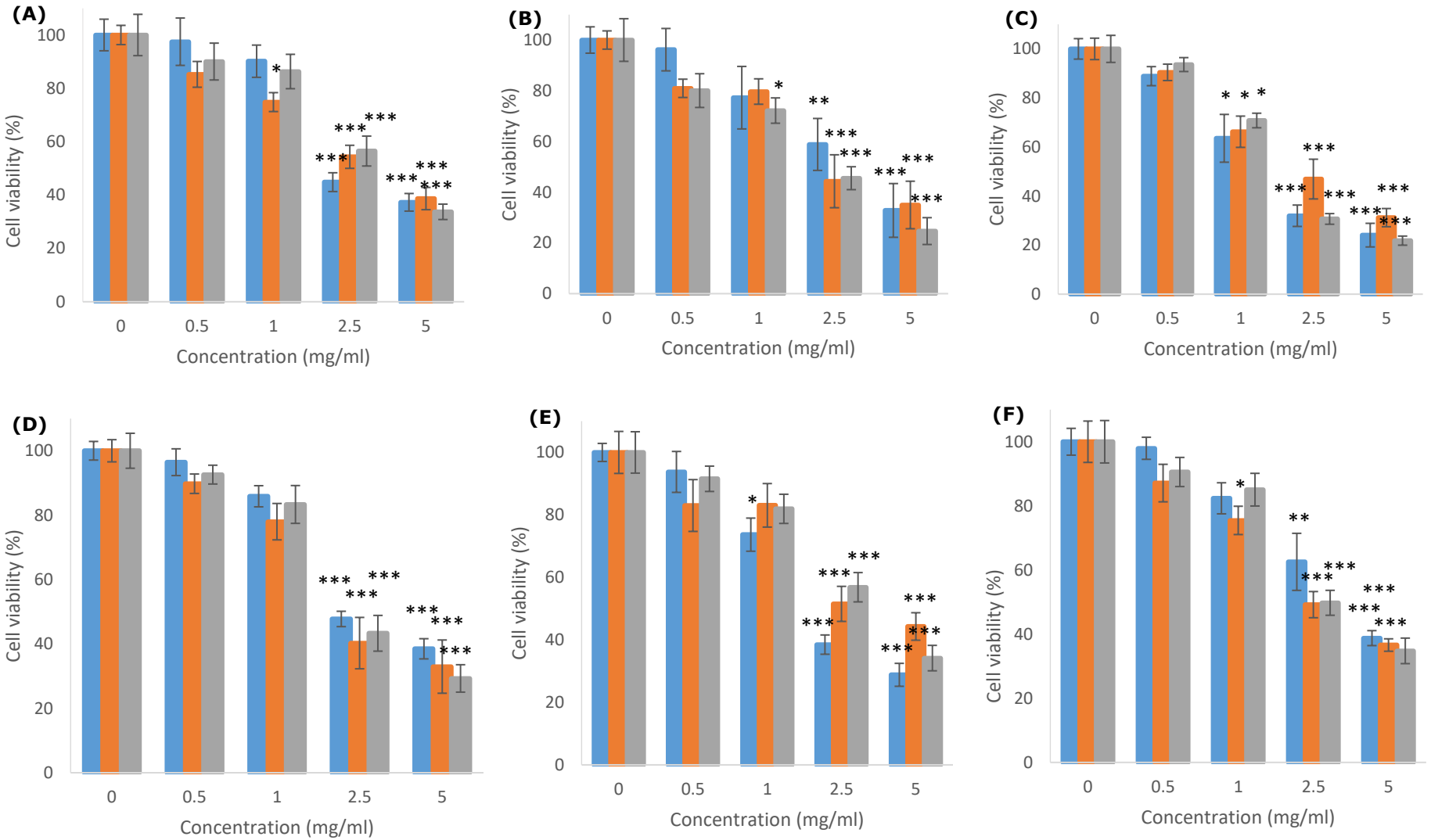


Figure 2.9: Effect of samples A, B and C on cell viability after 24 h treatment of Hep G2 cells. Sample extracted with (A) petroleum ether; (B) chloroform; (C) dichloromethane; (D) ethyl acetate; (E) acetone and (F) ethanol extract. Data are presented as mean ± SEM; n=3; blue bar is sample A; orange bar is sample B and grey bar is sample C; *p<0.05; **p<0.01 and ***p<0.001 as compared to DMSO control by ANOVA followed by Bonferroni's multiple comparison test.

2.3.4.4 Consistency in effect of TI samples (A – C) on cell growth.

Cells were treated with varying concentrations (0 – 5 mg/ml) for 24 h and cell growth was determined by cell count. Cell growth decreased as cells were exposed to increasing concentration of TI extracts. Samples (A – C) at concentrations of ≥1.0 mg/ml significantly ($p<0.05$) inhibited cell growth when compared to DMSO control (Figure 2.10).

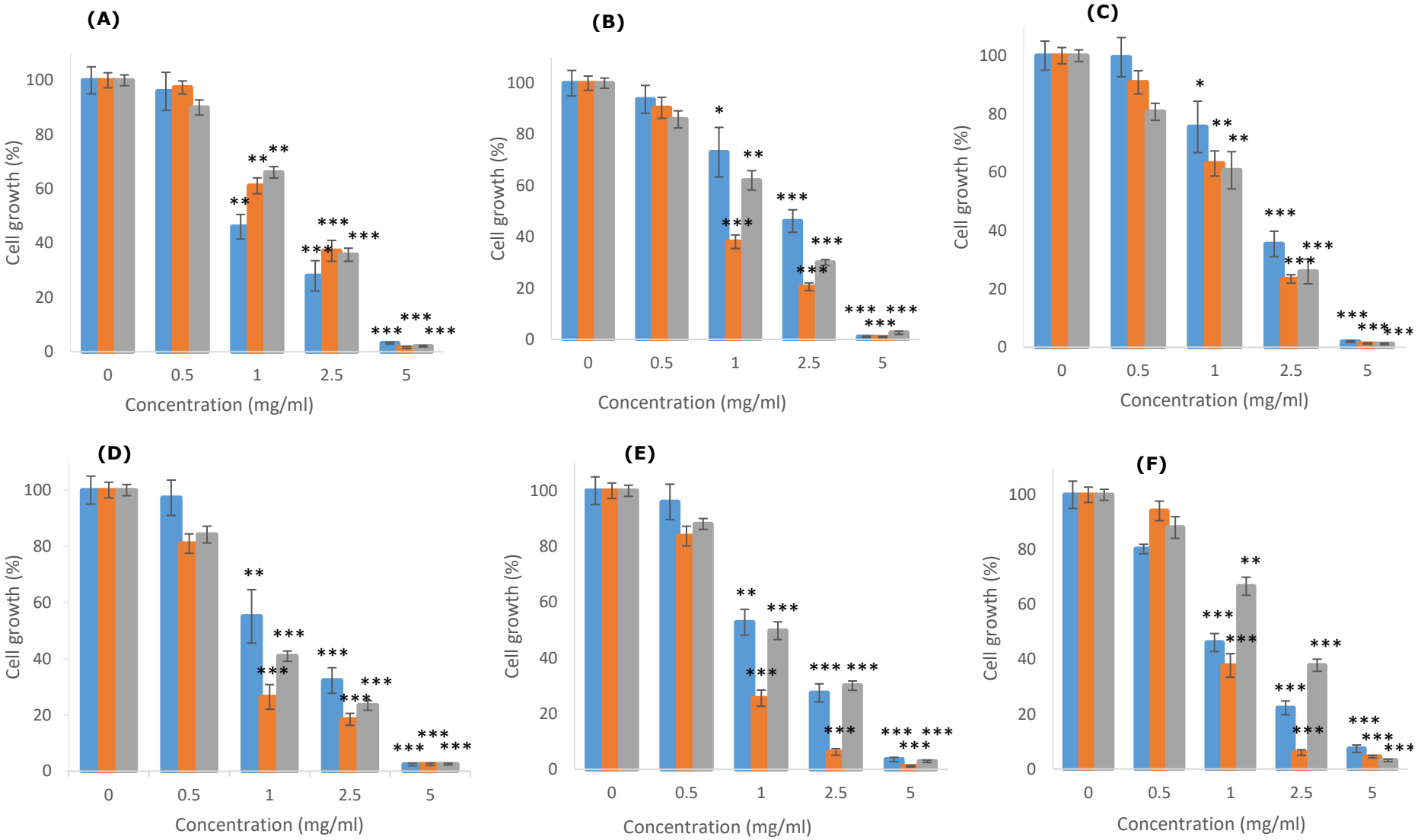


Figure 2.10: Effect of samples A, B and C on cell growth after 24 h treatment of Hep G2 cells. Sample extracted with (A) petroleum ether; (B) chloroform; (C) dichloromethane; (D) ethyl acetate; (E) acetone and (F) ethanol extract. Data are presented as mean ± SEM; n=3; blue bar is sample A; orange bar is sample B and grey bar is sample C; *p<0.05; **p<0.01 and ***p<0.001 as compared to DMSO control by ANOVA followed by Bonferroni's multiple comparison test.

Sample B extracted with chloroform significantly ($p<0.05$) inhibited cell growth at 1.0 mg/ml as compared to samples A and C, and at 2.5 mg/ml as compared to sample A. Treatment of cells with sample B extracted with ethyl acetate at 1.0 mg/ml significantly ($p<0.001$) inhibited cell growth as compared to sample A. Similarly, pre-treatment of cells with sample B extracted with acetone significantly ($p<0.001$) inhibited cell growth at 1.0 and 2.5 mg/ml as compared to samples A and C. Moreover, sample B extracted with ethanol significantly ($p<0.001$) inhibited cell growth at 1.0 mg/ml as compared to sample C, and at 2.5 mg/ml as compared to sample A and C (Table 2.9).

Table 2.9: Statistical comparison between varying concentrations of TI samples (A – C) on Hep G2 cell growth.

TI sample (mg/ml)	0	0.5	1.0	2.5	5.0
Petroleum ether	ns	ns	ns	ns	ns
Chloroform	ns	ns	0.011	0.014	ns
Dichloromethane	ns	ns	ns	ns	ns
Ethyl acetate	ns	ns	0.001	ns	ns
Acetone	ns	ns	0.001	0.001	ns
Ethanol	ns	ns	0.024	<0.001	ns

ns indicates not significant ($p>0.05$); n=3; *$p<0.05$; **$p<0.01$ and ***$p<0.001$ when samples were compared to each other by ANOVA followed by Bonferroni's multiple comparison test.

2.4 DISCUSSION

Scientific and medical advances are built on new knowledge that is reliable and robust and serves as a solid foundation for further development (Begley & Ioannidis, 2015). As part of method development and validation for this study, 3 serial sample extractions were carried out in order to assess the usefulness of the extraction procedure for all experiments throughout the research project and labelled the samples as A, B or C.

The study first examined the consistency and efficiency of the extraction procedure by comparing the yields between the TI samples (A – C). Sample A showed the highest degree of efficiency and inconsistency in yields compared with samples B or C. However, samples B and C showed greater consistency in yields.

Phytochemical analysis is of greater interest to the drug and food manufacturing industries because they provide a significant number of bioactive compounds used as drugs or as food supplements (Kinghorn et al., 2011). Some drugs that have been developed from plant bioactive compounds include the bronchodilator Chromolyn (sodium chromoglycate), developed from Khellin (a bioactive compound from *Ammi visnaga*) whereas Metformin and other bisguanidine-type antidiabetic drugs were developed from galegine (a bioactive compound from *Galega officinalis*) (Cragg & Newman, 2013). In this study, the qualitative data showed that the major phytochemical compounds identified from TI showed the presence of benzaldehydes, benzoic acids, cinnamic acids, flavonoids, indoles, lignans, phenolics and polyphenols. These findings agree with other studies which analysed qualitatively the phytochemical metabolites present in the stem bark extracts of TI and showed the presence of phytochemicals such as coumarins, flavonoids, phenolics and polyphenols (Adiko et al., 2013; Coulibaly et al., 2014). Alkaloids, saponins, tannins and triterpenes have also been shown to be present in stem bark of TI (Annan et al., 2012).

In assessing the consistency of the extraction procedure, Principal Component Analysis of the samples showed 3 groups of clusters. Extracts from petroleum ether was completely different from the other extracts, chloroform and dichloromethane extracts also clustered differently from acetone, ethanol and ethyl acetate extracts. The consistency was measured in terms of similarity index

which was calculated as a percentage of the amount of yield or phytochemical of sample A. On the other hand, inconsistency was defined as samples with low similarity index. The amounts of phytochemical compounds extracted from the replicate samples (A – C) showed a similarity index of at least 81.2 %. Samples extracted with dichloromethane showed the highest level of inconsistency (18.8 %) in the amounts of phytochemicals. Sample C of dichloromethane extract was farther away from samples A or B in the PCA plot, suggesting a significant difference in metabolite composition. The high level of variability between the samples extracted with dichloromethane was accounted for by the presence of significantly ($p<0.05$) higher levels of 4-hydroacetophenone, benzoic acid, ferulic acid, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, p-hydroxypropionic acid, protocatachaldehyde, syringic acid, syringin, vanillic acid and vanillin in sample C as compared to samples A and B. For a similarity index of at least 81.2 %, the samples were considered as similar and representative of each other.

Petroleum ether extract showed the highest amount of phenylpyruvic acids (particularly 4-hydroxyphenylpyruvic acid) and phenylpyruvic acid was detected in only petroleum ether extract. This could be responsible for the separation of the samples extracted with petroleum ether from acetone, chloroform, dichloromethane, ethanol or ethyl acetate extract. Similarly, chloroform and dichloromethane extracts showed the highest amounts of benzoic acids (particularly benzoic acid, salicylic acid, syringic acid and vanillic acid); benzaldehydes; cinnamic acids (particularly ferulic acid) and lignans (particularly secoisolariciresinol). On the other hand, benzoic acids like gallic acid and protocatechuic acid; and flavonoids like catechin, quercetin and taxifolin were detected only in acetone, ethanol and ethyl acetate extracts. These findings confirmed the different clustering of the extracts.

Hence, for subsequent extractions, petroleum ether extract is different and can be considered as solvent 1 due to the different composition. Either chloroform or dichloromethane can be used as similar phytochemical compounds will be extracted. However, due to the high variability (18.8%) in phytochemical metabolites in dichloromethane extracts, chloroform is chosen over dichloromethane for future extractions (solvent 2). Ethyl acetate extract showed the highest amount of catechin; hence, ethyl acetate can be used as solvent 3. Acetone and ethanol extracts showed a similar phytochemical profile, which was

characterised by high amounts of gallic acid, catechin and protocatechuic acid. However, ethanol extract showed higher levels of gallic acid and protocatechuic acid than acetone extract, hence, ethanol was chosen as solvent 4. Therefore, future extractions were carried out with only four solvents: petroleum ether, chloroform, ethyl acetate and ethanol respectively. These results can serve as a guide to isolate a phytochemical of interest in TI. The samples were further investigated to determine whether they show similar antioxidant activity and effects on cell viability and growth.

Determination of the antioxidant activity of a potential new drug is important because of the effect of oxidative stress in many pathological conditions e.g cancer, diabetes and cardiovascular diseases (Ponou et al., 2010). As a result, there is an increasing interest in finding NPs with antioxidant properties (Dini et al., 2009). Antioxidant activity is a widely used initial biomarker assessment for characterising medicinal plants, foods and their bioactive compounds (Houng et al., 1998).

DPPH assay is a widely used assay routinely used to assess the antioxidant status of new drugs (Sivakrishnan & Muthu, 2013). In this study, the samples (A – C) showed similar DPPH scavenging activity as shown by an overall coefficient of variation (CV) less than 20 %. With the exception of chloroform (CV=19.9 %) and ethanol (CV=16.9 %), all the other solvent extracts showed CV<5 %. Though sample C of dichloromethane extract showed 18.8 % variability from samples A and B, all the samples however showed similar DPPH scavenging activity. Other biomarkers (that is cell viability and proliferation) that were relevant to later chapters of the research were also used to assess robustness and validate the extraction procedure. Ethanol extract showed the strongest DPPH scavenging activity and chloroform extracts showed the weakest DPPH scavenging activity. For the individual extracts arranged in order of increasing DPPH scavenging activity: chloroform extract < dichloromethane extract < petroleum ether extract < ethyl acetate extract < acetone extract < ethanol extract.

In living organisms, heavy metals like iron and copper are known to catalyse oxidative processes (Liu et al., 2008). Due to the importance of metal ions in catalysing biological processes, the ferric reducing antioxidant potential (FRAP)

assay was also used to evaluate the consistency in extraction procedure. No statistically significant difference of consistency in FRAP activity was observed between TI samples A, B and C. For the individual extracts arranged in order of increasing FRAP activity: chloroform extract < petroleum ether extract < dichloromethane extract < ethanol extract < acetone extract < ethyl acetate extract.

The higher antioxidant activity shown by acetone, ethanol or ethyl acetate extract is likely to be as a result of the presence of higher amounts of flavonoids and phenolic phytochemical compounds than in extracts from chloroform, dichloromethane or petroleum ether. Flavonoids and polyphenols such as catechin, epicatechin, quercetin and resveratrol have been shown to possess high antioxidant activity (Iacopini et al., 2008). Moreover, benzoic acids like gallic acid possess a high DPPH scavenging activity (Liu et al., 2012). This is also in higher quantities in acetone, ethanol or ethyl acetate extract than in extracts from chloroform, dichloromethane or petroleum ether.

For robustness and reliability, cell-based assays were also used to assess consistency in the effect of extracts from the Soxhlet extraction. To investigate the consistency in the effect of extracts on cells, a suitable solvent was required for solubilisation and delivery of extracts into cells. For consideration of the most appropriate solvent system to use as vehicle for extract delivery into cells, the solubility of the extract in that solvent as well as the effect of the solvent on the cells was considered (Vandhana et al., 2010).

In this study, solubility of TI sample was tested in water, methanol, ethanol and DMSO. After 5 min of sonication, samples extracted with acetone, ethanol or ethyl acetate uniformly dissolved in water, ethanol, methanol and DMSO. On the other hand, samples extracted with chloroform, dichloromethane or petroleum ether were insoluble in water but partially soluble in methanol, ethanol or DMSO, as particles of extracts were found to be still present in solution. When the samples were sonicated for 15 min and incubated for 30 min at room temperature, all samples were completely soluble in methanol, ethanol or DMSO. However, after 30 min of incubation, samples extracted with chloroform, dichloromethane or petroleum ether precipitated out of solution of methanol or

ethanol but remained in solution with DMSO. Therefore, DMSO was chosen as the solvent to solubilize all samples for delivery into cells.

The effect of increasing the concentration of DMSO (0 – 5.0 %) was determined on cell viability and growth to select a non-toxic concentration of DMSO for delivery of TI extracts into cells. DMSO at a low concentration (0.5 – 1.0 %) was non-toxic to Hep G2 cells while higher concentration (2.5 – 5.0 %) significantly ($p<0.001$) decreased Hep G2 cell viability and growth. This agreed with the findings of Maes et al. (2012) and Timm et al. (2013) who showed that concentration of DMSO (≤ 1.0 %) was non-toxic to cells and concentration above 1.0 % inhibited cell growth (Maes et al., 2012; Timm et al., 2013). Therefore, DMSO at a non-toxic concentration of 0.5 % was used to solubilise and deliver samples into cells to test the effect of samples on cell viability and growth. TI extracts showed an inversely proportional relationship between concentration and cell viability or cell growth. No significant difference in effect was measured on Hep G2 cell viability and growth between the samples (A – C).

In summary, the extraction procedure consistently produced extracts with comparable composition and amounts of phytochemical, despite the difference in amounts of phytochemical compounds in sample C of dichloromethane extract, all the samples (A, B and C) showed consistent antioxidant activity and effects on cell viability and growth, and hence this process can be used to produce consistent extracts for future experiments. Due to the similar phytochemical profile for some of the solvent extracts, going forward, the extracts were grouped into four classes: petroleum ether extract as solvent 1, chloroform extract as solvent 2, ethyl acetate extract as solvent 3 and ethanol extract as solvent 4.

2.5 CONCLUSION

The Soxhlet extraction procedure is efficient in producing consistent and representative extracts with comparable amounts and composition of metabolites, which showed similar antioxidant activity, and effect on human liver cell viability and cell proliferation.

CHAPTER THREE

THE IMPACT OF TI ON HUMAN LIVER *IN VITRO*

3.1 INTRODUCTION

Due to the central role of the liver in the metabolism of both endogenous and exogenous substances in the body, it is prone to the toxic effects of these substances, or their metabolites and hence, it is very important to evaluate the impact of new drug agents on the liver during drug discovery (Almazroo et al., 2017). Toxicity of drugs to the liver is a frequent cause of drug withdrawal from the market resulting in huge financial loss to pharmaceutical industries (Rolf, 2009). For instance, drug-induced liver injury has been associated with over 1000 marketed drugs in the United States and is linked with liver conditions such as cholestasis, fibrosis, hepatitis, inflammation and necrosis (Abboud & Kaplowitz, 2007). Drugs such as nefadazone, troglitazone and trovafloxacin have been withdrawn from the market due to their toxicity to the liver, while acetaminophen and diclofenac are still in the market but pose a dose-related risk of causing liver toxicity (Abboud & Kaplowitz, 2007; Kaplowitz, 2004).

Over the past decades, several *in vitro* human liver models have been used in pharmacological investigation of liver toxicity and drug biotransformation and some of these models include: supersomes, microsomes, cytosol, cell lines, transgenic cell lines, primary hepatocytes, liver slices, and perfused liver (Almazroo et al., 2017). These *in vitro* methods allow for easier and cost-effective investigation of enzyme kinetics, evaluation of dose-response relationships, mechanisms of liver toxicity and xenobiotic metabolism than in *in vivo* studies (Soldatow et al., 2013). These *in vitro* models also have advantages such as small amount of a sample is needed for testing, shorter time is needed for testing, no requirement for reduction, refinement or replacement as in animal studies, and increase robustness in throughput for assessing multiple chemicals and their metabolites as well as reduced cost of animal care and maintenance (Almazroo et al., 2017; Soldatow et al., 2013). However, the use of the *in vitro* models is limited by a decrease in liver-specific function and loss of viability (Soldatow et al., 2013).

Furthermore, *in vitro* models have been used extensively to examine the toxic concentrations of tested samples and the impact on cell morphology and proliferation, enzyme activities and genomic damage (Goiato et al., 2015). According to Bural et al. (2011), a test sample can be classified as highly

cytotoxic if it causes reduction in cell proliferation by >75%, moderately cytotoxic (reduction in cell proliferation of 50–75%), slightly cytotoxic (reduction in cell proliferation of 25–50%), and non-cytotoxic (<25% reduction in cell proliferation) (Bural et al., 2011).

Moreover, immortalised cell lines and isolated hepatocytes are the most widely used *in vitro* models for liver toxicity testing (Soldatow et al., 2013). To ensure a safe use of TI as a therapeutic agent, assessment of cytotoxicity of TI against exposure to liver cells is necessary in order to prevent harmful off-target effects during treatment. Therefore, Hep G2 cell was adopted as the model of human liver cell for this research.

3.1.1 Model: human liver cells.

Human liver (Hep G2) cell is a human cancer cell line that was isolated from the liver biopsy of a 15-year-old male Caucasian from America (López-Terrada et al., 2009). These cells perform metabolic functions similar to a normal liver (Coatti et al., 2015). Hep G2 cells synthesize major plasma proteins (particularly albumin, transferrin, plasminogen and fibrinogen), bile acids and insulin-like growth factors as well as possessing receptors for low density lipoproteins and very low-density lipoproteins (Mitani et al., 1989). They also express various metabolic enzymes for xenobiotic biotransformation (Guillouzo et al., 2007). For example, they bioactivate procarcinogens, promutagens and procytotoxic compounds including aflatoxin B, 2-aminofluorene, benzo(a)pyrene and cyclopent(a)phenanthrene (Cao et al., 2008; Plazar et al., 2007). The DNA adducts formed by Hep G2 cells in metabolizing benzo(a)pyrene are like those found in normal human liver tissue (Doostdar et al., 1988). Just like normal hepatocytes, Hep G2 cells respond to stimulation by human growth factor (Coatti et al., 2015).

The main disadvantage of using Hep G2 cells is that they are obtained from a cancer cell line hence are immortal and some functions will be different (Guillouzo et al., 2007). However, they are a well-established cell line for *in vitro* human studies (Alía et al., 2006). This cell line has been widely used for studies involving hepatotoxicity, carcinogenesis, mutagenesis, cell development, liver metabolism and pharmacokinetic study of new drugs (Finegold et al., 2008;

López-Terrada et al., 2009). For these reasons, the Hep G2 cell model was adopted for this present study.

In this study, the impact of TI on human liver cells was determined as cell viability, proliferation and genomic stability and a non-toxic dose of TI was used subsequently to determine the impact of TI on drug metabolising enzymes in Hep G2 cells.

3.1.2 Aim:

To investigate the impact of TI on human liver cells *in vitro.*

3.1.2 Specific objectives:

- To determine the effect of TI on Hep G2 cell viability *in vitro*.
- To assess the effect of TI on Hep G2 cell growth *in vitro*.
- To assess the effect of TI on Hep G2 genomic stability *in vitro*.
- To evaluate the effect of TI on Phase 1 drug metabolising enzyme *in vitro* after short- and long-term exposure.
- To evaluate the effect of TI on Phase II drug metabolising enzymes *in vitro* after short- and long-term exposure.
- Using a mathematical model to understand the relationship between phytochemical compounds from TI and their impact on DMEs in Hep G2 cells.

3.2 METHODS

In this study, sample TI extracts used from each solvent (chloroform, ethanol, ethyl acetate or petroleum ether) were obtained as described previously (section 2.2.1.3).

3.2.1 The impact of TI on viability, proliferation and genomic stability in human liver cells

3.2.1.1 Impact of TI on viability and growth of Hep G2 cells *in vitro.*

Hep G2 cells were cultured and routinely maintained as described earlier (section 2.2.7.2). The impact of TI (0 – 5.0 mg/ml) for 24 h on Hep G2 cell viability was measured using the MTT assay and on cell growth for 72 h by cell count as both described previously (sections 2.2.3.5 and 2.2.3.6 respectively).

3.2.1.2 Impact of TI on genomic stability in Hep G2 cells *in vitro.*

DNA single strand breakage (SSB) was measured using the single cell gel electrophoresis (SCGE; comet) assay by the method of Duthie and Collins (1997). Hep G2 cells were grown on 24-well plates at a density of $7x10^5$ cells/well and incubated at 37 °C for 24 h. Cells were incubated with TI chloroform, ethanol, ethyl acetate or petroleum ether extract at a final concentration of 0 – 5.0 mg/ml at 37 °C for 24 h in 5% CO_2 / 95 % air. Cells were then harvested with trypsin-EDTA (0.2 ml/well) and counted using a haemocytometer. Cells were pipetted into PBS (1 ml final volume) and centrifuged at 200 g for 5 min at 4°C. The supernatant was discarded, and the pellet resuspended in 85 µL of 1% (w/v) low melting point agarose and pipetted onto a frosted microscope slide precoated with 1% (w/v) high melting point agarose. The gels were allowed to set at 4 °C for 10 min, and slides were then incubated in lysis solution [2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, NaOH to pH 10.0, and 1% (v/v) Triton X-100] at 4 °C to remove cellular protein. Where different concentrations of extracts had been applied to the cells, the slides were incubated in lysis solution in separate boxes to prevent potential leaching of higher concentrations of TI into lower concentrations. Slides were then aligned in a 260 mm wide horizontal electrophoresis tank containing electrophoresis solution (1 mM EDTA and 300 mM NaOH, pH 12.7) for 40 min before

electrophoresis at 4 °C for 40 min at 25 V and 999 mA. Slides were washed 3x5 min at 4 °C in neutralising buffer (0.4 M Tris-HCl, pH 7.5) and stained with 20 µL of 4,6-diamidine-2-phenylindol dihydrochloride (DAPI; 1 mg/mL stock solution). DNA SSB was analysed visually according to a scoring system where 100 images per gel were assigned a value of 0, 1, 2, 3, or 4 (from undamaged to maximally damaged) depending on the intensity of the fluorescence in the comet tail. Thus, the total score for 100 comets ranged from 0 – 400 from all undamaged cells to maximally damaged cells respectively (Duthie & Collins, 1997).

3.2.2 The impact of TI on drug metabolising enzyme (DME) activity in human liver cells *in vitro.*

3.2.2.1 Optimising cell homogenates for DME activity.

3.2.2.1.1 Effect of hand homogenisation on cell membrane breakage.

Hep G2 cells were grown for 7 days to approximately 80 – 90 % confluency as before. The medium was removed from the flasks and the cells were washed 4x5 ml PBS. The cells were scraped off the tissue culture flask with a cell scraper (FB11597692 Fisher brand, Loughborough, UK) into 5 ml ice-cold sodium phosphate (NaPi) buffer (0.1 M, pH 7.4) and distributed by pipetting several times to get a uniform cell suspension. The cell suspension (5 ml) was transferred into a hand homogeniser (5ml, Fisher brand, UK) on ice and homogenisation was carried out by upward and downward movement of a glass pestle, with the complete cycle of upward and downward movement of the pestle taken as one stroke. The cell homogenate (100 µl) was aliquoted into Eppendorf tubes (at intervals of 5 strokes) for up to 50 strokes. Additionally, 20 µl was sampled from each aliquot onto a haemocytometer to view if the cells had been disrupted. The cell homogenate was observed under a Leica DM IL microscope (Leitz Wetzlar, Germany) at a magnification of x10 for cell breakage and to ensure a homogeneous cell preparation. In addition, 10 µl of trypan blue (TB) was added to each sample of homogenate and incubated at room temperature for 30 sec. The cell homogenates at each stroke-point were observed under the microscope for TB-stained cells. The stained cells were counted using a haemocytometer and an average of 100 stained cells were assessed on a semi-

quantitative scoring scale of 10 – 100 %, where 10 is the least number of stained cells and 100 is the highest number of stained cells for 100 cells counted.

3.2.2.1.2 Effect of drill homogenisation on cell membrane breakage.

Hep G2 cell culture and scraping of cells were carried out as described previously (section 3.2.2.1A). Cell suspension (5 ml) was transferred into a homogeniser (50 ml, Fisher brand, UK) and homogenised on ice with a mixer drill (LC9/11624, Griffin and George Limited, London, UK). The homogeniser was slowly and carefully inserted into the Teflon pestle ensuring that the pestle was straight and well aligned with the homogeniser. The drill was switched on at the lowest power to begin with and the speed of the drill was slowly adjusted to a maximum of 5 rev/s. The homogeniser (at intervals of 5 strokes) was slowly moved up and down for up to 50 strokes. The cell homogenate was examined for cell disruption as described earlier (section 3.2.2.1.1).

3.2.2.1.3 Effect of sonication on cell membrane breakage.

Cell culture and cells scraping were carried out as described previously (section 3.2.2.1.1). Subsequently, cells (500 µl) were aliquoted into Eppendorf tubes and sonicated for 0, 30, 60 or 180 sec using an ultra-wave sonicator (U400, Ultra-wave limited, Cardiff, UK) on ice. At each time point of sonication, the cell homogenate was examined for cell disruption as described earlier (section 3.2.2.1A). The sonication was carried out for no more than 180 sec to avoid the generation of heat which could affect cells and enzyme activity.

3.2.2.1.4 Effect of drill homogenisation on cell membrane breakage.

Hep G2 cells were seeded in T75 cm^2 at a density of $1.5x10^6$ cells/flask (n=2) and grown for 7 days to be used for the estimation of lactate dehydrogenase (LDH) leakage in the cell homogenates. Medium (500 µl) was transferred from a flask of Hep G2 cells into an Eppendorf tube and 145 µl of Triton x-100 (1 % v/v) was added to the flask, shaken gently, and incubated at room temperature for 2 min. Medium (500 µl) was then transferred into an Eppendorf tube and used to assess the total cell viability in the flask. The medium was removed from a

separate flask of cells and the cells were washed 4x5 ml with ice cold Krebs-Hepes buffer (pH 7.4). The cells were scraped (on ice) into 5 ml ice cold Krebs-Hepes buffer (pH 7.4) and the cell suspension was transferred into a glass homogeniser (on ice). The cell suspension was then slowly and carefully homogenised using a mixer drill and 500 µl aliquots were taken for up to 50 strokes at intervals of 10 strokes. At stroke 50, an additional 500 µl was transferred into an Eppendorf tube, 5 µl of Triton x-100 (1 % v/v) was added, shaken gently, and incubated at room temperature for 2 min.

LDH leakage was measured by modifications of the method of Anuforo et al. (1978). The modifications were that the volumes of the reaction solutions were proportionally adjusted to µl, cell homogenates were used, and the procedure was carried out in plain 96-well plates.

Cell homogenate (10 µl) was pipetted into a 96-well plate containing 180 µl Krebs-Hepes buffer and 10 µl pyruvic acid (1.36 mM final concentration). The reaction was started by the addition of 10 µl NADH (0.2 mM final concentration) and the absorbance was read at 340 nm at 37 °C.

To evaluate the effect of cell particle interference on the efficiency of homogenate preparation, the assay was repeated using cell homogenates that were centrifuged at 1500 g for 5 min and the supernatant used to set up 96-well plates as described above.

The percentage LDH leakage was calculated using the formula below, and a graph was plotted with % LDH against number of homogenisation strokes and comparing centrifugated and non-centrifugated homogenate (Anuforo et al., 1978).

a) Optical density of dead cells in solution = absorbance of sample - absorbance of blank.
b) %LDH = [optical density of sample/optical density of ctrl +T], Where the samples were control (ctrl), strokes 0 – 50 (S_0 – S_{50}), S_{50T} (stroke 50 + Triton x-100) and ctrl +T.

3.2.2.1.5 Optimising buffer conditions for preparation of cell homogenates for cytochrome P450 activity.

Cytochrome P450 enzymes are challenging to measure in cells due to their unstable nature and hence, require a special buffer for efficient extraction. To optimise buffer conditions for assessing total cytochrome P450 activity, Hep G2 cells were seeded at $5.2x10^5$ cells in $25cm^2$ tissue culture ($T25cm^2$) flasks and grown for 7 days to approximately 80 – 90 % confluency. Hep G2 cells were then recovered using 3 different buffers. The cells were washed (3x2 ml) and scraped in 2 ml ice-cold NaPi buffer (0.1 M, pH 7.4) or Tris – HCl buffer (10 mM supplemented with 0.25 M sucrose, pH 7.25) or cytochrome P450 buffer (0.1 M NaPi buffer containing 100 ml glycerol, 186.1 mg EGTA, 77.2 mg dithiothreitol and 0.5 % v/v Triton N101, pH 7.4). Cell suspensions were homogenised by using a mixer drill for 10 strokes, transferred into centrifuge tubes, and centrifuged at 1500 g for 5 min at 4 °C.

Total cytochrome P450 activity in the different buffers was measured as described previously by Schenkman and Jansson (2006) with modification. Hep G2 cell homogenate was divided equally into two quartz cuvettes (1 ml) and scanned at 390 – 500 nm in a dual-beam ultraviolet spectrophotometer (Helios α, Thermo Scientific, UK) to obtain a baseline activity. In a fume hood, CO was gently bubbled through both sample and reference cuvettes for 30 sec and a few grains of sodium dithionite was added to the reference cuvette. The reference cuvette was covered with a paraffin film and shaken gently to dissolve the sodium dithionite. The cuvettes were rescanned at 390 – 500 nm to obtain the cytochrome P450 activity measurement. The concentration of cytochrome P450 in the cuvette was calculated from the absorbance at 450 nm relative to the absorbance at 490 nm using the Beer's Law equation: $A = \varepsilon \cdot c \cdot L$ and the extinction coefficient ($\varepsilon 450–490nm$) = 91 mM^{-1} cm^{-1} (Schenkman & Jansson, 2006).

3.2.2.2 The short- and long-term effects of TI on cytochrome P450 activity in human liver cells *in vitro.*

To assess the short-term effect of TI on cytochrome P450, Hep G2 cells were seeded at $5.2x10^5$ cells in T25cm^2 flasks and grown for 7 days to 80 – 90 % confluency. The medium in the flasks was changed and 0.5 mg/ml (0.5 % final DMSO concentration) of TI extract (petroleum ether, chloroform, ethyl acetate or ethanol) or DMSO control was added to the respective flasks. The cells were incubated at 37 °C for 4, 8, 16 or 24 h.

To assess the longer-term effect of TI on cytochrome P450, Hep G2 cells were seeded and grown as above for 7 days. These cells were then incubated with 0.5 mg/ml of TI extracts (as above) for 0, 1, 3 or 7 days.

Hep G2 cell homogenates for both short- and long-term exposures were prepared in cytochrome P450 buffer (pH 7.4) and the effect of TI on total cytochrome P450 activity was measured as described earlier (section 3.2.2.1.5).

3.2.2.3 The effect of TI on Phase II DME activities in human liver cells *in vitro*

3.2.2.3.1 Administration of TI and preparation of cells for Phase II DME activities in human liver cells *in vitro*.

To assess the short- (0 – 24 h) and long-term (0 – 7 days) effect of TI on Phase II DME, Hep G2 cell homogenates were prepared in NaPi buffer (pH 7.4) as described previously (section 3.2.2.1.5). These cell homogenates were used for the assessment of catalase, GPx and GSR activities.

3.2.2.3.2 Assessment of catalase activity in Hep G2 cells

Catalase activity was measured by the method of Aebi (1984). Hep G2 cell homogenate (0.1 ml) was added to a cuvette containing 0.4 ml H_2O_2 (30 mM final concentration) and 1 ml of 0.1 M sodium phosphate buffer supplemented with 0.08% (v/v) Triton-X100, pH 7.0. Decrease in absorbance was read at 37 °C at 240 nm at 1 min intervals for 3 min against a blank (buffer and cell homogenate). The rate of reaction was calculated using the H_2O_2 molar extinction coefficient of 39.1 $mol^{-1}cm^{-1}$ (Aebi, 1984).

3.2.2.3.3 Measurement of GSR activity in Hep G2 cells

GSR activity was measured as described previously by Calberg and Mannervik (1975). Cell homogenate (100 µl) was added to 50 µl of 1 mM oxidised glutathione (GSSG) and 1 ml of 0.2 M sodium phosphate buffer supplemented with 2 mM EDTA, pH 7.0. The reaction was initiated by the addition of 50 µl of 2mM NADPH and the absorbance was read at 37 °C at 340 nm at 1 min intervals for 3 min against a blank (buffer and cell homogenate). The rate of reaction was calculated using the NADPH molar extinction coefficient of 62.2 $mol^{-1}cm^{-1}$ (Carlberg & Mannervik, 1975).

3.2.2.3.4 Assessment of GPx activity in Hep G2 cells.

GPx activity was measured as described previously by Paglia and Valentine (1967). In a cuvette, cell homogenate (50 µl) was added to 915 µl of reaction mixture and 35 µl of 2.2 mM H_2O_2. The reaction mixture composed of 5 mg NADPH, 46 mg reduced glutathione, 3 ml distilled water, 24 ml of NaPi buffer (50 mM supplemented with 5 mM EDTA, pH 7.6), 1 ml of sodium azide (112.5 mM) and 20 U glutathione reductase. The components of the reaction mixture were prepared separately and added to the buffer. The change in absorbance was read at 340 nm for 2 min. A unit of GPx is defined as GPx that oxidases 1 µmol of NADPH/min and the rate of reaction was calculated using the NADPH molar extinction coefficient of 62.2 $mol^{-1}cm^{-1}$ (Paglia & Valentine, 1967).

3.2.2.3.5 Assessment of GSH in Hep G2 cells.

GSH was measured as described previously by Hissin and Hilf (1976) with modification. To assess the short- and long-term effect of TI on GSH levels, Hep G2 cells were treated with TI as previously described (section 3.2.2.2). The cells were washed (3x2 ml) with PBS and scraped in 2 ml ice-cold 6.5 % (w/v) trichloroacetic acid (TCA).

GSH stock (1 mM) was prepared and used to make serial dilutions (0 – 1 mM) with distilled water in Eppendorf tubes on ice. The reaction was carried out in black 96-well plates containing 180 µl of 0.1 M NaPi buffer (supplemented with 2 mM EDTA, pH 8.0), 10 µl O-phthaldiaaldehyde (7.5 mM) and 10 µl of cell homogenate or GSH. The reaction mixture was incubated in the dark at room temperature for 15 min. The fluorescence was read at 350 nm excitation (360/40 nm) and 420 nm emission (460/40 nm). Standard curve (0 – 1 mM) was prepared with GSH (Hissin & Hilf, 1976).

3.2.2.4 Effect of TI phytochemical metabolites on Phase I and Phase II DME in Hep G2 cells.

Partial least squares – discriminant analysis (PLS-DA) was used to determine the relationship between phytochemical compounds within each TI extract and their effect on DME or GSH levels in Hep G2 cells. Based on the actual measured effects, a plot was made for TI extract at a concentration of 0.5 mg/ml to show the phytochemicals with the strongest impact on DME or GSH. A 50-cm diameter circle was drawn at the side (positive or negative) of the graph showing the strongest measured enzyme activity or GSH levels. Phytochemicals located within each circle were selected as those with greatest impact on enzyme activity or GSH. The plot was colour coded as: deep blue for enzyme activity or GSH; green for phytochemical compounds; and orange for solvent extracts.

3.2.2.5 Measurement of total protein concentration in Hep G2 cells.

Protein concentration in Hep G2 cell homogenate was measured to express the cytochrome P450 and Phase II DME activities per mg protein. Protein estimation was carried out using a commercially available kit (Sigma-Aldrich, Bicinchoninic acid protein assay kit, BCA1 and B9643, Gillingham, UK). The protein determination reagent was prepared by adding 1ml of copper sulphate pentahydrate (4% w/v) solution to 49 ml Bicinchoninic acid (BCA) solution (1:50). Stock (2 mg/ml) bovine serum albumin (BSA) protein standards were made by serial dilutions (0 – 1 mg/ml) with de-ionized water in Eppendorf tubes on ice.

Dilutions of cell homogenates (1:10 and 1:100) were made from Hep G2 homogenates prepared in sections 3.2.2.2 and 3.2.2.3. The protein assay was carried out in 96-well plates on ice and the reaction was made using protein assay reagent (200 µl) and 25 µl of BSA or cell homogenates (1, 1:10 and 1:100). The plates were wrapped in tin foil and incubated at 37 °C for 30 min. The absorbance was read at 563 nm and a standard curve was prepared using BSA.

3.3 RESULTS

3.3.1 The impact of TI on cell viability, cell proliferation and genomic stability in human liver cells.

3.3.1.1 Impact of TI on viability of human liver cells *in vitro*.

Cells were treated with varying concentrations (0 – 5 mg/ml) of TI extracts for 24 h and cell viability was determined by MTT assay. As the concentration of all TI extracts increased, there was a corresponding decrease in the viability of Hep G2 cells. TI extracts showed no significant toxicity at concentrations of ≤ 1.0 mg/ml. Conversely, all four TI extracts significantly ($p<0.001$) decreased cell viability at concentrations between 2.5 – 5.0 mg/ml when compared to DMSO control (Figure 3.1).

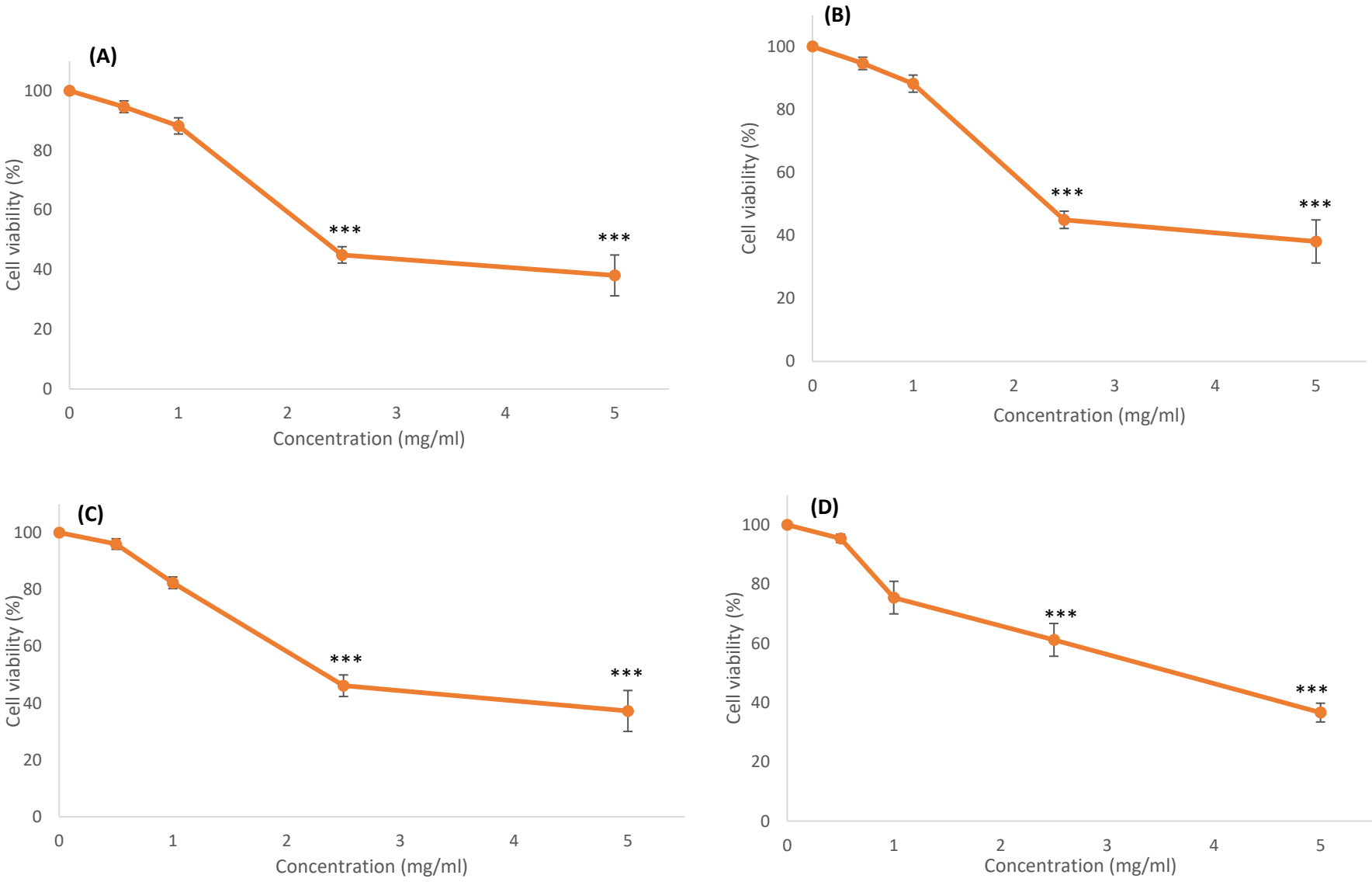


Figure 3.1: Effect of TI extracts on Hep G2 cell viability after 24 h treatment. (A) petroleum ether extract; (B) chloroform extract; (C) ethyl acetate extract and (D) ethanol. Data are presented as mean ± SEM; n=3; ***p<0.001 as compared to DMSO control by One-way ANOVA followed by Bonferroni's multiple comparison test.

TI extracts concentrations between 3.5 – 3.8 mg/ml were required to reduce the viability of Hep G2 cells by 50 % (IC_{50}) (Table 3.1).

Table 3.1: Comparison of IC_{50} (mg/ml) for TI extracts on Hep G2 cell viability.

Extract	IC_{50} (mg/ml)
Petroleum ether	3.5
Chloroform	3.8
Ethyl acetate	3.5
Ethanol	3.7

3.3.1.2 Impact of TI on growth of human liver cells *in vitro.*

Hep G2 cell growth decreased as cells were exposed to increasing concentration of TI for 24 h and cell growth was determined by cell count. All four TI extracts significantly ($p<0.001$) inhibited cell growth at concentrations ≥1 mg/ml when compared to control. No significant effect on cell growth was observed for TI extracts at 0.5 mg/ml (Figure 3.2).

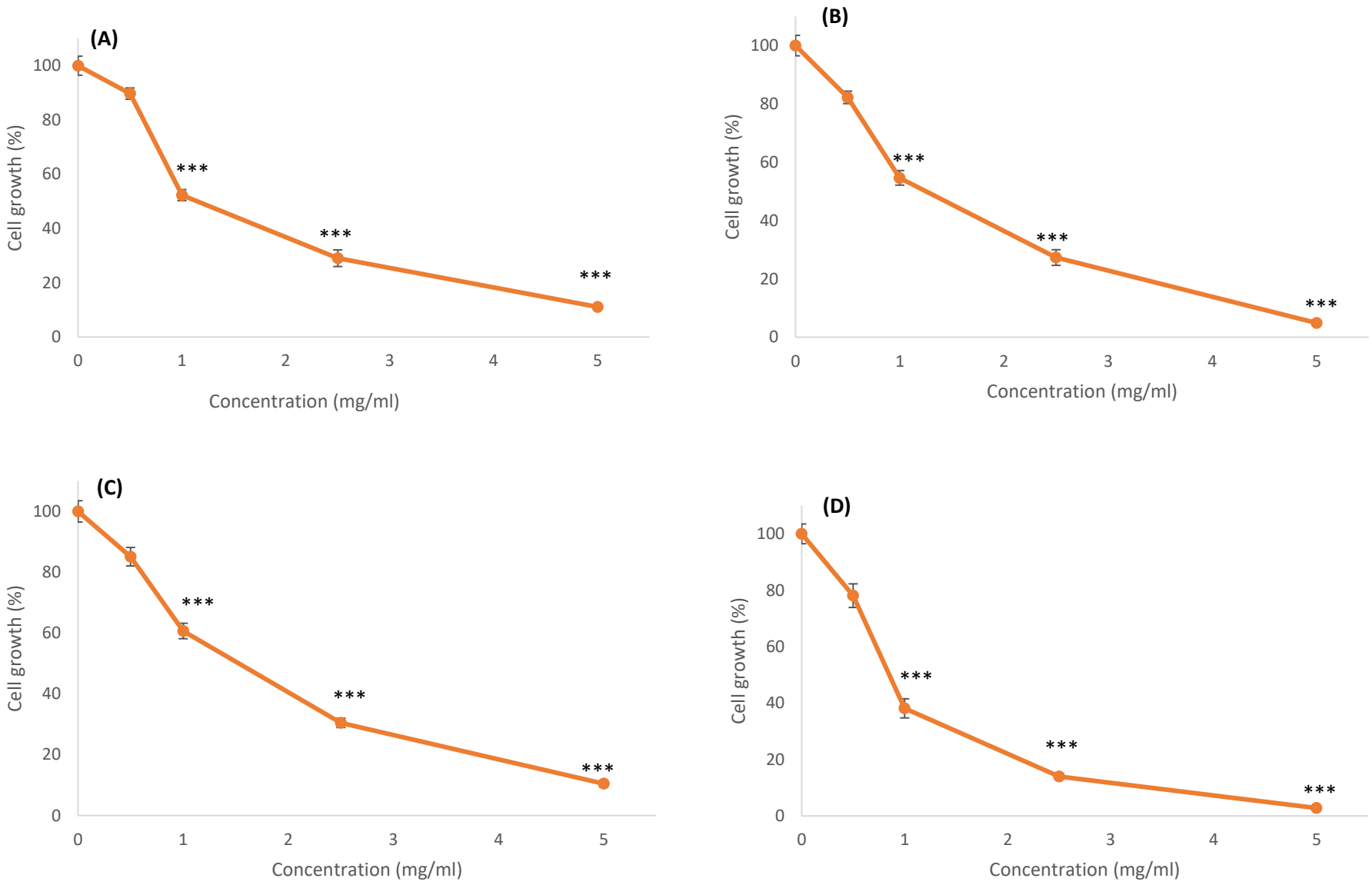


Figure 3.2: Effect of TI extracts on Hep G2 cell growth after 24 h treatment. (A) petroleum ether extract; (B) chloroform extract; (C) ethyl acetate extract and (D) ethanol. Data are presented as mean ± SEM; n=3; ***p<0.001 as compared to DMSO control by One-way ANOVA followed by Bonferroni's multiple comparison test.

Despite a similar effect measured for the extracts, different concentrations of the extracts were required to inhibit the growth of Hep G2 cells by 50 % (IC_{50}). Chloroform and ethyl acetate extracts showed a higher IC_{50} (2.2 mg/ml), whereas a lower concentration (IC_{50} = 1.6 mg/ml) of ethanol extract was required to reduce the growth of Hep G2 cells by 50 % (Table 3.2).

Table 3.2: Comparison of IC_{50} (mg/ml) for TI extracts on Hep G2 cell growth.

Extract	IC_{50} (mg/ml)
Petroleum ether	2.2
Chloroform	2.0
Ethyl acetate	2.2
Ethanol	1.6

3.3.1.3 Impact of TI on genomic stability in Hep G2 cells *in vitro.*

Genomic stability in Hep G2 cells was assessed by measuring DNA SSB. There was a dose-dependent increase in DNA SSB with increasing concentration of all four TI extracts. A significant ($p<0.05$) increase in DNA SSB was observed after treatment of cells with TI extracts at a concentration of 1.0 mg/ml or above when compared to DMSO control (Figure 3.3).

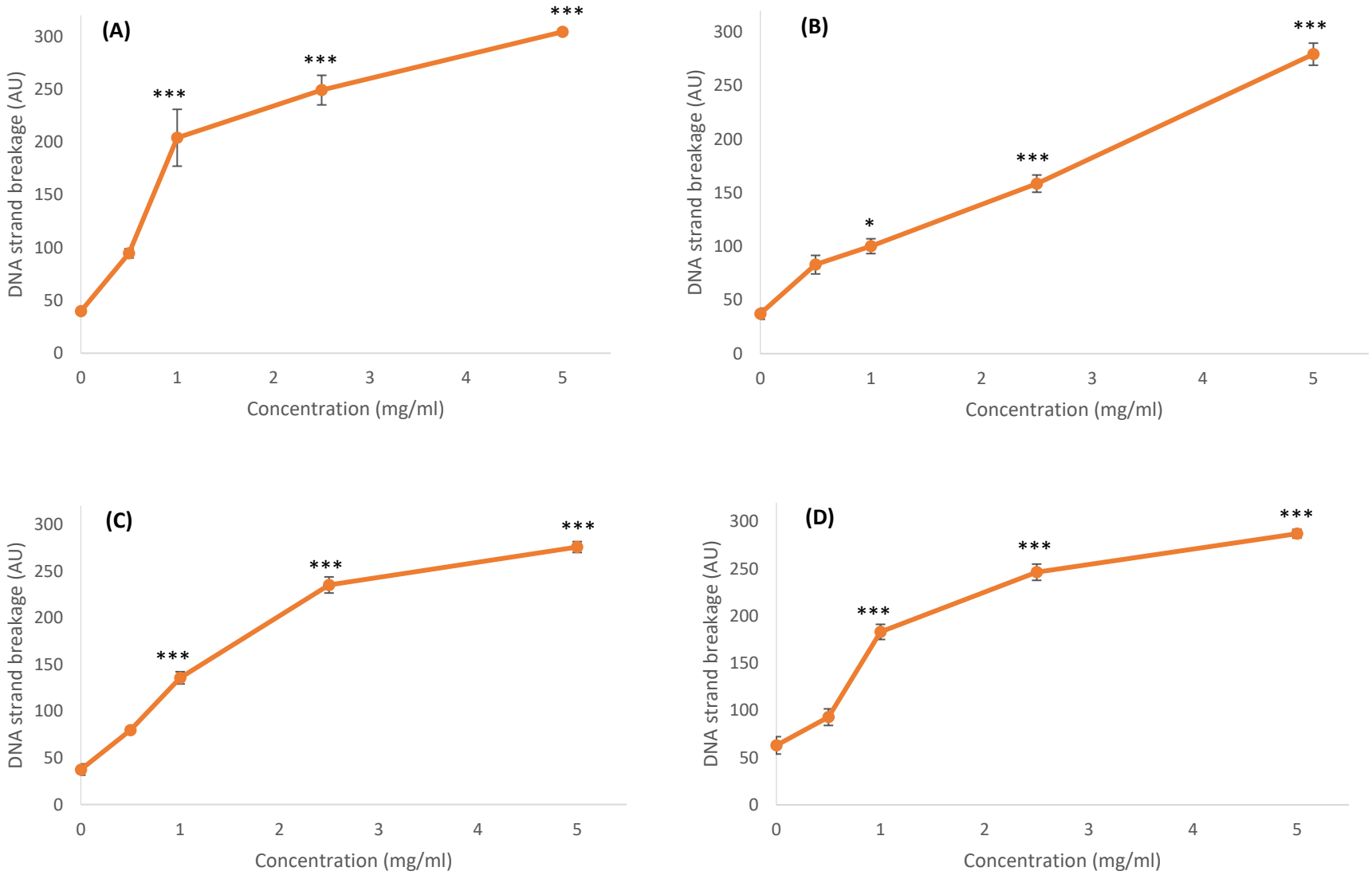


Figure 3.3: Effect of TI extracts on Hep G2 DNA strand breakage after 24 h treatment. (A) petroleum ether extract; (B) chloroform extract; (C) ethyl acetate extract and (D) ethanol. Data are presented as mean ± SEM; n=3; *p<0.05 and ***p<0.001 as compared to DMSO control by One-way ANOVA followed by Bonferroni's multiple comparison test.

The lowest concentration of TI that caused a 50 % increase in DNA SSB (LC_{50}) was measured. Chloroform extract showed the least effect on DNA stability (LC_{50} = 2.0 mg/ml) while ethanol extract showed the highest genotoxicity (LC_{50} = 1.1 mg/ml) in Hep G2 cells (Table 3.3).

Table 3.3: Comparison of LC_{50} (mg/ml) for TI extracts on Hep G2 DNA strand breakage (AU).

Extract	LC_{50} (mg/ml)
Petroleum ether	1.3
Chloroform	2.0
Ethyl acetate	1.5
Ethanol	1.1

3.3.2 The impact of TI on DME activity in human liver cells *in vitro.*

3.3.2.1 Optimising preparation of cell homogenates for DME activity

3.3.2.1.1 Effect of hand and drill homogenisations on cell membrane breakage

For assessing cell membrane breakage, TB-stained cells were counted and scored on a semi-quantitative scale of 10 – 100 % for an average of 100 stained cells. With no homogenisation (stroke 0), there were numerous cell clusters observed under the microscope. Approximately 10 – 20 % cells were observed to be TB stained after 30 seconds, and the level of TB staining increased as the number of homogenisation strokes increased. Few cell clusters were observed for strokes 5 – 15 while numerous single cells were observed at strokes 20 – 50. Cells at stroke 50 showed the highest level of TB uptake (90 – 100 %) when the homogenisation was carried out by hand (Table 3.4).

For drill homogenisation (as for hand homogenisation), at stroke (0), numerous cell clusters were observed and very low levels of TB-stained cells (10 – 20 %). At strokes 5, many single cells were observed, and this increased TB uptake from 10 – 20 % to 50 – 60 %. No difference in the level of TB uptake was observed between strokes 10 and 15. Homogenisation strokes between 20 and 50 showed

the highest TB uptake (90 – 100 %) when the homogenisation was carried out using a drill homogeniser (Table 3.4).

Table 3.4: Hep G2 cell membrane breakage when homogenised with hand or drill homogeniser.

Number of strokes	Hand homogenisation TB staining (%)	Drill homogenisation TB staining (%)
0	10 – 20	10 – 20
5	10 – 20	50 – 60
10	30 – 40	70 – 80
15	50 – 60	70 – 80
20	50 – 60	90 - 100
25	50 – 60	90 - 100
30	70 – 80	90 - 100
35	70 – 80	90 - 100
40	70 – 80	90 - 100
50	90 - 100	90 - 100

Cell homogenates were prepared using either hand or drill homogenisation, n=3.

3.3.2.1.2 Effect of sonication on cell membrane breakage.

As the time of sonication increased, the number of TB-stained cells increased. The level of TB-stained cells increased from 10 – 20 % for 0 sec of sonication to 30 – 40 % for 30 sec and then peaked at 70 – 80 % for 180 sec.

Table 3.5: Hep G2 cell membrane breakage when homogenised by sonication.

Time of sonication (s)	TB staining (%)
0	10 – 20
30	30 – 40
60	50 – 60
90	50 – 60
180	70 – 80

Cell homogenates were prepared using sonication for 0 – 180 s, n=3.

3.3.2.1.3 Effect of drill homogenisation on cell membrane breakage.

A semi-linear increase in LDH leakage was observed as the number of homogenisation strokes increased. The more the cell membrane breakage, the greater the LDH leakage, and the more efficient the homogenate preparation. Before centrifugation of cell homogenate, LDH leakage was observed to increase from 23 % (stroke 0) to 58 % (stroke 50). Centrifugation caused a higher LDH level of 40 % at stroke 0, 68 % at stroke 10 and 85 % at stroke 50 (Figure 3.4).

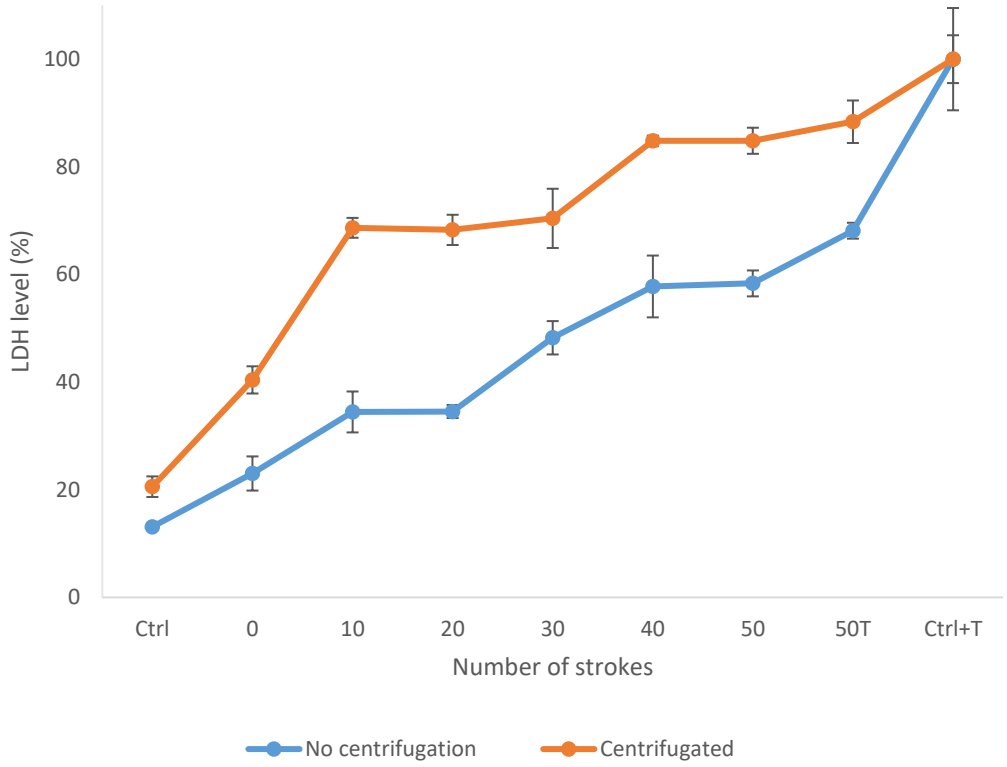


Figure 3.4: Level of LDH in cell homogenate prepared at various strokes with or without centrifugation. Data are presented as mean % LDH ± SEM, n=3, T=Triton-X100.

3.3.2.1.4 Optimising buffer conditions for preparation of cell homogenates for cytochrome P450 activity.

Hep G2 cells prepared in cytochrome P450 buffer showed 63.2 % higher levels of total cytochrome P450 as compared to cells prepared in NaPi buffer ($p<0.05$). There was no significant difference in total cytochrome P450 activity in cells prepared using Tris – HCl or NaPi buffers (Figure 3.5).

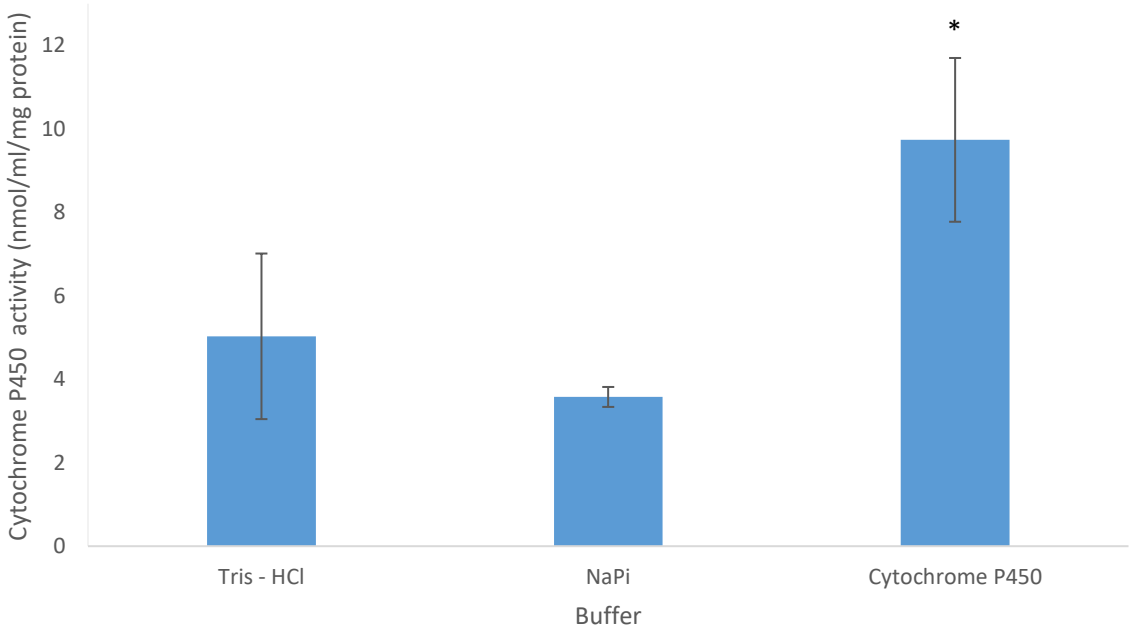


Figure 3.5: Effect of different buffers on total cytochrome P450 activity in Hep G2 cells. Data are presented as mean ± SEM, n = 3. *$p<0.05$ as compared to NaPi buffer by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.2 The effect of TI on cytochrome P450 activity in human liver cells *in vitro*.

3.3.2.2.1 Short term effect of TI extracts on total cytochrome P450 activity in Hep G2 cells.

Total cytochrome P450 activity increased with time up to 24 h in control cells. A similar effect was seen in cells treated with ethyl acetate or ethanol extracts at 24 h. However, pre-treatment with petroleum ether or chloroform extract significantly induced total cytochrome P450 activity at 4 h but inhibited the increase in cytochrome P450 levels seen in the control cells at 24 h (Figure 3.6).

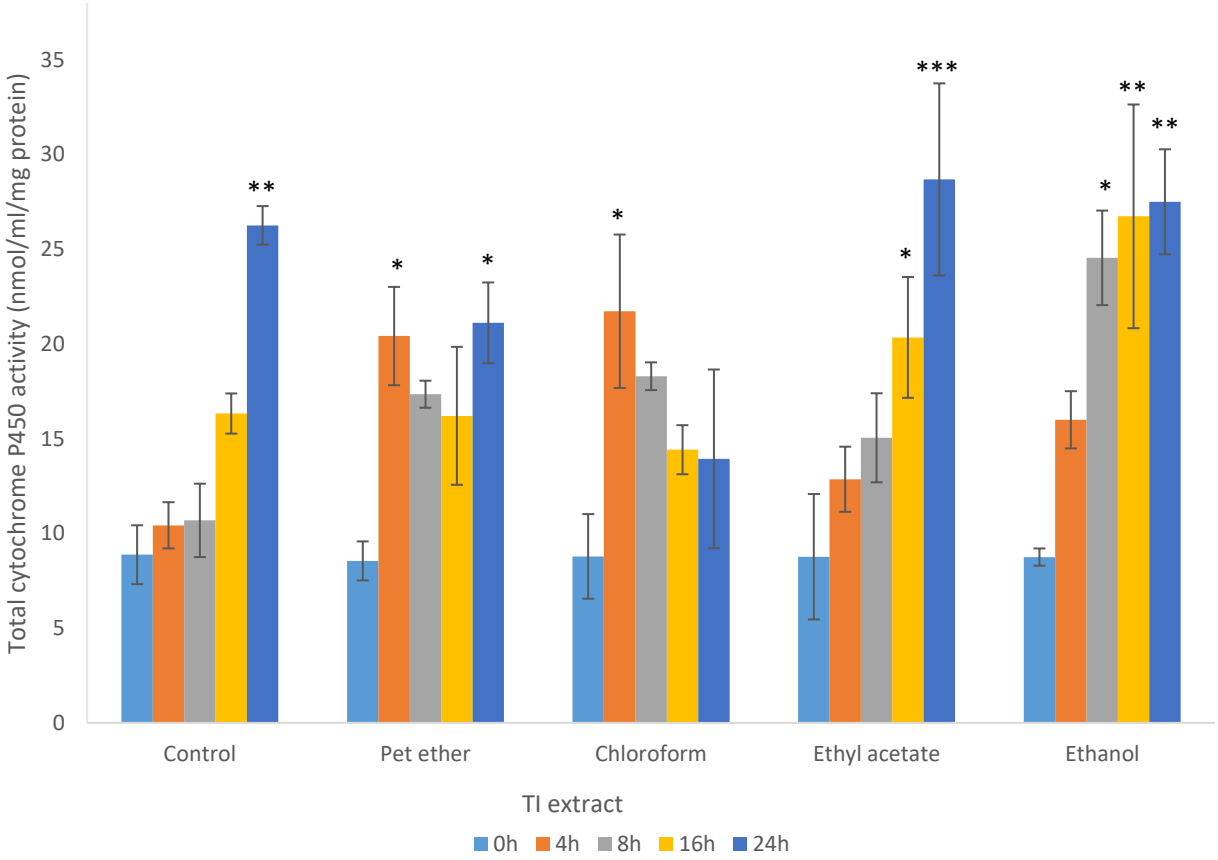


Figure 3.6: Short-term effect of 0.5 mg/ml of TI on total cytochrome P450 levels in Hep G2 cells. Data are presented as mean ± SEM, n = 3. *p<0.05, **p<0.01 and ***p<0.001 as compared to 0 h within each extract by ANOVA followed by Bonferroni's multiple comparison test.

There was a significant ($p<0.05$) increase in total cytochrome P450 activity for cells pre-treated with petroleum ether or chloroform extract at 4 h as compared to ethyl acetate or ethanol extracts (Table 3.6).

Table 3.6: One-way comparison of short-term effect of TI extracts on total cytochrome P450 levels in Hep G2 cells.

Treatment	Time (h)				
	0	4	8	16	24
Ctrl vs pet	ns	0.043	ns	ns	ns
Ctrl vs chlo	ns	0.046	ns	ns	0.045
Ctrl vs eth ace	ns	ns	ns	ns	ns
Ctrl vs etOH	ns	ns	ns	ns	ns
Pet vs chlo	ns	ns	ns	ns	ns
Pet vs eth ace	ns	ns	ns	ns	0.028
Pet vs etOH	ns	0.044	ns	ns	0.034
Chlo vs eth ace	ns	0.042	ns	ns	0.018
Chlo vs etOH	ns	0.039	ns	0.035	0.023
Eth ace vs etOH	ns	ns	ns	ns	ns

ns indicates not significant ($p>0.05$), ctrl is DMSO control, Pet is petroleum extract, Chlo is chloroform extract, Eth ace is ethyl acetate extract, and EtOH is ethanol extract, n=3, by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.2.2 Long term effect of TI extracts on total cytochrome P450 activity in Hep G2 cells.

Total cytochrome P450 activity significantly ($p<0.05$) increased with time in control cells and in Hep G2 cells pre-treated with ethyl acetate or ethanol extracts. Conversely, there was a transient increase (1 day) and a subsequent decrease in total cytochrome P450 activity in Hep G2 cells pre-treated with petroleum ether extract but no increase in P450 with cells treated with chloroform extract. Hence, pre-treatment of cells with chloroform or petroleum ether extracts showed significant ($p<0.001$) inhibition in total cytochrome P450 activity after 1 – 7 days treatment as compared to the DMSO control (Figure 3.7).

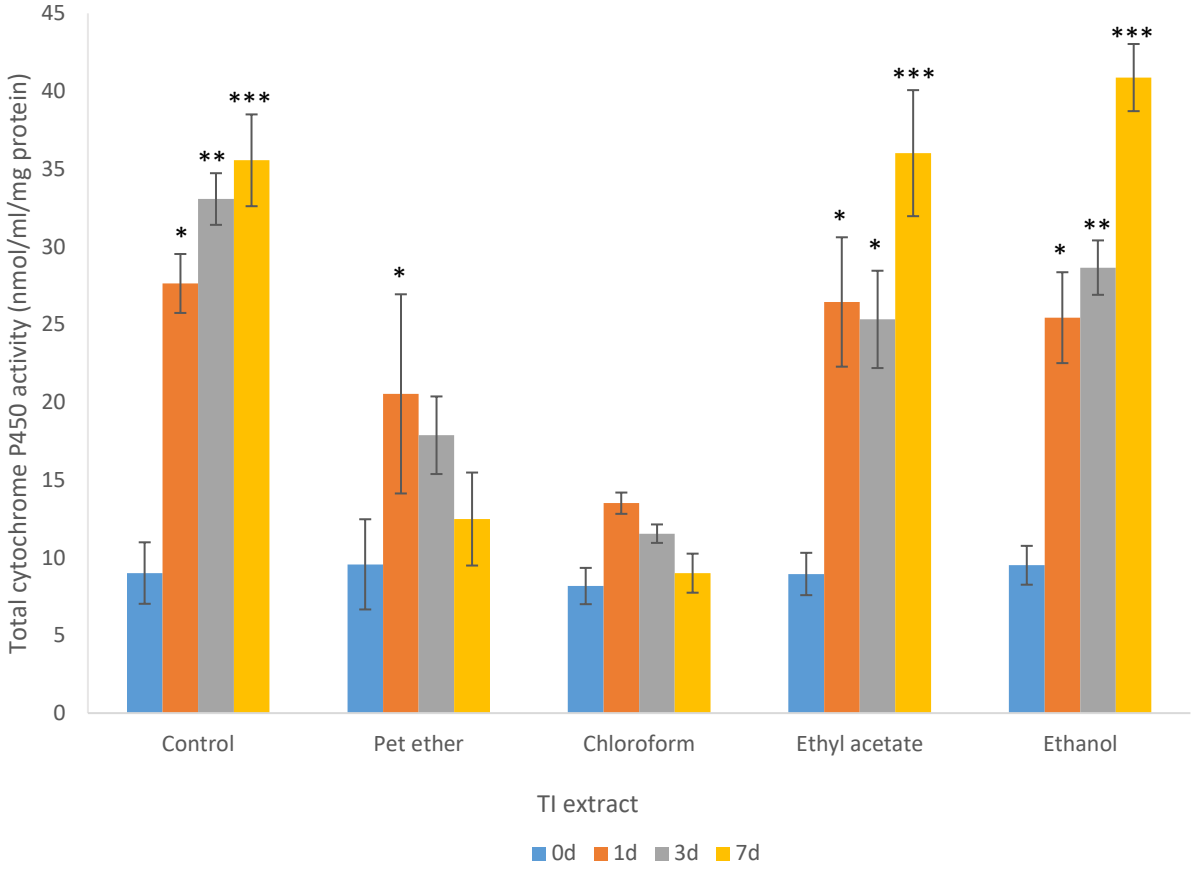


Figure 3.7: Long-term effect of 0.5 mg/ml of TI on total cytochrome P450 levels in Hep G2 cells. Data are presented as mean ± SEM; d=day; n = 3. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ as compared to day 0 within each extract by ANOVA followed by Bonferroni's multiple comparison test.

For the 7-day time course, total cytochrome P450 activity were significantly ($p<0.01$) reduced by chloroform extract as compared to ethyl acetate or ethanol extract. Similarly, 3 – 7 days treatment of Hep G2 cells with petroleum ether extract showed a significant ($p<0.05$) reduction in cytochrome P450 levels as compared ethyl acetate or ethanol extract (Table 3.7).

Table 3.7: One-way comparison of long-term effect of TI extracts on total cytochrome P450 levels in Hep G2 cells.

Treatment	Time (day)			
	0	1	3	7
Ctrl vs pet	ns	ns	0.018	0.001
Ctrl vs chlo	ns	0.042	0.003	< 0.001
Ctrl vs eth ace	ns	ns	ns	ns
Ctrl vs etOH	ns	ns	ns	ns
Pet vs chlo	ns	ns	ns	ns
Pet vs eth ace	ns	0.031	0.018	< 0.001
Pet vs etOH	ns	0.036	0.012	< 0.001
Chlo vs eth ace	ns	0.022	0.001	< 0.001
Chlo vs etOH	ns	0.032	0.014	< 0.001
Eth ace vs etOH	ns	ns	ns	ns

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.2.3 Effect of TI phytochemical metabolites on total cytochrome P450 activity in Hep G2 cells.

The measured effects of TI extracts on total cytochrome P450 levels showed that treatment with chloroform or petroleum ether extract inhibited total cytochrome P450 activity after 3 days as compared to the control (Table 3.7). A 50-cm circle was drawn at the negative side of the graph of TI phytochemicals versus total cytochrome P450 activity to show the phytochemicals were strongly associated with the inhibitory effects shown by chloroform or petroleum ether extract on total cytochrome P450 activity. Total cytochrome P450 activity was inhibited at 7 days of treatment by 3,4-dimethoxybenzaldehyde; 4-hydroxyphenylpyruvic acid and p-asinic acid which were found in chloroform or petroleum ether extract but not ethanol or ethyl acetate extract (Figure 3.8). List of all phytochemicals associated with inhibitory effect from the graph are shown in appendix 2 (Table 8.1). However, some phytochemicals were found in the TI extracts (for instance ethanol or ethyl acetate) that showed no effect on total cytochrome P450 activity and hence, are excluded from the main findings.

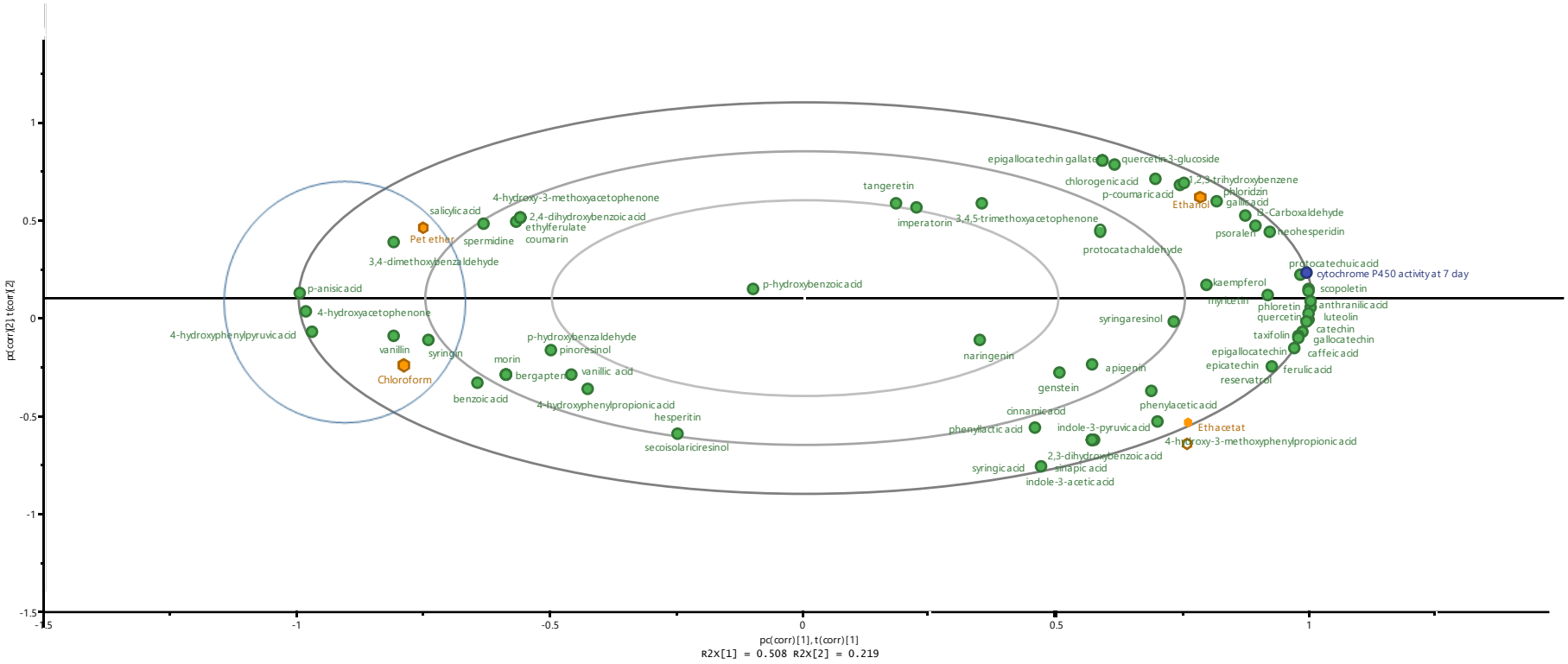


Figure 3.8: PLS-DA plot showing phytochemical compounds isolated from TI using chloroform, ethanol, ethyl acetate or petroleum ether and the relationship with total cytochrome P450 activity at 7 days. The plot was colour coded as: deep blue for total cytochrome P450 activity; green for phytochemical compounds; and orange for TI extracts.

3.3.2.3 The effect of TI on phase II DMEs activities in human liver cells *in vitro*.

3.3.2.3.1 Short-term effect of TI on catalase activity in Hep G2 cells.

Catalase activity significantly ($p<0.01$) increased in control cells after 24 h in culture. A similar increase was seen in cells treated with ethanol extract at 24 h. Conversely, treatment of cells with chloroform extract induced catalase activity at 4, 8 and 16 h but showed a significant ($p<0.01$) inhibition at 24 h as compared to DMSO control (Figure 3.9).

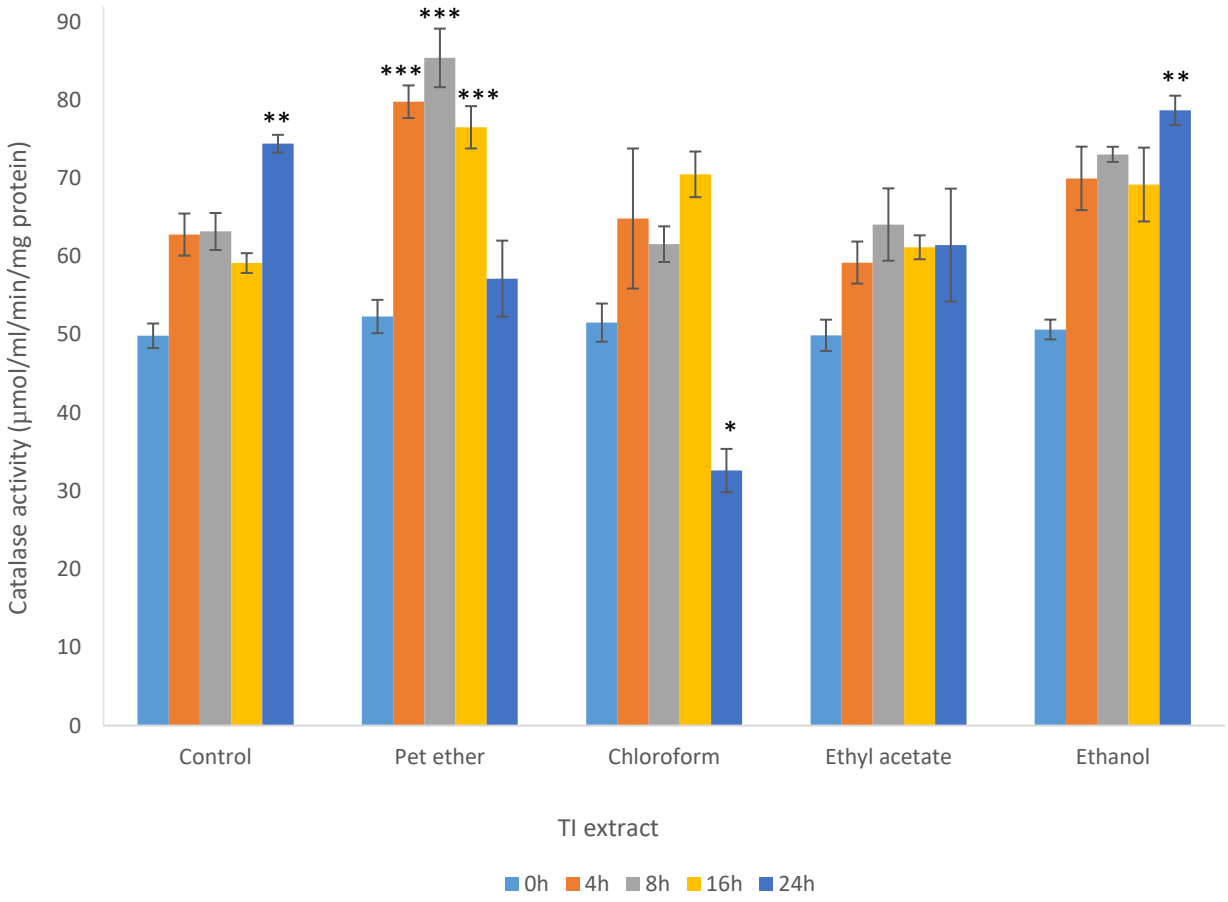


Figure 3.9: Short-term effect of 0.5 mg/ml of TI on catalase activity in Hep G2 cells after 24 h treatment. Data are presented as mean ± SEM, n=3, **p<0.01 and ***p<0.001 as compared to time 0 h within each extract by ANOVA followed by Bonferroni's multiple comparison test.

Comparing TI extracts after 4 h of treatment, petroleum ether extract showed a significantly elevated catalase activity as compared to ethyl acetate extract. After 8 h of treatment, petroleum ether extracts also showed significantly ($p<0.05$) higher catalase activity as compared to chloroform extract or ethyl acetate extract. At 24 h treatment, chloroform extract showed a significantly ($p<0.01$) decreased catalase activity as compared to the petroleum ether extract, ethyl acetate extract or ethanol extract. At 24 h, ethanol extract significantly ($p<0.05$) increased catalase activity as compared to petroleum ether extract (Table 3.8).

Table 3.8: One-way ANOVA comparison of short-term effect of TI extracts on catalase activity in Hep G2 cells.

Treatment	Time (h)				
	0	4	8	16	24
Ctrl vs pet	ns	0.032	0.015	ns	0.030
Ctrl vs chlo	ns	ns	ns	ns	0.003
Ctrl vs eth ace	ns	ns	ns	ns	ns
Ctrl vs etOH	ns	ns	ns	ns	ns
Pet vs chlo	ns	ns	0.005	ns	0.003
Pet vs eth ace	ns	0.043	0.026	ns	ns
Pet vs etOH	ns	ns	ns	ns	0.023
Chlo vs eth ace	ns	ns	ns	ns	0.001
Chlo vs etOH	ns	ns	ns	ns	0.001
Eth ace vs etOH	ns	ns	ns	ns	ns

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.2 Long-term effect of TI on catalase activity in Hep G2 cells.

A transient increase at 1 day ($p<0.001$) then decrease in catalase activity was observed in control and in Hep G2 cells pre-treated with either ethyl acetate or ethanol extracts. Moreover, treatment of cells with ethanol extract significantly ($p<0.001$) induced catalase activity after 7 days as compared to the control. Conversely, treatment with chloroform or petroleum ether extract inhibited catalase activity after 1 day exposure (Figure 3.10).

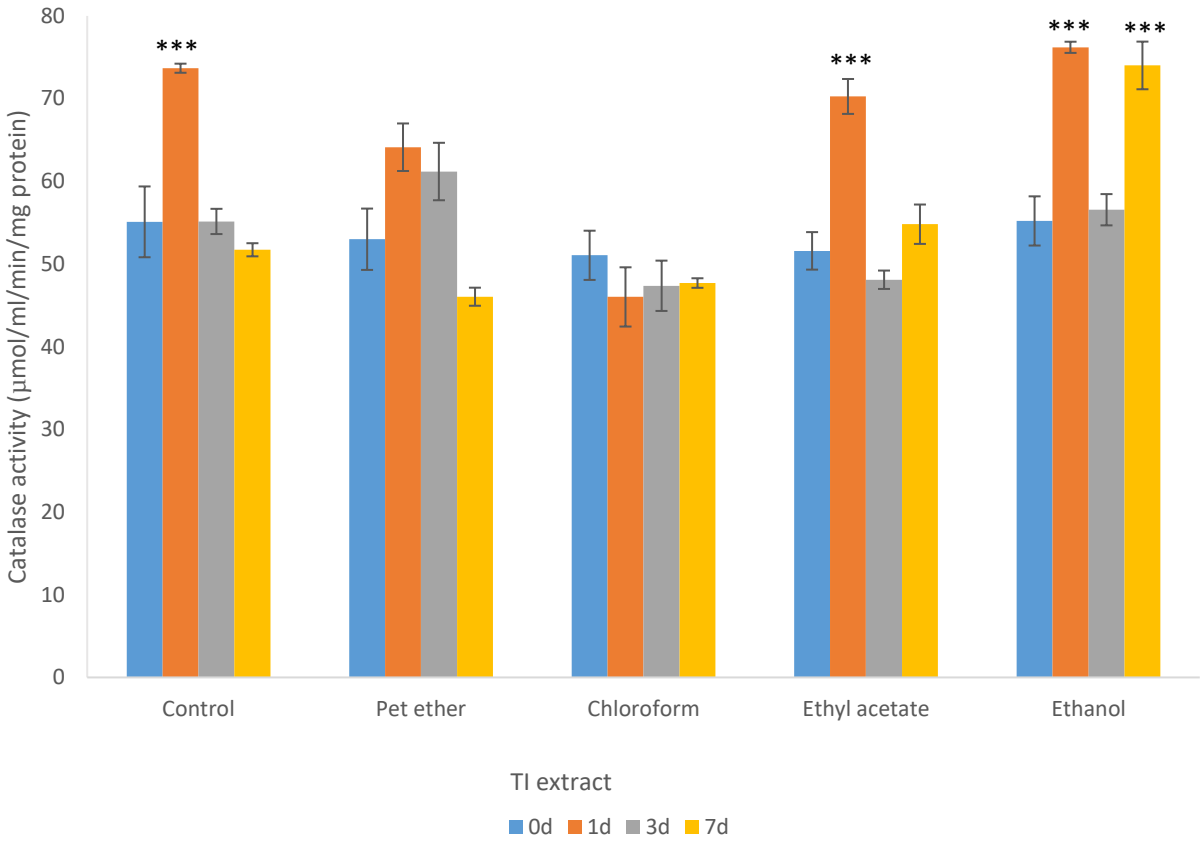


Figure 3.10: Long-term effect of 0.5 mg/ml of TI on catalase activity in Hep G2 cells after 7-day treatment. Data are presented as mean ± SEM; d=day; n=3; **p<0.01 and ***p<0.001 as compared to day 0 within each extract by ANOVA followed by Bonferroni's multiple comparison test.

Comparing TI extracts at day 1, treatment of cells with chloroform extract showed a significantly ($p<0.01$) inhibited catalase activity as compared to ethyl acetate and ethanol extracts. At day 7, ethanol extract showed a significantly ($p<0.001$) induced catalase activity as compared chloroform, petroleum ether and ethyl acetate extracts (Table 3.10).

Table 3.9: One-way ANOVA comparison of long-term effect of TI extracts on catalase activity in Hep G2 cells.

Treatment	Time (day)			
	0	1	3	7
Ctrl vs pet	ns	0.001	ns	ns
Ctrl vs chlo	ns	<0.001	ns	ns
Ctrl vs eth ace	ns	ns	ns	ns
Ctrl vs etOH	ns	ns	ns	<0.001
Pet vs chlo	ns	ns	ns	ns
Pet vs eth ace	ns	ns	ns	ns
Pet vs etOH	ns	<0.001	ns	<0.001
Chlo vs eth ace	ns	<0.001	ns	ns
Chlo vs etOH	ns	<0.001	ns	<0.001
Eth ace vs etOH	ns	ns	ns	<0.001

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; **$p<0.01$ and ***$p<0.001$ by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.3 Effect of TI phytochemical metabolites on catalase activity in Hep G2 cells.

Based on the measured effects of the TI extracts on catalase activity, treatment with ethanol extract induced catalase activity at 7 days, while chloroform or petroleum ether extract inhibited catalase activity at 24 h as compared to the control (Tables 3.8 and 3.9). Similar phytochemicals were linked with the induction in catalase activity shown by ethanol extract; hence, two 50-cm circles were drawn at the negative and positive sides of the graph of TI phytochemicals versus catalase activity at 24 h to show the inhibitory effect of chloroform or petroleum ether extracts, and the induction shown by ethanol extract (Figure 3.11).

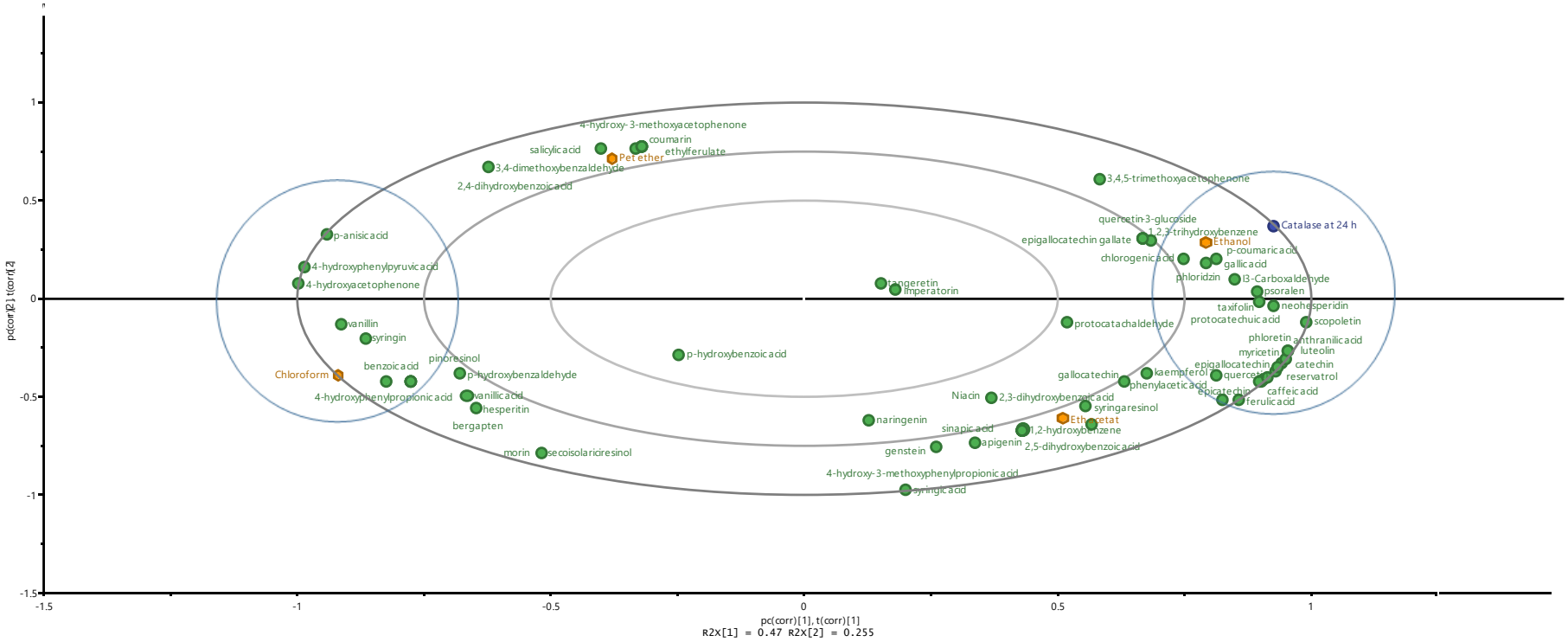


Figure 3.11: PLS-DA plot showing phytochemical compounds isolated from TI using chloroform, ethanol, ethyl acetate and petroleum ether and their relationship with catalase activity after treatment for 24 h. The plot was colour coded as: deep blue for catalase activity; green for phytochemical compounds; and orange for TI extracts.

Catalase activity at 24 h of exposure to TI extracts was induced by metabolites such as: 1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol which were present in ethanol or ethyl acetate extract and not detected in chloroform or petroleum ether extract.

Conversely, catalase activity was inhibited at 24 h of treatment by 4-hydroxyphenylpyruvic acid and p-asinic acid which were found in chloroform or petroleum ether extract but not in ethanol or ethyl acetate extracts. 4-hydroxyphenylpropionic acid also inhibited catalase activity and was present in chloroform extract but not in ethanol, ethyl acetate or petroleum ether extract (Table 3.10).

Table 3.10: Relationship between phytochemical compounds isolated from TI extracts and the impact on catalase activity in Hep G2 cells after treatment for 24 h.

TI	24 h
Petroleum ether extract	4-hydroxyphenylpyruvic acid and p-asinic acid.
Chloroform extract	4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid and p-asinic acid.
Ethanol extract	1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol.

3.3.2.3.4 Short-term effect of TI extracts on GSR activity in Hep G2 cells.

GSR activity was significantly increased ($p<0.001$) at 24 h in control cells. A similar effect was seen in cells treated with petroleum ether, ethyl acetate or ethanol extracts at 24 h. Contrary to this, pre-treatment of cells with chloroform extract showed a significantly lower GSR activity at 24 h as compared to the control (Figure 3.12).

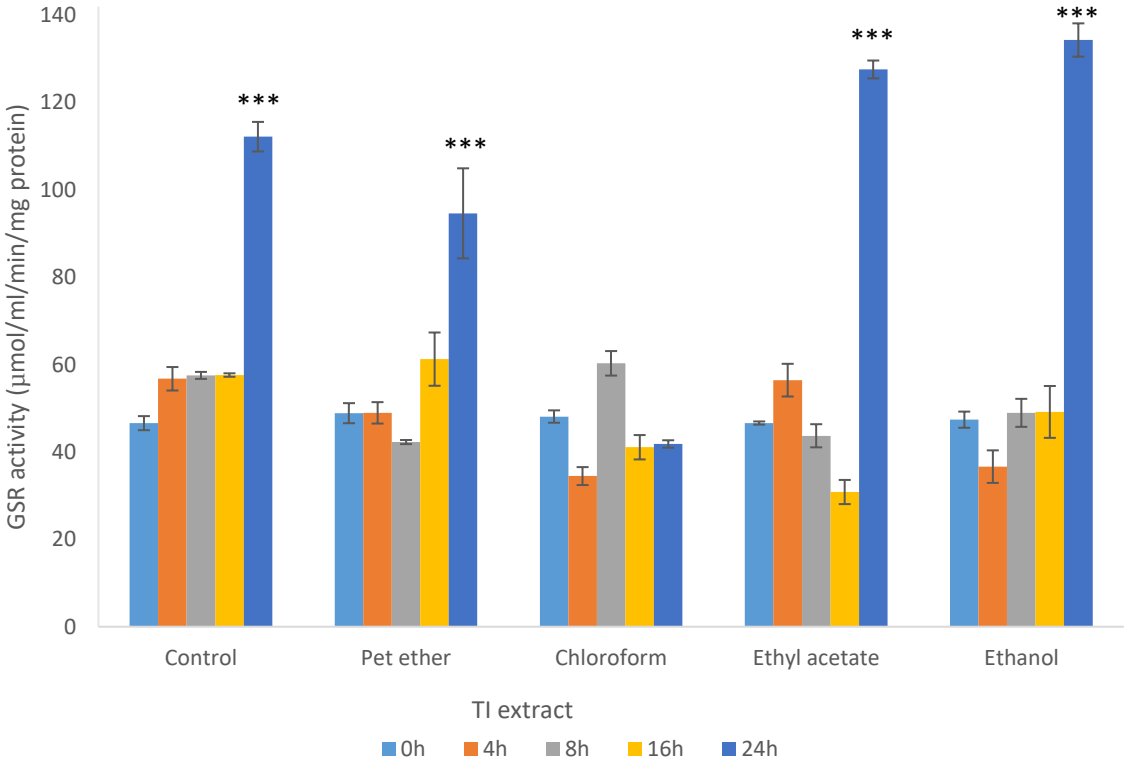


Figure 3.12: Short-term effect of 0.5 mg/ml of TI on GSR activity in Hep G2 cells.
Data are presented as mean ± SEM; n = 3. ***$p<0.001$ as compared to 0 h for each extract by ANOVA followed by Bonferroni's multiple comparison test.

Comparing the TI extracts after 16 h of treatment, a significantly ($p<0.001$) inhibited GSR activity was seen in cells treated with ethyl acetate extract as compared to those treated with petroleum ether extract. After 24 h, GSR activity was also significantly ($p<0.001$) inhibited in cells treated with chloroform extract as compared to the other TI extracts (Table 3.11).

Table 3.11: One-way comparison of short-term effect of TI extracts on GSR activity in Hep G2 cells.

Treatment	Time (h)				
	0	4	8	16	24
Ctrl vs pet	ns	ns	ns	ns	ns
Ctrl vs chlo	ns	0.013	ns	ns	< 0.001
Ctrl vs eth ace	ns	ns	ns	0.001	ns
Ctrl vs etOH	ns	ns	ns	ns	ns
Pet vs chlo	ns	ns	ns	ns	< 0.001
Pet vs eth ace	ns	ns	ns	0.001	ns
Pet vs etOH	ns	ns	ns	ns	ns
Chlo vs eth ace	ns	0.016	ns	ns	< 0.001
Chlo vs etOH	ns	ns	ns	ns	< 0.001
Eth ace vs etOH	ns	ns	ns	ns	ns

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.5 Long-term effect of TI extracts on GSR activity in Hep G2 cells.

GSR activity significantly increased ($p<0.001$) with time in control cells and in Hep G2 cells pre-treated with petroleum ether, ethyl acetate or ethanol extracts. Conversely, treatment of cells with chloroform extract for the 7-day time course significantly ($p<0.001$) inhibited GSR activity as compared to the control (Figure 3.13).

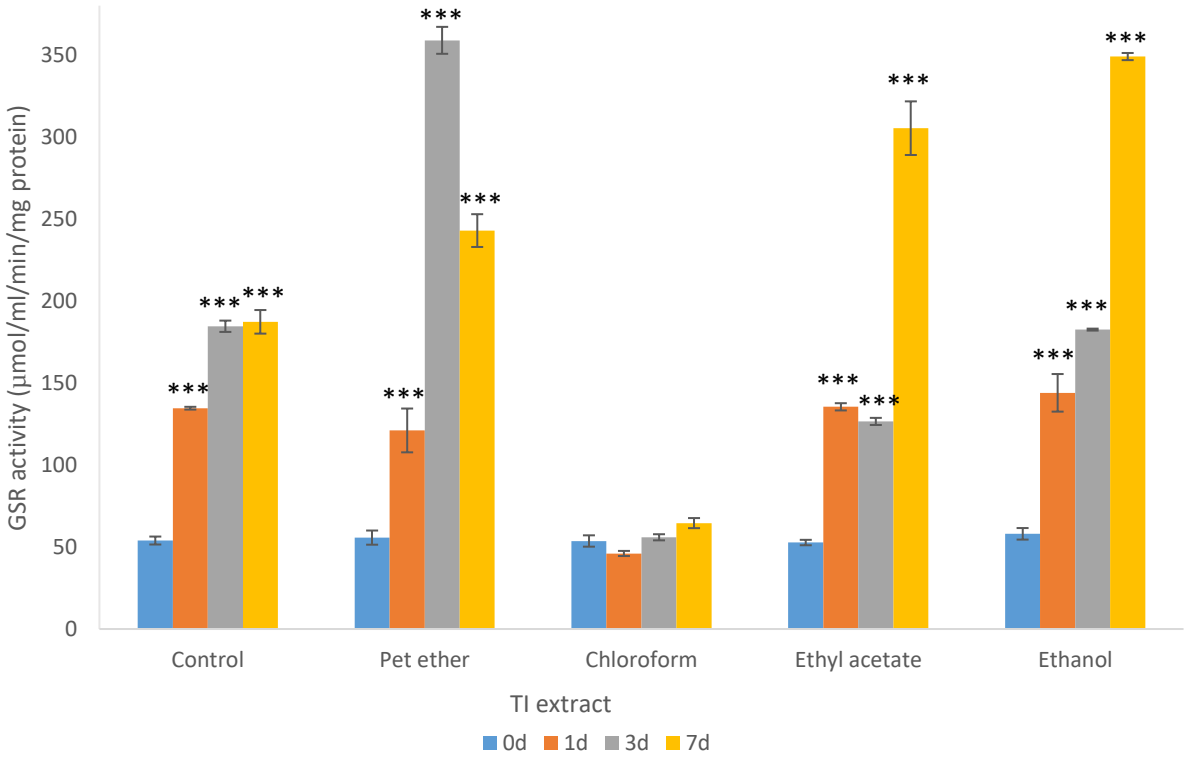


Figure 3.13: Long-term effect of 0.5 mg/ml of TI on GSR activity in Hep G2 cells.
Data are presented as mean ± SEM; d=day; n = 3. ***$p<0.001$ as compared to day 0 for each extract by ANOVA followed by Bonferroni's multiple comparison test.

Comparing the TI extracts, treatment of cells with chloroform extract for the 7-day time course significantly ($p<0.001$) inhibited GSR activity as compared to the other TI extracts. Hep G2 cells also showed a significantly ($p<0.001$) induced GSR activity after 3 days treatment with petroleum ether extract as compared to those treated with ethanol or ethyl acetate extracts (Table 3.12).

Table 3.12: One-way ANOVA comparison of long-term effect of TI extracts on GSR activity at different time points in Hep G2 cells.

Treatment	Time (day)			
	0	1	3	7
Ctrl vs pet	ns	ns	< 0.001	< 0.001
Ctrl vs chlo	ns	< 0.001	< 0.001	< 0.001
Ctrl vs eth ace	ns	ns	0.001	< 0.001
Ctrl vs etOH	ns	ns	ns	< 0.001
Pet vs chlo	ns	< 0.001	< 0.001	< 0.001
Pet vs eth ace	ns	ns	< 0.001	< 0.001
Pet vs etOH	ns	ns	< 0.001	< 0.001
Chlo vs eth ace	ns	< 0.001	< 0.001	< 0.001
Chlo vs etOH	ns	< 0.001	< 0.001	< 0.001
Eth ace vs etOH	ns	ns	< 0.001	ns

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.6 Effect of TI phytochemical metabolites on GSR activity in Hep G2 cells.

Treatment of Hep G2 cells with TI ethanol, ethyl acetate or petroleum ether extract induced GSR activity at day 7, while chloroform extract inhibited GSR activity as compared to the control (Tables 3.12). Therefore, two 50-cm circles were drawn at the negative and positive sides of the graph of TI phytochemicals versus GSR activity at 7 days to show the phytochemicals possibly linked with the measured effects (Figure 3.14).

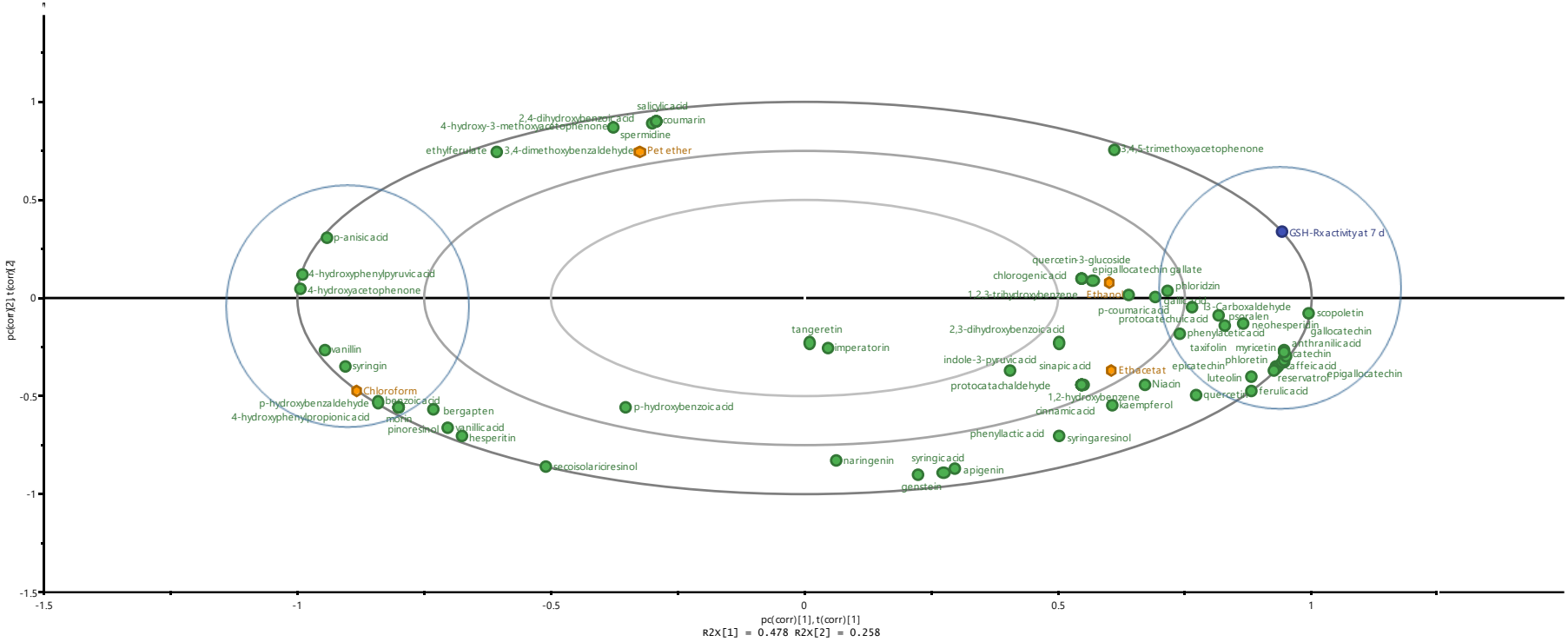


Figure 3.14: PLS-DA plot showing phytochemical compounds isolated from TI using chloroform, ethanol, ethyl acetate and petroleum ether and the relationship with GSR activity after treatment for 7 days. The plot was colour coded as: deep blue for GSR activity; green for phytochemical compounds; and orange for TI extracts.

The metabolites showing the closest relationship with induction of GSR activity include: anthranilic acid; caffeic acid; epicatechin; epigallocatechin; gallocatechin, luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol which were present in only ethanol or ethyl acetate extract but not in chloroform or petroleum ether extract induced GSR activity at 7 days.

Conversely, GSR activity was inhibited at 7 days of exposure by 4-hydroxyphenylpropionic acid, morin, p-hydroxybenzaldehyde and pinoresinol which were present in chloroform extract but not in ethanol, ethyl acetate or petroleum ether extract (Table 3.13). All phytochemicals showing impact on GSR activity are shown in appendix 2 (Table 8.3).

Table 3.13: Relationship between phytochemical compounds isolated from TI extracts and the impact on GSR activity in Hep G2 cells after treatment for 7 days.

TI	7 day
Chloroform extract	4-hydroxyphenylpyruvic acid; benzoic acid; morin; p-hydroxybenzaldehyde and pinoresinol.
Ethyl acetate extract	anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; phloretin; psolaren; and resveratrol.
Ethanol extract	anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; phloretin; psolaren; and resveratrol.

3.3.2.3.7 Short-term effect of TI extracts on GPx activity in Hep G2 cells.

In control cells, GPx activity was significantly increased at 24 h and a similar effect was seen in cells pre-treated with petroleum ether extract. Pre-treatment with ethanol extract significantly induced GPx activity earlier at 8 – 24 h. However, treatment with either chloroform or ethyl acetate extract inhibited the increase in GPx activity seen at 24 h in control cells (Figure 3.15).

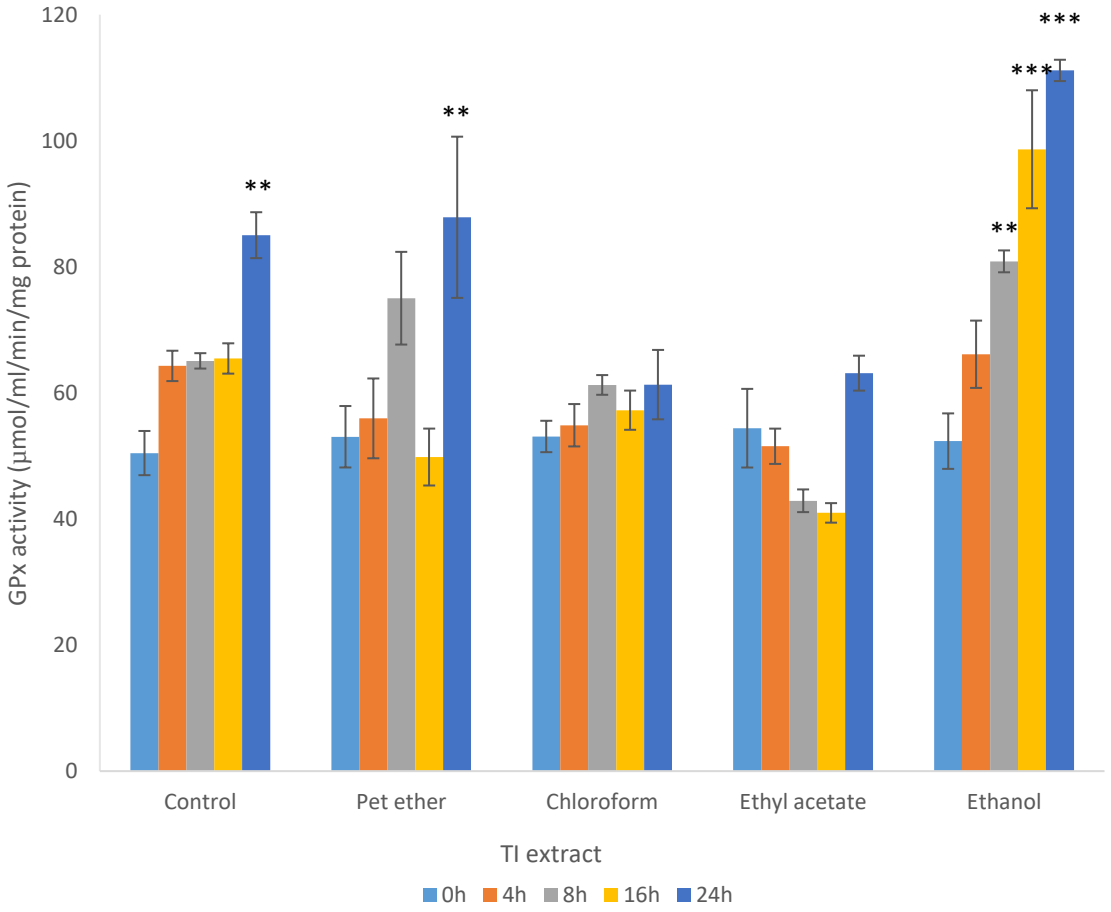


Figure 3.15: Short-term effect of 0.5 mg/ml of TI on GPx activity in Hep G2 cells.
Data are presented as mean ± SEM, n = 3. **p<0.01 and ***p<0.001 as compared to 0 h for each extract by ANOVA followed by Bonferroni's multiple comparison test.

For a 24-h treatment of Hep G2 cells with TI extracts, no significant difference in GPx activity was measured in the cells as compared to the control. However, comparison between the TI extracts showed that after 8 h treatment of Hep G2 cells with ethyl acetate extract, GPx activity was significantly ($p<0.01$) inhibited as compared to the cells treated with petroleum ether or ethanol extracts. After 16 h, a significant ($p<0.001$) induction in GPx activity was seen in cells treated with ethanol extract as compared to the TI chloroform, ethyl acetate and petroleum ether extracts. A similar effect was seen after 24 h treatment of cells with ethanol extract as compared to chloroform and ethyl acetate extracts (Table 3.14).

Table 3.14: One-way comparison of short-term effect of TI extracts on GPx activity in Hep G2 cells.

Treatment	Time (h)				
	0	4	8	16	24
Ctrl vs pet	ns	ns	ns	ns	ns
Ctrl vs chlo	ns	ns	ns	ns	ns
Ctrl vs eth ace	ns	ns	ns	ns	ns
Ctrl vs etOH	ns	ns	ns	ns	ns
Pet vs chlo	ns	ns	ns	ns	ns
Pet vs eth ace	ns	ns	0.007	ns	ns
Pet vs etOH	ns	ns	ns	< 0.001	ns
Chlo vs eth ace	ns	ns	ns	ns	ns
Chlo vs etOH	ns	ns	ns	< 0.001	< 0.001
Eth ace vs etOH	ns	ns	< 0.001	< 0.001	< 0.001

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.8 Long-term effect of TI extracts on GPx activity.

GPx activity was significantly ($p<0.001$) increased in control cells after 1 day and remained elevated by day 7. A similar increased GPx activity was measured in Hep G2 cells pre-treated with petroleum ether or ethanol extract (Figure 3.16). Similarly, a significant ($p<0.001$) induction in GPx activity was seen in cells treated with ethanol extract after 3 – 7 days as compared to the control. Conversely, a 7-day time course treatment of cells with chloroform extract showed a significant ($p<0.01$) inhibition in GPx activity as compared to the control (Table 3.15).

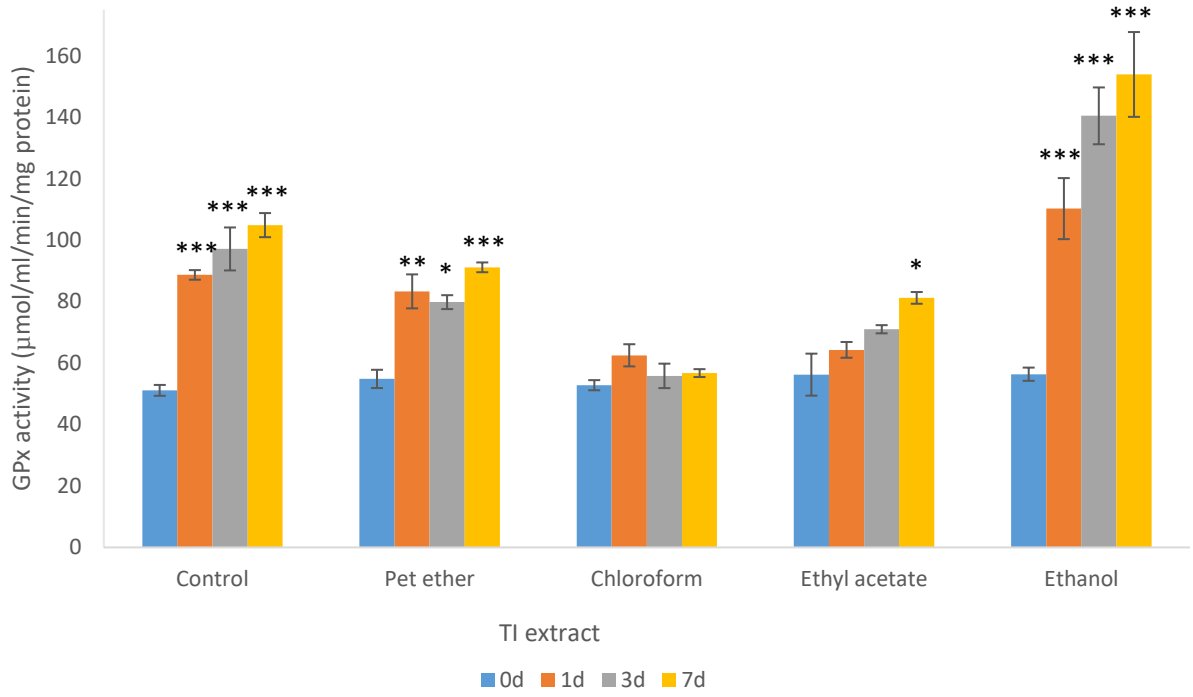


Figure 3.16: Long-term effect of 0.5 mg/ml of TI on GPx activity in Hep G2 cells.
Data are presented as mean ± SEM; d=day; n = 3. *p<0.05; **p<0.01 and ***p<0.001 as compared to day 0 for each extract by ANOVA followed by Bonferroni's multiple comparison test.

After 7 days, GPx activity was significantly ($p<0.001$) induced in cells treated with ethanol extract as compared to cells treated with petroleum ether, chloroform or ethyl acetate extract. Contrary to this, GPx activity was significantly ($p<0.05$) inhibited in cells treated with chloroform extract as compared to those treated with petroleum ether or ethyl acetate extract (Table 3.15).

Table 3.15: One-way ANOVA comparison of long-term effect of TI extracts on GPx activity at different time points in Hep G2 cells.

Treatment	Time (day)			
	0	1	3	7
Ctrl vs pet	ns	ns	ns	ns
Ctrl vs chlo	ns	0.010	< 0.001	< 0.001
Ctrl vs eth ace	ns	0.026	0.011	ns
Ctrl vs etOH	ns	ns	< 0.001	< 0.001
Pet vs chlo	ns	ns	0.032	< 0.001
Pet vs eth ace	ns	ns	ns	ns
Pet vs etOH	ns	0.006	< 0.001	< 0.001
Chlo vs eth ace	ns	ns	ns	0.025
Chlo vs etOH	ns	< 0.001	< 0.001	< 0.001
Eth ace vs etOH	ns	< 0.001	< 0.001	< 0.001

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.9 Effect of TI phytochemical metabolites on GPx activity in Hep G2 cells.

Based on the measured effects of TI extracts on GPx activity, treatment of Hep G2 cells with TI extracts showed that ethanol extract induced GPx activity at 7 days, while chloroform extract inhibited GPx activity at 7 days as compared to the control (Table 3.15). The phytochemicals associated with the impact of TI extracts on GPx activity were selected based on the analysis of the PLS-DA graph (Figure 3.17).

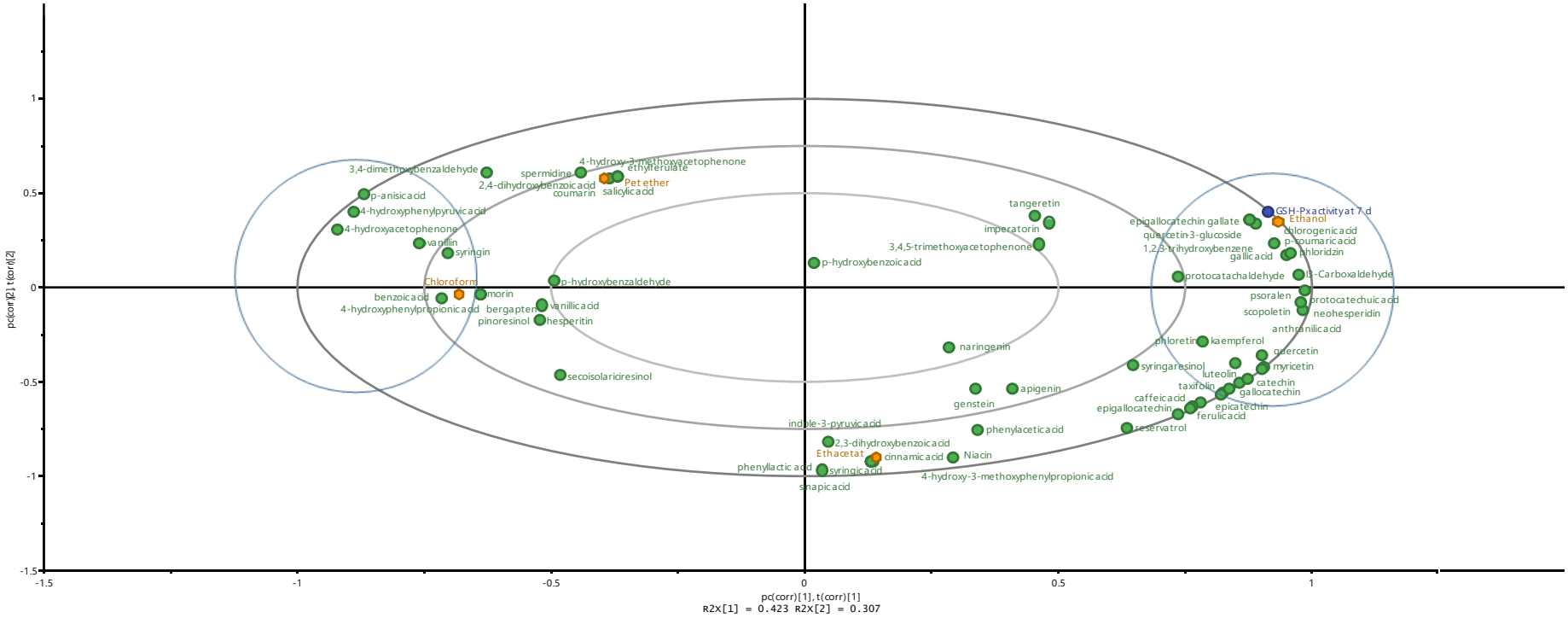


Figure 3.17: PLS-DA plot showing phytochemical compounds isolated from TI using chloroform, ethanol, ethyl acetate and petroleum ether and the relationship with GPx activity after treatment for 7 days. The plot was colour coded as: deep blue for GPx activity; green for phytochemical compounds; and orange for TI extracts.

GPx activity at 7 days of exposure to TI extracts was induced by 1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; chlorogenic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; p-coumaric acid; phloretin; protocatachaldehyde; psolaren; quercetin-3-glucoside and resveratrol induced GPx activity and were present in ethanol or ethyl acetate extract and not detected in chloroform or petroleum ether extract.

Conversely, GPx activity was inhibited at 3 - 7 days of treatment by 4-hydroxyphenylpyruvic acid and p-asinic acid and were found in chloroform or petroleum ether extract but not ethanol or ethyl acetate extract. Similarly, 4-hydroxyphenylpropionic acid and bergapten which also inhibited GPx activity were present in chloroform extract but not in ethanol, ethyl acetate or petroleum ether extract (Table 3.16).

Table 3.16: Relationship between phytochemical compounds isolated from TI extracts and the impact on GPx activity in Hep G2 cells after treatment at 7 days.

TI	7 day
Chloroform extract	4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid; bergapten and p-asinic acid.
Ethanol extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; protocatachaldehyde; psolaren; quercetin-3-glucoside and resveratrol.

3.3.2.3.10 Short-term effect of TI extracts on GSH levels in Hep G2 cells.

A significant increase in GSH was seen in control cells after 24 h and in cells treated with ethyl acetate or ethanol extracts. An earlier (at 4 h) increase in GSH was observed in cells treated with petroleum ether or chloroform extracts, which returned to control levels after 4 h (Figure 3.18).

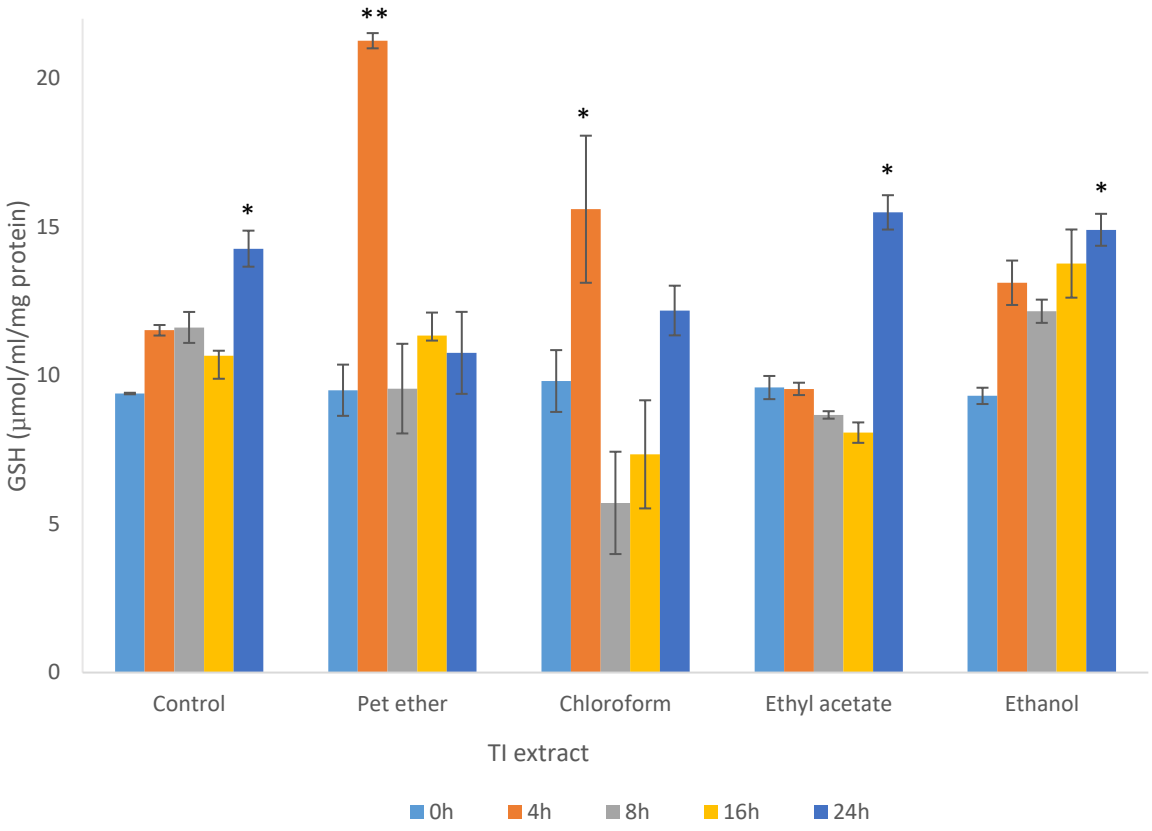


Figure 3.18: Short-term effect of 0.5 mg/ml of TI on GSH in Hep G2 cells.
Data are presented as mean ± SEM; n = 3. *p<0.05 and **p<0.01 as compared to day 0 for each extract by ANOVA followed by Bonferroni's multiple comparison test.

At 4 h, treatment of cells with petroleum ether extract showed a significantly ($p<0.05$) higher GSH as compared to chloroform, ethanol or ethyl acetate extract (Table 3.17).

Table 3.17: One-way comparison of short-term effect of TI extracts on GSH in Hep G2 cells.

Treatment	Time (h)				
	0	4	8	16	24
Ctrl vs pet	ns	0.012	ns	ns	ns
Ctrl vs chlo	ns	ns	ns	ns	ns
Ctrl vs eth ace	ns	ns	ns	ns	ns
Ctrl vs etOH	ns	ns	ns	ns	ns
Pet vs chlo	ns	0.041	ns	ns	ns
Pet vs eth ace	ns	0.008	ns	ns	ns
Pet vs etOH	ns	0.024	ns	ns	ns
Chlo vs eth ace	ns	ns	ns	ns	ns
Chlo vs etOH	ns	ns	ns	ns	ns
Eth ace vs etOH	ns	ns	ns	ns	ns

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.11 Long-term effect of TI extracts on GSH levels in Hep G2 cells.

A significant increase in GSH levels was seen in control cells after 1 days which later returned to day 0 control levels. Ethanol extract also caused an increase in GSH levels after 7 days exposure as compared to the control. Contrary, cells treated with petroleum ether or chloroform extract did not show the same increase in GSH after 1 day. In the case of chloroform extract, GSH levels were significantly decreased after 7 days as compared to control liver cells (Figure 3.19).

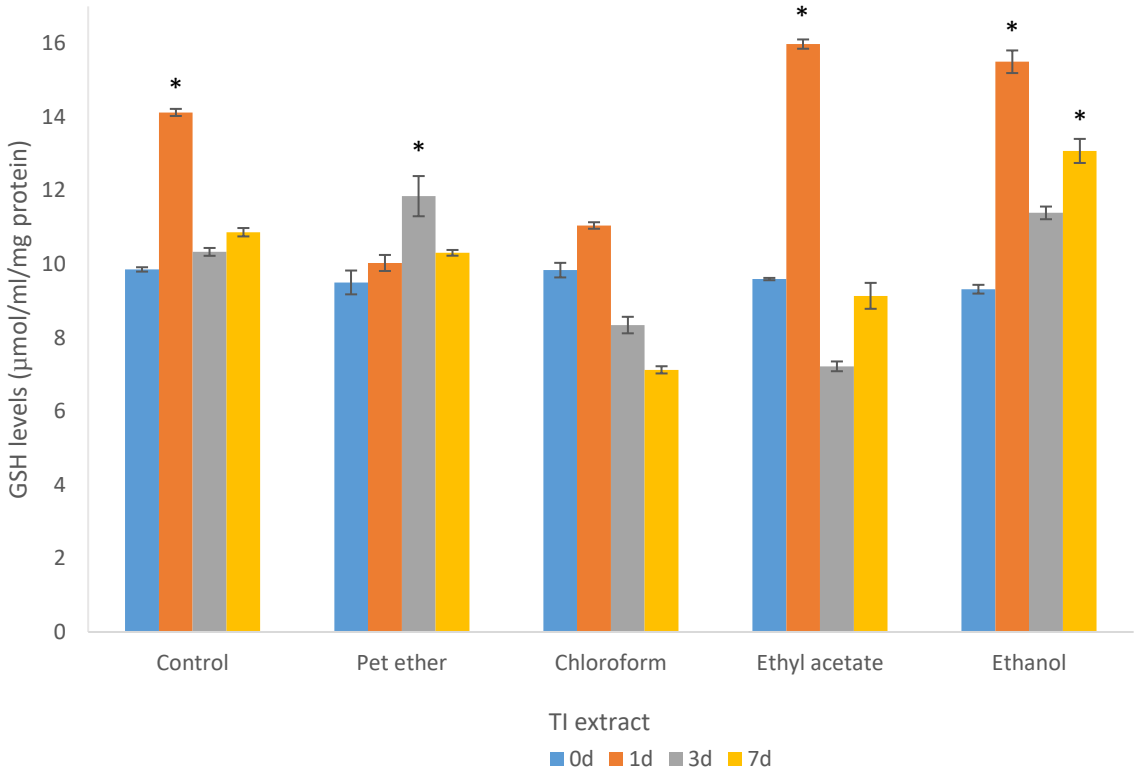


Figure 3.19: Long-term effect of 0.5 mg/ml of TI on GSH levels in Hep G2 cells.
Data are presented as mean ± SEM; d=day; n = 3. *p<0.05 as compared to day 0 for each extract by ANOVA followed by Bonferroni's multiple comparison test.

Comparing the TI extracts, cells treated with chloroform extract showed a significantly ($p<0.05$) lower GSH at 1 day as compared to those treated with ethyl acetate or ethanol extracts. A similar diminished GSH levels was measured in cells treated with petroleum ether extract as compared to those treated with ethyl acetate or ethanol extracts (Table 3.18).

Table 3.18: One-way ANOVA comparison of long-term effect of TI extracts on GSH in Hep G2 cells.

Treatment	Time (day)			
	0	1	3	7
Ctrl vs pet	ns	ns	ns	ns
Ctrl vs chlo	ns	ns	ns	0.001
Ctrl vs eth ace	ns	ns	0.032	ns
Ctrl vs etOH	ns	ns	ns	0.039
Pet vs chlo	ns	ns	0.023	0.034
Pet vs eth ace	ns	0.001	0.006	ns
Pet vs etOH	ns	0.009	ns	0.044
Chlo vs eth ace	ns	0.001	ns	ns
Chlo vs etOH	ns	0.014	ns	0.005
Eth ace vs etOH	ns	ns	0.037	0.036

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

3.3.2.3.12 Effect of TI phytochemical metabolites on GSH levels in Hep G2 cells.

It was found that all four extracts showed no effect on GSH levels at 24 h as compared to the control. However, longer treatment with TI extracts showed that ethanol extract induced GSH levels at 7 days, ethyl acetate extract induced GSH levels at 3 days while chloroform extract inhibited GSH levels at 7 days as compared to the control (Tables 3.18 and 3.19). The possible associated phytochemicals impacting on GSH levels on day 7 were analysed by PLS-DA (Figure 3.20).

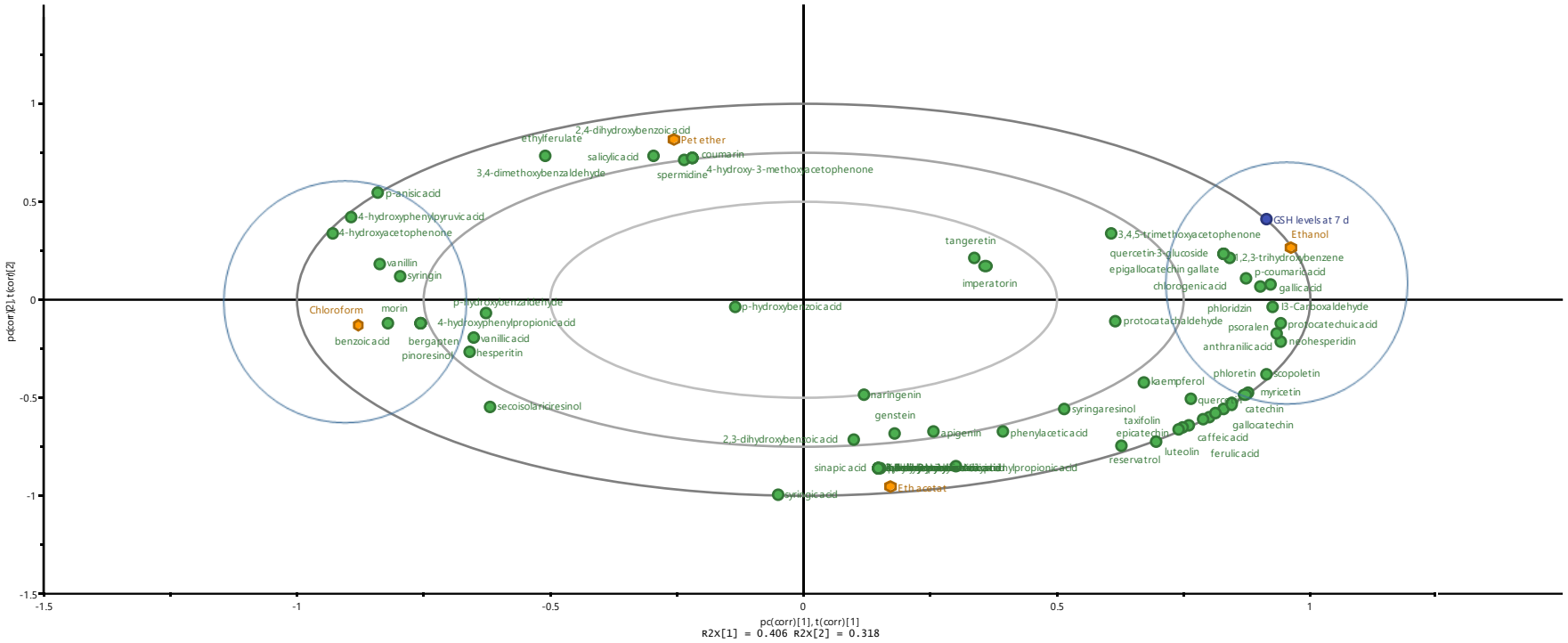


Figure 3.20: PLS-DA plot showing phytochemical compounds isolated from TI using chloroform, ethanol, ethyl acetate and petroleum ether and the relationship with GSH levels after treatment for 7 days. The plot was colour coded as: deep blue for GSH levels; green for phytochemical compounds; and orange for TI extracts.

GSH levels at 7 days of exposure to TI extracts were induced by metabolites which include 1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; chlorogenic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; p-coumaric acid; phloretin; psolaren; quercetin-3-glucoside and resveratrol and were detected in ethanol or ethyl acetate extract and not detected in chloroform or petroleum ether extract.

However, GSH levels was inhibited at 7 days of treatment by 4-hydroxyphenylpyruvic acid and p-asinic acid which were detected in chloroform or petroleum ether extract but not in ethanol or ethyl acetate extract. Similarly, 4-hydroxyphenylpropionic acid, bergapten, morin and pinoresinol which also inhibited GSH levels were present in chloroform extract only but not in ethanol, ethyl acetate or petroleum ether extract (Table 3.19).

Table 3.19: Relationship between phytochemical compounds isolated from TI extracts and the impact on GSH levels in Hep G2 cells after treatment for 7 days.

TI	7 day
Chloroform extract	4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid; bergapten; morin; p-asinic acid and pinoresinol.
Ethanol extract	1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; phloretin; psolaren; quercetin-3-glucoside and resveratrol.

3.4 DISCUSSION

Isolated human liver cell lines retain the differentiated parenchymal functions and provide an *in vitro* system for studying drug metabolism and cytotoxicity directly in man (Coatti et al., 2015; Doostdar et al., 1988). Due to the importance of human liver in drug metabolism, cell lines of liver origin (for instance Hep G2, Hep 3B, Huh-1 and SK- Hep-l) are widely used in biomedical research as models for xenobiotic metabolism (Coatti et al., 2015) This is because of the strong relationship isolated liver cells provide as *in vitro* model to the liver *in vivo,* and the only model that expresses enzymes that can produce metabolites of a given drug comparable to that produced by humans *in vivo* (Plazar et al., 2007).

Furthermore, the Hep G2 cell line is one of the most often used cell lines in xenobiotic and metabolism research because it is characterised in detail biochemically and physiologically (Doostdar et al., 1988). Primary human hepatocytes also provide an excellent model for acute *in vitro* studies of xenobiotic metabolism but unlike Hep G2 cells, they quickly lose their enzyme activities and viability (Donohue et al., 2006). To study the impact of TI on human liver cells *in vitro*, the Hep G2 cell model was adopted because it is immortalised and retains the key functions of the normal human liver (Donohue et al., 2006; Doostdar et al., 1988). To ensure a safe use of TI as a potential anticancer agent, assessment of cytotoxicity of TI sample against the exposed cells is necessary to exclude negative off-target effects during treatment. Therefore, the impact of TI was determined on cell viability, proliferation, genomic stability and drug metabolising enzymes in Hep G2 cells.

In this study, Hep G2 cells were treated with varying concentrations (0 – 5 mg/ml) of four extracts of TI namely: chloroform, ethanol, ethyl acetate or petroleum ether extract for 24 h and the impact on viability and growth were measured. All four TI extracts showed inversely proportional relationship on cell viability and growth. TI concentration of ≥3.5 mg/ml was required to reduce the viability of Hep G2 cells by 50 % (IC_{50}) and all the four extracts were non-toxic at ≤1 mg/ml. TI also showed an IC_{50} of 1.6 – 2.2 mg/ml on cell growth with ethanol extract (IC_{50}=1.6 mg/ml) showing the highest toxicity and all the four extracts were non-toxic at <1 mg/ml on growth.

There are no previous data on the impact of TI on Hep G2 cells and hence, other studies involving Hep G2 cell viability and proliferation have been used to support the present study. Thus, in other studies, the cytotoxicity of potassium dichromate was investigated in Hep G2 cells and potassium dichromate was found to significantly ($p<0.05$) decrease the viability of Hep G2 cells after exposure of the cells to 25 µM of potassium dichromate for 24 h (Patlolla et al., 2009). The effect of quercetin was also studied by treating Hep G2 cells with 50 – 100 µM of quercetin and quercetin was shown to cause a significant ($p<0.05$) inhibition in Hep G2 cell growth after 24 h (Alía et al., 2006). Similarly, treatment of Hep G2 cells with varying concentrations of methanolic extract of *Trillium govanianum* for 24 h showed cytotoxicity in the cells at IC_{50}= 7 µg/ml (Khan et al., 2016). Moreover, a significant ($p<0.05$) cytotoxic effect of aspidospermine was also shown in Hep G2 cells after treatment with 50 – 100 µM of aspidospermine for 24 h (Coatti et al., 2015). These studies show the reliability and sensitivity of Hep G2 cells in response to change in viability and proliferation after exposure to small concentrations of chemicals or natural products.

In addition, acute *in vivo* studies of the ethanol extract of TI also showed that administering 100 – 5000 mg/kg body weight of TI for 24 h to mice or rats showed the median lethal dose (LD_{50}) of 2236.06 mg/kg body weight in mice and estimated to be above 5000 mg/kg body weight in rats (Ben-Azu et al., 2016; Moomin et al., 2020; Zaza et al., 2016). As reported previously, any substance with an LD_{50} beyond 1000 mg/kg body weight should be considered safe or of low toxicity when administered orally (Rolf, 2009). Additionally, treatment of Sprague – Dawley rats with 300 – 1000 mg/kg of TI ethanol extract for 14 days was shown to protect liver architecture against damage caused by gentamicin and significantly ($p<0.01$) reduced the increase in creatinine, serum electrolytes (for instance chloride, potassium and sodium) and urea induced by gentamicin (Ansah et al., 2016).

The impact of TI on genomic stability in Hep G2 cells was also determined using varying concentrations (0 – 5 mg/ml) of the four TI extracts. All four extracts were shown to be non-toxic at 0.5 mg/ml with the lowest concentration of TI that caused a 50 % increase in DNA SSB (LC_{50}) at the concentrations of 1.1 – 2 mg/ml. Considering the sensitivity of Hep G2 cells to respond to micromolar

concentrations of NPs, TI extracts showed low concerns with DNA damage and toxicity on human liver cells thus, a potential good drug candidate for drug discovery.

The human body has a complex system of natural enzymatic and non-enzymatic defences which eliminate the detrimental effects of foreign substances from the body (Alam et al., 2013). Many of these enzymes are produced by the liver and are normally distributed in the cytoplasm and other organelles of the cell (Ingawale et al., 2014). The presence of various liver enzymes in serum is used to evaluate the functional status of the liver and to detect liver injury (Rang et al., 2014).

As part of method development to determine the impact of TI on DME in the liver, Hep G2 cells were homogenised by using a hand homogeniser, drill homogeniser or a sonicator to break the cell membrane and release the enzymes from the cells. The preparation of cell homogenate in the cells was optimised by assessing the uptake of trypan blue (TB) or release of lactate dehydrogenase (LDH) from the cells. The TB test is used to determine the viability of cells in a cell suspension, and it is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue and Eosin, whereas dead cells do not have intact cell membranes and lack the ability to pump out the dyes (Strober, 2015). Similarly, leakage of intracellular LDH into the culture medium is also used as an indicator of cell membrane breakage for measuring cytotoxicity and an increase in LDH leakage shows higher cell membrane damage and hence cytotoxicity (Alía et al., 2006).

Drill homogenisation at 20 strokes showed a highest amount (90-100 %) of TB uptake as compared to TB uptake of 50-60 % and 70-80 % for hand homogenisation and sonication respectively. Drill homogenisation at 20 strokes was then used to compare LDH leakage in centrifuged and non-centrifuged cell homogenate. Centrifugation showed higher LDH level than in non-centrifuged cell homogenate. The difference in LDH levels could be due to centrifugation resulting in a clearer homogenate that showed less interference in the assay. It was concluded that cell homogenate to be used to determine the impact of TI on DME in Hep G2 cells, as a model for the human liver, would be prepared with drill homogeniser at 20 strokes and then centrifuged.

Cytochrome P450 enzymes (CYP) are the principal enzymes involved in Phase I metabolism of xenobiotics in the body (Meyer, 1996). They are a superfamily of haem–containing enzymes that metabolise a vast array of compounds in the body (Gonzalez, 2005). These group of enzymes are quite challenging to extract from cells due to their unstable nature, hence, require a special buffer for efficient extraction and quantification (Schenkman & Jansson, 2006). To optimise buffer conditions for assessing total cytochrome P450 enzymes, cell homogenate was prepared in NaPi, Tris-HCl or cytochrome P450 buffer and the total cytochrome P450 levels were measured. The cytochrome P450 buffer showed the highest amount of total cytochrome P450, hence, was used to prepare the cell homogenate for assessing Phase I DME.

TI at a concentration of 0.5 mg/ml showed no significant cyto- and genotoxicity on Hep G2 cells and thus, the Hep G2 cells were treated with TI chloroform, ethanol, ethyl acetate or petroleum ether extract at a concentration of 0.5 mg/ml for short (0 – 24 h) or long term (0 – 7 day) to determine the impact of TI on Phase I DME in human liver cells. There was an increase in total cytochrome P450 levels in control cells with time and a similar increasing effect was observed with pre-treatment of cells with ethyl acetate or ethanol extract. On the other hand, a short-term pre-treatment of cells with chloroform or petroleum extract showed an initial increase in total cytochrome P450 activity after 4 h and a drastic decline afterwards (up to 7 days) when compared to the control. Hep G2 cells have shown both culture medium- and time-dependent changes in DME activities such as P450-mixed function oxidases, epoxide hydrolase, glutathione transferase and glucuronyltransferase (Duthie & Collins, 1997). This shows that DME from Hep G2 cells are very sensitive to changes in medium and time of detection. In this study, though the short- and long-term treatments were carried out on different days, the total cytochrome P450 activity was similar for the TI extract treatments at 24 h in short-term and 1 day in long-term. Therefore, the effect recorded was a real impact of the TI extracts and the results were reproducible.

Based on the measured effects of the TI extracts on total cytochrome P450 activity, treatment with chloroform or petroleum ether extract reduced total cytochrome P450 levels at 7 days by 75 % and 65 % respectively as compared to the control. Assessment of the relationship between the total cytochrome

P450 activity and the isolated phytochemicals showed that 3,4-dimethoxybenzaldehyde; 4-hydroxyphenylpyruvic acid and p-asinic acid which were found in chloroform or petroleum ether extract but not ethanol or ethyl acetate extract could account for the inhibitory effect shown by TI chloroform and petroleum ether extracts.

No previous data are available on the impact of TI on total cytochrome P450 activity and therefore, other data showing the effects of natural products or compounds on cytochrome P450 enzymes are used to discuss the present findings. Thus, in previous studies, chemical compounds such as cimetidine, fluconazole and metyrapone are known inhibitors of cytochrome P450 enzymes and cause a decrease in metabolism consequently prolonging the half-lives of drugs that depend on the cytochrome P450 enzymes for their degradation and excretion (Zhao et al., 2006). Moreover, compounds found in cruciferous vegetables, garlic, grapefruits and green tea have also been shown to significantly ($p<0.05$) inhibit Phase I metabolising enzymes (particularly CYP1A1, CYP1A2, CYP2C19, CYP2E1, and CYP3A4) and a drug transporter protein (P-glycoprotein) and hence, have been linked with chemo-preventive effects (Foster et al., 2002; Ondieki et al., 2017; Sprouse & van Breemen, 2016). On the other hand, cigarette smoking has been shown to induce cytochrome P450 enzymes (CYP1A1 and CYP1A2) and elevated levels of these enzymes have been closely associated with a high risk of colon and lung cancer (Sprouse & van Breemen, 2016; Zhou et al., 2010). Induction of metabolising enzyme levels would shorten half-lives of drugs and possibly lead to subtherapeutic levels in the body (Sprouse & van Breemen, 2016). Furthermore, bergapten (TI phytochemical found in chloroform extract) at a concentration of 1 µM was shown to significantly ($p<0.05$) inhibit CYP3A4 activity in liver microsomes (Ohnishi et al., 2000). Additionally, administration of a TI phytochemical (5 mM benzoic acid) for 6 h has been shown to significantly ($p<0.05$) induce cytochrome P450 in *P. chrysosporium* (Ning et al., 2010). Furthermore, subacute toxicity studies show that feeding mice with 1000 ppm bergapten has been shown to significantly (p,0.05) reduce liver total cytochrome P450 activity in female mice (Diawara et al., 2000). Seven-day consecutive administration of 8 – 128 µm of ethyl vanillin or vanillin (found in TI extracts) in rats showed a significant ($p<0.05$) induction in CYP2E1 activity and inhibition in CYP1A2 activity (Chen et al., 2012).

Reactive oxygen species (ROS), such as H_2O_2 and superoxide ions, are produced in all cells from mitochondrial and enzymatic sources and if left unchecked, they can cause oxidative damage to DNA, membrane lipids and proteins (Lubos et al., 2011). GSH and intracellular antioxidant enzymes, such as catalase, superoxide dismutase and GPx protect cells from these harmful effects (Wu & Batist, 2013). These antioxidants get oxidised when they act to neutralise free radicals and thus, need to be frequently restored to prevent toxicity resulting from the accumulation of free radicals (Adejuwon et al., 2014; Aslam et al., 2013). This study determined the impact of TI on these cellular defence mechanisms (Phase II DME) in human liver cells by treating Hep G2 cells with the four TI extracts in a similar way as described above. These cell homogenates were used for the assessment of GSH, catalase, GSR and GPx activities.

Catalase, GSH and superoxide dismutase are the primary defence mechanisms against ROS and they act to neutralise free radicals from the body (Adejuwon et al., 2014). This study assessed the effect of TI on the body's Phase II enzymes: catalase, GPx and GSR activities and GSH. An increase in catalase activity was recorded up to 24 h in the DMSO control cells which returned to day 0 levels as observed in the DMSO control cells or cells treated with petroleum ether or ethyl acetate extract. On the contrary, TI chloroform extract inhibited catalase activity by 56 % at 24 h while ethanol extract induced catalase activity by 43 % on the 7^{th} day as compared to the DMSO control.

Similarly, treatment of cells with TI petroleum ether extract showed 84 % increase in GSH at 4 h which returned to day 0 control levels after 4 h. However, GSH levels were increased by 20 % at day 7 for treatment with TI ethanol extract and diminished by 34 % for treatment with TI chloroform extract. Though the short- and long-term treatments were carried out on different days, similar reproducible effect on GSH or catalase activity was also measured for the short- and long-term treatment with TI extracts.

No previous data have been reported on the effects of TI on catalase or GSH in Hep G2 cells. However, in *in vivo* studies, treatment of Sprague – Dawley rats with 300 mg/kg of TI ethanol extract for 7 days was shown to significantly ($p<0.001$) increase GSH and catalase activity against potassium dichromate-induced toxicity in the kidney (Moomin et al., 2020). Moreover, TI ethanol

extract (300 – 1000 mg/kg) has also been shown to ameliorate the hepatotoxicity and nephrotoxicity in rats induced by gentamicin after 14 days as evidenced by the profound reduction in hepatic and renal injury and induced a significant ($p<0.001$) increase in activities of antioxidant enzymes such as catalase, superoxide dismutase and GSH (Ansah et al., 2016). A study by Ben-Azu et al. (2016) also showed the neuroprotective effect of TI when mice were exposed to ketamine for 14 days and treated with 125 mg/kg of TI ethanol extract for 7 days. This was shown by the increased activities of catalase and superoxide dismutase in brain tissues.

Moreover, assessment of the relationship between catalase activity and the isolated phytochemicals showed that despite the TI extracts being crude (containing several compounds), similar phytochemicals were observed to be associated with the effects on catalase activity at both short- and long-term treatment. As a result, only a single time-point (24 h) treatment was analysed to identify the possible phytochemicals responsible for the effects shown by the TI extracts. Only chloroform and ethanol extracts showed impact on catalase activity while ethyl acetate and petroleum ether extracts showed no obvious effect. The induction of catalase activity shown by ethanol extract was linked with phytochemicals such as: 1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol as these were present in ethanol extract and not detected in chloroform extract. On the contrary, the presence of 4-hydroxyphenylpropionic acid, 4-hydroxyphenylpyruvic acid and p-asinic acid in chloroform extract but not in ethanol extract was also closely associated with the inhibitory effect on catalase activity.

Furthermore, it was observed that the TI extracts only showed effect on GSH after a prolonged treatment but not in short term treatment for 24 h when compared to the DMSO control. Hence, the relationship between GSH and isolated phytochemicals was analysed for only 7 days of exposure to TI extracts. The induction of GSH shown by ethanol extract could be attributed to 1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; chlorogenic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; p-coumaric acid; phloretin; psolaren; quercetin-3-glucoside and

resveratrol because they were not detected in chloroform or petroleum ether extract. Similarly, the inhibitory effect of chloroform extract on GSH was possibly due to the presence of 4-hydroxyphenylpropionic acid, 4-hydroxyphenylpyruvic acid bergapten, morin, p-asinic acid and pinoresinol as these phytochemicals were found to be absent in ethanol or ethyl acetate extract.

In previous studies involving similar phytochemicals detected in TI and in agreement with these findings, treatment of HeLa cells with 250 μM scopoletin for 15 min has been shown to increase GSH and catalase activity by 1.5-fold as compared to the control cells (Pradhan et al., 2020). Exposure of HL60 cells to 25 μg/mL of epigallocatechin or epigallocatechin gallate for 16 h was shown to significantly ($p<0.05$) induce catalase activity (Monobe et al., 2014). Additionally, Yokomizo and Moriwaki (2006) also reported that treatment of Caco-2 cells with 50 μM of apigenin, kaempferol, luteolin or quercetin for 1 h showed a significant ($p<0.05$) increase in catalase activity. They also showed that apigenin significantly ($p<0.05$) inhibited catalase activity. Boadi et al. (2016) also administered 5 – 25 μM of genistein, kaempferol or quercetin to 3T3-L1 cells for 24 h and found that GSH levels were sustained for the flavonoid-treated samples when compared to their respective controls (Boadi et al., 2016; Dixon & Paiva, 1995). Furthermore, administration of 5 mg of N-(p-amylcinnamoyl)anthranilic acid to male Sprague-Dawley rats for 24 h significantly ($0<0.01$) increased catalase activity as compared to the control (Çakır et al., 2019). Administration of myricetin or quercetin to humans aged 18 – 82 years resulted in significantly ($p<0.05$) increasing GSH in their red blood cells (Maurya et al., 2016).

Contrary to the above earlier findings, spectrophotometric studies of the effects of epigallocatechin gallate, myricetin and other flavonoids on catalase activity have shown significant ($p<0.05$) inhibition (Krych & Gebicka, 2013). In *in vitro* studies, CaCo-2 and Hep G2 cells were treated with 50 – 200 μM of myricetin, quercetin or rutin for 24 h followed by treatment with 50 μM H_2O_2 for 30 min and the catalase activity was not significantly affected by the flavonoids (Aherne & O'brien, 1999).

Furthermore, the body also employs various antioxidant enzymes as its defence against peroxide species and therefore, their activities are important for the

intracellular neutralisation of cell-damaging peroxides (Duthie et al., 2000). GPx catalyses the elimination of hydroperoxide in the presence of GSH and is found widely in tissues such as the liver and blood (Kamata et al., 1994). On the other hand, GSR catalyse the reduction of GSH after it has been used by GPx to reduce free radicals (Brigelius-Flohé & Kipp, 2009). Physiologically, GPx is essential for the reduction of peroxides *in vivo* (Brigelius-Flohé & Kipp, 2009; Kamata et al., 1994). Hydroperoxides are shown to induce cell proliferation, migration, invasion and angiogenesis, but at higher levels promote apoptosis and thus, can act as both pro- and anti-carcinogen (Brigelius-Flohé & Kipp, 2009). It is therefore important to assess the activities of these enzymes in cancer drug discovery.

In this study, GSR activity in liver cells was increased after 24 h - 7 days treatment with TI ethanol, ethyl acetate or petroleum ether extracts by 86, 63 and 30 % respectively as compared with the control. Conversely, chloroform extract decreased GSR activity by 65 % in the liver cells when compared to the control. This study also showed that exposure of the liver cells to TI ethanol extract induced GPx activity by 47 % at 7 days while TI chloroform or ethyl acetate extract inhibited GPx activity by 46 and 23 % respectively when compared to the control. The induction of these enzymes by the TI extracts may increase the ability of the liver to resist damage due to oxidative stress.

Several studies have also shown the effect of various NPs on cellular redox homeostasis. For instance, administering 10 μM of Honokiol (a bioactive compound from *Magnolia officinalis*) to human kidney-2 (HK-2) cells for 24 h has been shown to significantly ($p<0.01$) increase the levels of GSH as compared to the control cells (Park et al., 2020). Malik et al. (2019) also treated Hep G2 cells with Livotrit (an Indian polyherbal preparation) for 16 h and found that lower concentration of 0.05% Livotrit significantly ($p<0.05$) induced the gene expression and activities of catalase, GPx and GSR, while higher concentration of 0.5% Livotrit showed no significant effect on catalase, GPx or GSR activities as compared to the respective controls with values of 1311.27 ± 101 units/mg protein, 6205.51 ± 117 units/mg protein and 15781.96 ± 795 units/mg protein respectively. The activities of antioxidant enzymes such as catalase, GPx and superoxide dismutase were significantly ($p<0.05$) induced in Hep G2 cells after exposure to 25 – 50 μg/ml of fermented black ginseng extract for 24 h (Bak et al., 2014).

In addition, the present study also investigated the relationship between GSR or GPx and isolated phytochemicals from TI extracts and showed that both enzymes were induced by ethanol extract which could be due to the presence of 1,2,3-trihydroxybenzene; anthranilic acid; caffeic acid; chlorogenic acid; epicatechin; epigallocatechin; epigallocatechin gallate; gallocatechin; luteolin; myricetin; neohesperidin; p-coumaric acid; phloretin; protocatachaldehyde; psolaren; quercetin-3-glucoside and resveratrol in the TI ethanol extract but not in chloroform extract. Moreover, inhibition of both GSR and GPx activities were linked to 4-hydroxyphenylpropionic acid, morin and pinoresinol which were present in chloroform extract but not in ethanol, ethyl acetate or petroleum ether extract.

In agreement to these findings, Boadi et al. (2016) administered 5 – 25 μM of genistein, kaempferol or quercetin (phytochemicals found in TI) to 3T3-L1 cells for 24 h and found that GSR and GPx enzyme activities remained unchanged from 0–10 μM but significantly ($p<0.01$) increased from 15–25 μM of the different flavonoids when compared to their respective. Nagata et al. (1999) also showed that administering 20 μM of catechin or quercetin (bioactive compounds found in TI) to cultured rat hepatocytes (BL-9) significantly ($p<0.01$) enhanced the activity of GPx activity and showed no significant changes to GSR activity with the flavonoid treatments as compared to the respective controls. Moreover, pre-treatment of Hep G2 cells with 50 – 100 μM of epicatechin for 20 h has been shown to significantly ($p<0.05$) increase the GSH and the activities of both GSR and GPx to act against oxidative stress caused by tert-butylhydroperoxide (Martín et al., 2010).

However, Iio et al. (1993) spectrophotometrically measured the effect of epicatechin gallate, epigallocatechin gallate kaempferol, myricetin or quercetin on GSR activity and found that the flavonoids inhibited GSR. In the present study, phenols (particularly hydroxytyrosol) were observed to show no effect on GSH, GSR and GPx activities in Hep G2 cells. In disagreement to these findings, treatment of Hep G2 cells with 10 – 40 μM of hydroxytyrosol for 2 or 20 h significantly ($p<0.05$) increased GSH and GPx activity (Goya et al., 2007).

In summary, different extracts of TI have shown differential activity on DMEs activity with TI chloroform extract inhibiting the activity of DMEs whereas ethanol

extract induced the activities. The difference in effects on both Phase I and II DMEs shown by the four TI extracts is a consequence of the different phytochemical composition as earlier shown in the PCA and the major phytochemical profiles of the TI extracts. The presence of 4-hydroxyphenylpropionic acid, bergapten, morin, p-hydroxybenzaldehyde and pinoresinol exclusively in chloroform extract but not in ethanol, ethyl acetate or petroleum ether extract could possibly be the contributing phytochemicals responsible for the inhibitory effects of chloroform extract on DME. On the other hand, anthranilic acid; caffeic acid; epicatechin; epigallocatechin; gallocatechin, luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol which were present in only ethanol or ethyl acetate extract but not in chloroform or petroleum ether extract could be responsible for the induction of DME by TI ethanol extract. Due to the low toxicity in human liver and ability to induce antioxidant enzymes, low concentrations of TI extract are well tolerated by liver cells and could be used for cyto- and genotoxicity linked with the damaging effects of free radicals while higher concentration of TI extract could be used for liver cell toxicity studies. Therefore, the activity shown by TI extract may be as a consequence of its ability to induce antioxidants in the system and its potential use as an anticancer agent.

3.5 CONCLUSIONS

TI extracts were equally cyto- and genotoxic to normal human liver cells at high concentrations and well tolerated at lower concentrations. TI also showed differential time-dependent effect on DMEs which may impact on subsequent metabolism in hepatic cells and different phytochemicals account for the different effects shown by the TI extracts.

CHAPTER FOUR

THERAPEUTIC EFFICACY OF TI IN COLON CANCER

4.1 INTRODUCTION

Cancer is one of the leading causes of death worldwide and accounts for 8.2 million death/year and is increasing gradually (Prakash et al., 2013). The survival rate of cancer patients is closely related to the stage of cancer at diagnosis (Ludvigsen et al., 2020). Early diagnosis and various medical treatments such as chemotherapy and surgery can be used to reduce the fatality of cancer (Lindhorst & Hummon, 2020). Several chemotherapy medications available on the market such as 5-fluorouracil, anthracycline and methotrexate have been used for the treatment of various cancers such as breast, colon, leukaemia, lung and stomach cancers, but no drug has been found to be entirely effective and safe (Basmadjian et al., 2014; Prakash et al., 2013). The toxicity of drugs is the major problem with established cancer therapy. For example, synthetic drugs and radiotherapy are associated strongly with serious side effects such as high risk of bleeding, blood clots, headache, hypertension, mouth, secondary cancers, skin inflammation and gut perforations (Du & Tang, 2014; Prakash et al., 2013). Cancer cells also rapidly develop resistance to therapeutic drugs (Du & Tang, 2014). Although a lot of research has been carried out to advance the control and treatment of cancer, significant work and need for improvement remain in the search for safer alternatives (Prakash et al., 2013).

NPs have been studied extensively for cost-effective and safer alternative treatments for cancer. These products have exhibited anticancer properties by interfering with the initiation, development and progression of cancer and by modulating various developmental mechanisms including apoptosis, cellular proliferation, angiogenesis and metastasis (Rajesh et al., 2015).

CRC is the third-most prevalent, and second-most deadly, cancer worldwide with 2.2 million newly diagnosed cases and 940 thousand deaths in 2020 (World Health Organization, 2020). In this study, the use of TI as a potential anticancer agent was determined in human colon cells by adopting CaCo-2 and NCM460 cells as models for cancerous and normal colon cells respectively.

NCM460 cells are human colon epithelial cells which were originally isolated from the normal colonic mucosa of a 68-year-old Hispanic male (Moyer et al., 1996). Primary normal colon mucosal epithelial cells have a relatively short lifespan of only a few days but NCM460 cells have become immortalised as they have a

mutant TP53 gene (Duthie et al., 2008; Moyer et al., 1996). However, the NCM460 cells are not malignantly transformed, and retained many normal mucosal colonocytes characteristics including expression of epithelial cell antigens including cytokeratins and villin, thus, provide a perfect model for normal colonic epithelium (Moyer et al., 1996). The NCM460 cell line has been used previously to investigate the absorption and metabolism of folate and other B vitamins, and to investigate how protective phytochemical compounds in the diet act differentially against normal and cancerous colon cells *in vitro* (Duthie et al., 2008; Kumar et al., 1997). The NCM460 cells have also been used as normal colon cells in studies using SW480 to identify possible diagnostic proteomic biomarkers for colorectal cancer and protein markers such as calumenin, protein SET, reticulocalbin and S100A6 were found to show an increased expression in the colon cancer cells compared with the normal cells (Ludvigsen et al., 2020). The NCM460 has also been used to study the molecular kinetics of enzymes such as protein-tyrosine kinase and the transport of vitamins such as riboflavin (Kumar et al., 1997; Said et al., 2000).

On the other hand, CaCo-2 cells are an established model for human colon cancer cells, and they were isolated from colon carcinoma of a 72-year-old Caucasian (Pinto et al., 1983). When CaCo-2 cells are cultured under certain conditions, they become differentiated and polarised such that their function and morphology resemble the enterocyte lining of the small intestine (Lea, 2015). They express functional microvilli, tight junctions, digestive enzymes and transporters (such as esterases, peptidases and P-glycoprotein) and retain the ability to transport ions, proteins and vitamins (Baker & Baker, 1993; Duthie et al., 1997; Lea, 2015). The CaCo-2 cell line has been used for a range of applications among the following: to study the effects of microbiota or their metabolites on the barrier function of the intestinal epithelium; to explain pathways involved in the transport of drugs or food components across the intestinal epithelium; in studying potential toxicity of drugs or food metabolites in the intestinal mucosa (Knipp et al., 1997; Shimizu, 2010; Tang et al., 1993).

In this study, the impact of TI on biomarkers of DNA stability in normal and cancer human colon cells was determined as cell viability, proliferation and genomic stability and a non-toxic dose of TI was used to determine the impact of TI on DNA SB repair (SBR) and colon cell migration.

4.1.2 Aim:

To investigate the therapeutic impact of TI on human colon cells *in vitro* against biomarkers of cancer.

4.1.3 Specific objectives:

- To determine the effect of TI on CaCo-2 and NCM460 cell viability and growth.
- To assess the effect of TI on CaCo-2 and NCM460 cells DNA single strand breakages (SSB).
- To assess the effect of TI on CaCo-2 and NCM460 cells DNA SBR.
- To assess the effect of TI on CaCo-2 cell migration.

4.2 METHODS

In this study, sample TI extracts used from each solvent (chloroform, ethanol, ethyl acetate or petroleum ether) were obtained as described previously (section 2.2.1.3).

4.2.1 Maintenance and routine culture of human colon cells *in vitro*

CaCo-2 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC No. 86010202, lot 17H003, passage 8) and grown as monolayer or multilayer culture in low glucose (1 g/L) DMEM supplemented with 20 % (v/v) FBS, 1 % (v/v) NEAAs and 1 % (v/v) penicillin (100 U/ml): streptomycin (100 µg/ml). The routine culture of CaCo-2 cells was carried out as described by Duthie and Collins (1997).

NCM460 cells were obtained from INCELL (San Antonio, TX, passage 3-10) under a Material Transfer Agreement and were grown as monolayer culture in high glucose (4.5 g/L) DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) NEAAs and 1 % (v/v) penicillin (100 U/ml): streptomycin (100 µg/ml). The routine culture of NCM460 cells was carried out as described by Duthie et al. (2008). The culture medium was changed on both cell lines every 3 – 4 days, and the cell stocks were sub-cultured into 75 cm^2 flasks at a ratio of 1:3 and incubated at 37 °C in a humidified atmosphere of 5% CO_2 / 95 % air (Duthie & Collins, 1997; Duthie et al., 2008).

4.2.2 Impact of TI on cell viability and cell growth in human colon cells *in vitro*.

The impact of TI on cell viability in CaCo-2 or NCM460 cells was measured using the MTT assay as described previously (section 2.2.3.5) and cell growth was determined by cell count as described previously (section 2.2.3.6) using TI extract (0 – 5.0 mg/ml) for 24 h.

4.2.3 Impact of TI on DNA single strand breakage in human colon cells *in vitro.*

The impact of TI on DNA SSB in CaCo-2 or NCM460 cells was measured using the SCGE assay as described previously (section 3.2.1.2) using TI extract (0 – 5.0 mg/ml) for 24 h.

4.2.4 Impact of TI on DNA strand break repair (SBR) in colon cells *in vitro*.

4.2.4.1 Establishing a dose-response for H_2O_2-induced DNA SSB in human colon cells.

CaCo-2 or NCM460 cells were grown in T25 cm^2 flasks at a density of $5x10^5$ cells/flask for 7 days at 37 °C in a humidified atmosphere of 5% CO_2 / 95 % air. Cells were harvested with 1 ml of trypsin-EDTA and counted using a haemocytometer. To induce DNA damage, cells at a density of $5x10^4$ cells/ml were incubated with H_2O_2 (0 – 100 µM) in Eppendorf tubes for 5 min on ice. Cells were then used to assess DNA SSB as described earlier (section 3.2.1.2).

4.2.4.2 Impact of TI on DNA SBR in colon cells.

The impact of TI on DNA SBR of H_2O_2–induced DNA damage was measured using the method of Duthie and Collins (1997). CaCo-2 or NCM460 cells were pipetted into 24-well plates at a density of $7.4x10^5$ cells/well and incubated at 37 °C for 24 h. Based on the data obtained from the dose-response with TI to get a concentration of 0.25 mg/ml (section 4.2.3), cells were then incubated with TI extract (final concentration of 0.25 mg/ml in medium) at 37 °C for 24 h. DMSO (0.5 %) was used as control. Culture medium was removed from the flasks which were then washed twice with 2 ml PBS before exposure of cells to 30 µM H_2O_2 for 5 min on ice. H_2O_2 was pipetted off the cells and any residual H_2O_2 was removed by washing the cells twice with 2 ml PBS. Cells were then incubated with serum-free medium at 37 °C in a humidified atmosphere of 5% CO_2 / 95 % air. Cells were harvested with 0.2 ml trypsin-EDTA after 0, 30 min and 60 min, and DNA SBR capacity analysed by SCGE (section 3.2.1.2).

4.2.5 Impact of TI on human colon cancer cell migration *in vitro*.

4.2.5.1 Establishing a dose-response for the effect of hydroxyurea on CaCo-2 cell viability and growth.

A stock solution of 10 mM hydroxyurea (HU) was made in complete medium with serial concentrations of HU (0 – 10 Mm) prepared subsequently. The procedures for assessing the impact of HU on CaCo-2 cell viability and growth were as described previously (sections 2.2.3.5 and 2.2.3.6 respectively).

4.2.5.2 Optimising seeding density and incubation time for CaCo-2 cell migration.

The cell seeding density and incubation time were optimised for determining cell migration in CaCo-2 cells by modifying the method of Gadad et al. (2013). CaCo-2 cells were grown for 7 days to 70 – 90 % confluency in low glucose (1 g/L) DMEM supplemented with 20 % (v/v) FBS, 1 % (v/v) NEAAs and 1 % (v/v) penicillin (100 U/ml): streptomycin (100 µg/ml). Cells were harvested with 1 ml of trypsin-EDTA and counted using a haemocytometer. A 6-well plate (Nunc, Denmark) was marked with a dot on the bottom of each well using a marker pen to serve as a reference point during image analysis. Sterile cloning rings (6x8 mm, Fisher Scientific, UK) were placed centrally within each well of the plate. Cells (40 µl) were pipetted into the cloning rings at a density of $1x10^2$ – $4x10^5$ cells/ring and incubated at 37 °C for 4 – 24 h. The cloning rings were removed at 4, 6, 8 or 24 h and 2 ml fresh medium was added to the wells. Images of cells were acquired digitally using a Leica DMI 4000B microscope (Leitz Wetzlar, Germany) at a magnification of x2.5 to observe cells for attachment and formation of monolayers (Gadad et al., 2013).

4.2.5.3 Impact of TI on CaCo-2 cell migration

The impact of TI on CaCo-2 cell migration was measured using the modified method of Gadad et al. (2013) (Figure 4.1). CaCo-2 cells were grown for 7 days to 70 – 90 % confluency. Cells were harvested with 1 ml of trypsin-EDTA and counted using a haemocytometer. Cells (40 µl) at a density of $4x10^5$ cells/ring were pipetted into 6-well plates with cloning rings (as prepared above) and incubated at 37 °C for 4 – 6 h. The cloning rings were removed, and 2 ml medium was added to the cells. Images of cells were acquired at the start of each experiment to observe for cell attachment and formation of monolayers (as above) and the cells were incubated at 37 °C for a further 24 h. TI (petroleum ether, chloroform, ethyl acetate or ethanol extracts) and HU were added together to cells at a final concentration of 0.25 mg/ml and 5 mM respectively. DMSO (0.5 %) and HU (5 mM) were used as controls. Images of cells were acquired after the addition of TI extracts at day 0, 1, 2 or 7 days of treatment (Gadad et al., 2013).

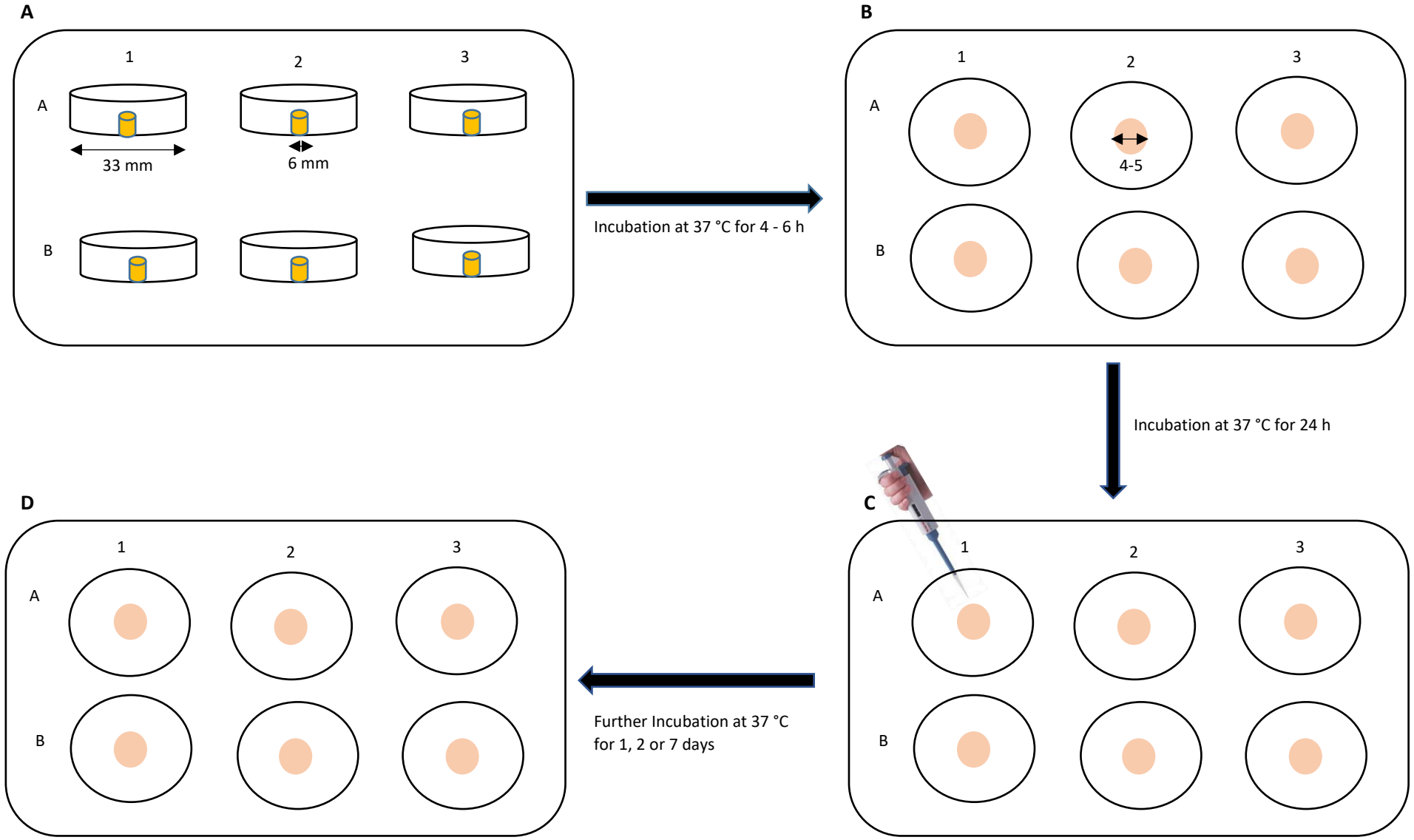


Figure 4.1: Formation of circular monolayers of CaCo-2 cells and the acquisition of cellular images for migration.
(A) Cloning rings placed centrally in wells, cells pipetted into rings and incubated at 37 °C for 4-6 h, (B) Aerial view of cells after removal of cloning rings and addition of 2 ml of medium for a further incubation at 37 °C for 24 h, (C) Addition of DMSO, or HU or TI extract and HU, acquisition of images at Day 0 of experiment and a further incubation at 37 °C for 1, 2 or 7 days and (D) Acquisition of cellular images after 1, 2 or 7 days of treatment with DMSO and HU or TI extract and HU.

4.2.5.4 Image Analysis for the effect of TI on CaCo-2 cell migration

The acquired photomicrographs were analysed for cell migration using an image processing and analysis software (ImageJ software version 1.51r). Briefly, a 90 ° angle was generated by the software on the image acquired at day 0 and ten radii were manually drawn from a central reference mark on the image of cells (Figure 4.1). The same procedure was repeated for the same well after incubation for 1, 2 or 7 d. The net migration was calculated using the difference between the average radii at 1, 2 or 7 days, and the average radii 0 d and the data were presented as mean ± SEM (Gadad et al., 2013).

Net migration = r2 – r1, where r1 is the average of radii of circular image (green outline) at day 0 and r2 is the average of radii of image (orange outline) after treatment with control or extract for 1, 2 or 7 days (Figure 4.2).

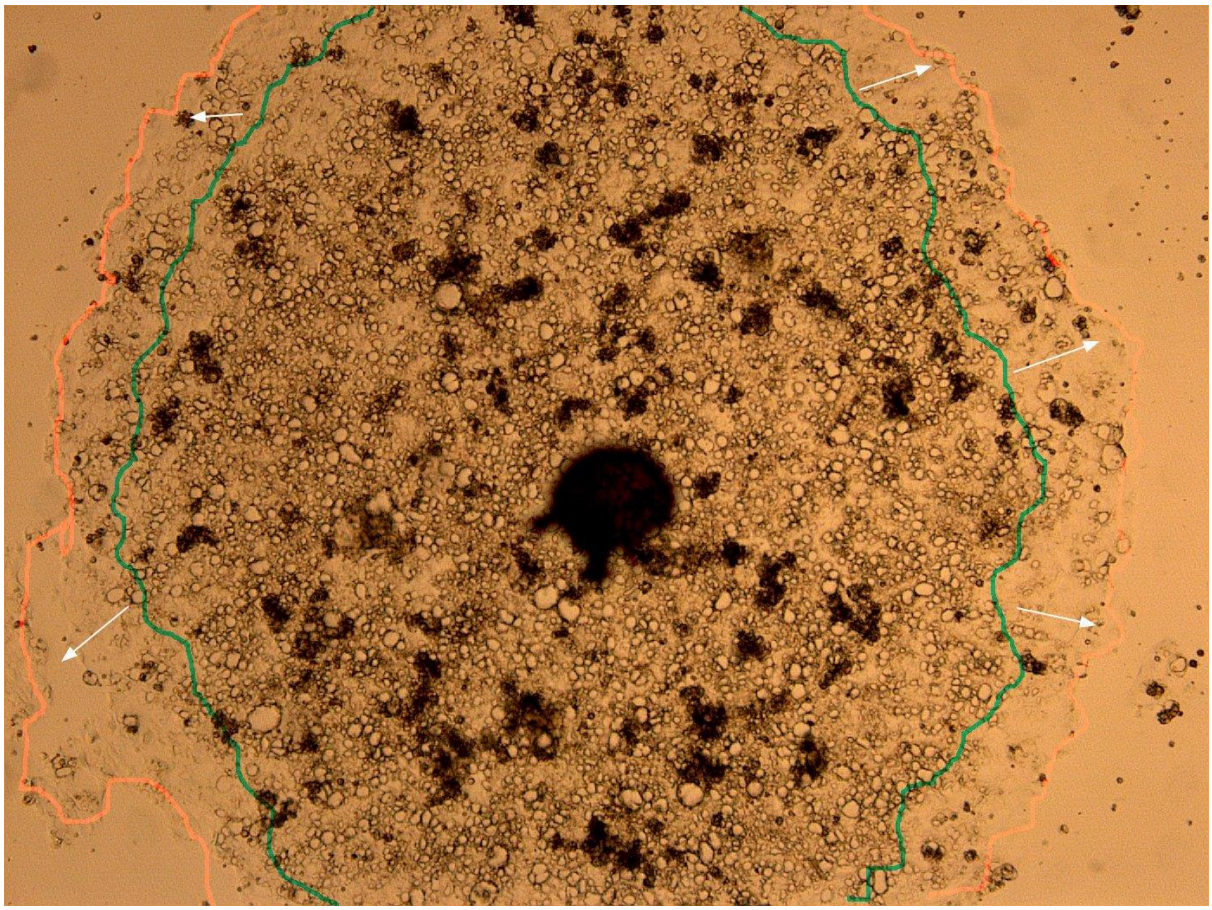


Figure 4.2: Radial migration after treatment of CaCo-2 cells with DMSO and HU or TI extract and HU. The green outline is the image at day 0, white arrows showing the direction of cell migration and the red outline is the image after treatment for 1, 2 or 7 days.

4.2.6 Effect of TI phytochemical metabolites on colon cell DNA SBR and CaCo-2 cell migration

PLS-DA was used to determine the relationship between phytochemicals and their effect on colon cell DNA SBR or CaCo-2 cell migration as described previously (section 3.2.2.4).

4.3 RESULTS

4.3.1 Impact of TI on cell viability in human colon cells *in vitro.*

Cells were treated with varying concentrations (0 – 5 mg/ml) for 24 h and cell viability was determined by MTT assay. As the concentration of all four TI extracts increased, there was a corresponding decrease in the viability of both CaCo-2 and NCM460 cells. TI extracts showed no significant toxicity at concentrations ≤0.5 mg/ml in NCM460 cells when compared to DMSO control. Treatment with chloroform or ethyl acetate extracts at a concentration of 0.5 mg/ml significantly decreased ($p<0.05$) viability only in CaCo-2 cells while ethanol extract at 0.5 mg/ml significantly ($p<0.01$) decreased viability in only NCM460 cells. Chloroform extract showed the greatest toxic effect on viability in both colon cells followed by petroleum ether extract, and ethyl acetate and ethanol extracts showed the least toxicity on viability (Figure 4.3).

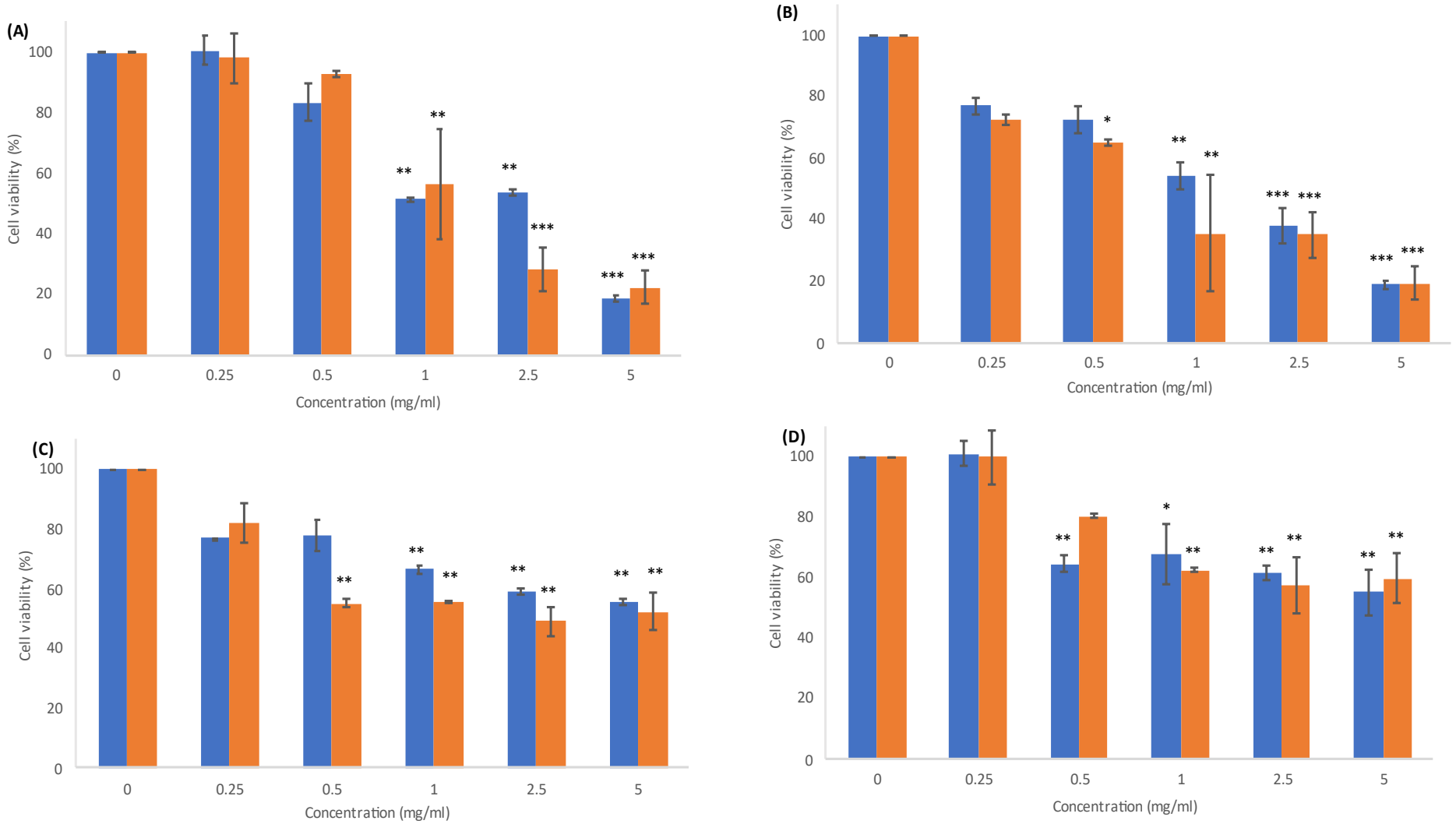


Figure 4.3: Effect of TI extract on CaCo-2 or NCM460 cell viability after 24 h treatment. (A) petroleum ether extract; (B) chloroform extract; (C) ethyl acetate extract and (D) ethanol extract. Data are presented as mean ± SEM; blue bars are NCM460 and orange bars are CaCo-2 cells; n=3; *P<0.05; **p<0.01 and ***p<0.001 as compared to the appropriate DMSO control by ANOVA followed by Bonferroni's multiple comparison test.

ANOVA was used to determine whether TI extract was more or less toxic to CaCo-2 (cancer) or NCM460 cells (normal). Ethyl acetate extract at 0.5 mg/ml or petroleum ether extracts at 2.5 mg/ml significantly ($p<0.05$) decreased CaCo-2 cell viability when compared with NCM460 cells. Ethanol extract at 0.5 mg/ml significantly ($p<0.05$) reduced the viability of NCM460 cells as compared to CaCo-2 cells (Table 4.1).

Table 4.1: Statistical comparison of viability of CaCo-2 versus NCM460 cells after treatment with TI extracts.

	CaCo-2 vs NCM460 cell viability					
TI Extract (mg/ml)	0	0.25	0.5	1.0	2.5	5.0
Petroleum ether	ns	ns	ns	ns	0.021	ns
Chloroform	ns	ns	ns	ns	ns	ns
Ethyl acetate	ns	ns	0.013	ns	ns	ns
Ethanol	ns	ns	0.032	ns	ns	ns

ns indicates not significant ($p>0.05$), n=3, by One-way ANOVA followed by Bonferroni's multiple comparison test.

TI concentrations of 1.69 – 7.66 mg/ml were required to reduce the viability of colon cells by 50 % (IC_{50}). CaCo-2 cells were more susceptible to TI extract at lower concentrations while NCM460 cells were unaffected. Pre-treatment of cells with petroleum ether or chloroform extracts showed higher toxicity (IC_{50} = 1.69 – 2.54 mg/ml) in both colon cells when compared with ethyl acetate or ethanol extracts (IC_{50} = 3.05 – 7.66 mg/ml). Treatment with TI ethanol or ethyl acetate extract showed approximately 2-fold toxicity in CaCo-2 cells as compared to NCM460 cells (Table 4.2).

Table 4.2: Comparison of IC_{50} (mg/ml) for TI extract on CaCo-2 and NCM460 cell viability.

	IC_{50} (mg/ml)	
Extract	NCM460	CaCo-2
Petroleum ether	2.54	1.96
Chloroform	2.21	1.69
Ethyl acetate	7.66	3.05
Ethanol	6.74	3.99

4.3.2 Impact of TI on cell growth in human colon cells *in vitro*.

Cells were treated with varying concentrations (0 – 5 mg/ml) for 24 h and cell growth was determined by cell count. TI at increasing concentrations inhibited human colon cell growth. All four TI extracts significantly ($p<0.001$) inhibited cell growth at concentrations ≥2.5 mg/ml when compared to DMSO control. Treatment with lower concentrations (0.5 mg/ml) of petroleum ether significantly ($p<0.05$) inhibited growth in both NCM460 and CaCo-2 cells. Conversely, treatment with chloroform or ethyl acetate extracts at a concentration of 0.5 mg/ml significantly decreased ($p<0.05$) growth only in NCM460 cells while ethanol extract at 1 mg/ml significantly ($p<0.01$) decreased growth only in CaCo-2 cells (Figure 4.4).

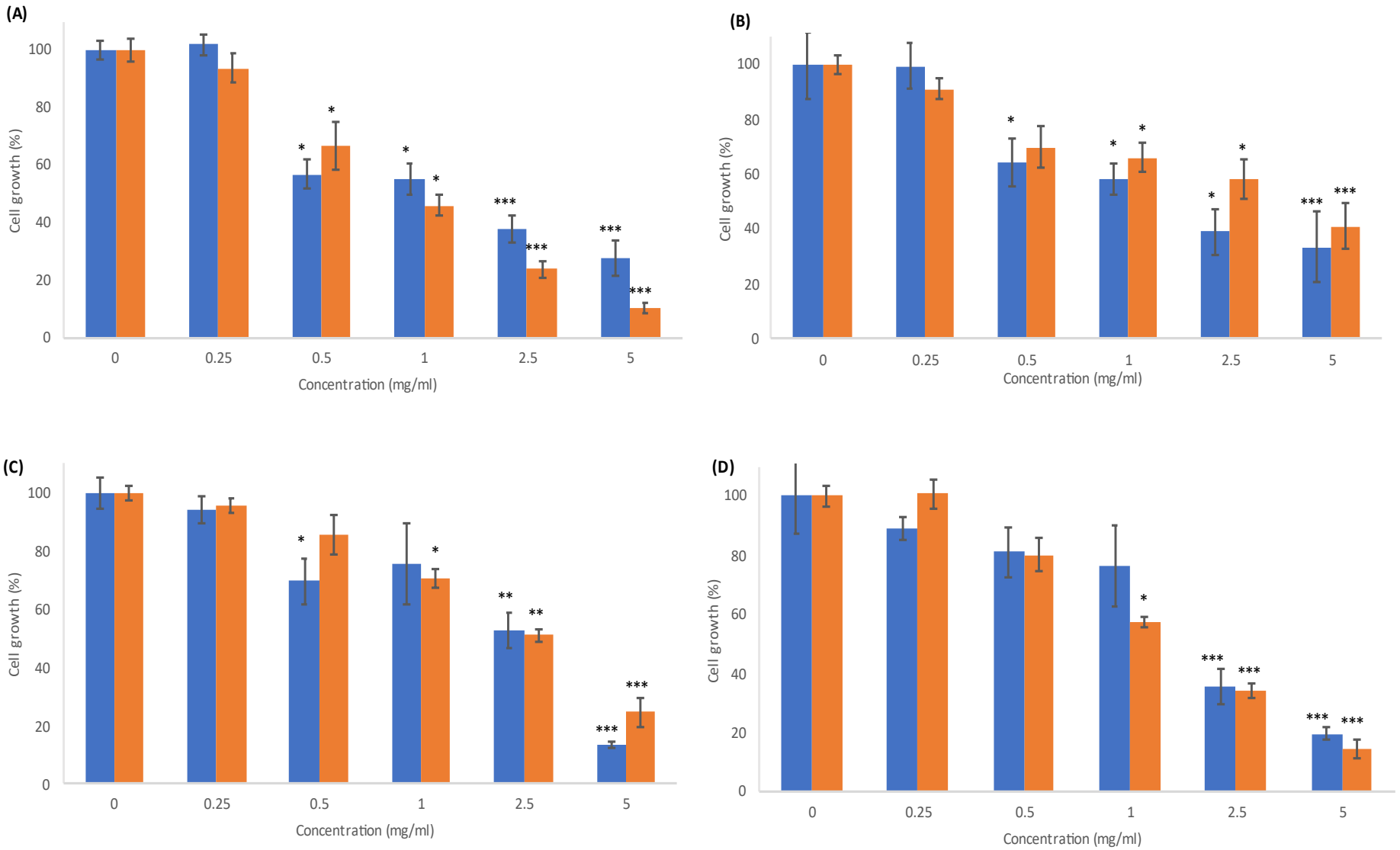


Figure 4.4: Effect of TI extract on CaCo-2 or NCM460 cell growth after 24 h treatment. (A) petroleum ether extract; (B) chloroform extract; (C) ethyl acetate extract and (D) ethanol extract. Data are presented as mean ± SEM; blue bars are NCM460, and orange bars are CaCo-2 cells; n=3; *P<0.05; **p<0.01 and ***p<0.001 as compared to the appropriate DMSO control by ANOVA followed by Bonferroni's multiple comparison test.

ANOVA was used to investigate whether TI extract was more or less toxic in CaCo-2 (cancer) or NCM460 cells (normal). Petroleum ether extract at 5 mg/ml or ethanol extract at 1 mg/ml significantly ($p<0.05$) decreased CaCo-2 cell growth when compared with NCM460 cells. Conversely, treatment with chloroform extract at 2.5 mg/ml significantly ($p<0.05$) decreased NCM460 cell growth when compared with CaCo-2 cells (Table 4.3).

Table 4.3: Statistical comparison of growth of CaCo-2 versus NCM460 cells after treatment with TI extracts.

	CaCo-2 vs NCM460 growth viability					
TI Extract (mg/ml)	0	0.25	0.5	1.0	2.5	5.0
Petroleum ether	ns	ns	ns	ns	ns	0.031
Chloroform	ns	ns	ns	ns	0.032	ns
Ethyl acetate	ns	ns	ns	ns	ns	ns
Ethanol	ns	ns	ns	0.028	ns	ns

ns indicates not significant ($p>0.05$), n=3, by ANOVA followed by Bonferroni's multiple comparison test.

A higher concentration (IC_{50} = 3.65 mg/ml) of chloroform extract was required to reduce the growth of CaCo-2 cells by 50 % (IC_{50}) whereas a lower concentration (IC_{50} = 1.95 mg/ml) of petroleum ether extract was required to reduce the growth of CaCo-2 cells by 50 %. CaCo-2 cells were more susceptible to petroleum ether extract at lower concentrations while NCM460 cells were unaffected at these concentrations. Ethyl acetate and ethanol extracts affected both cell lines equally (Table 4.4).

Table 4.4: Comparison of IC_{50} for TI extract on CaCo-2 and NCM460 cells growth.

	IC_{50} (mg/ml)	
Extract	NCM460	CaCo-2
Petroleum ether	2.53	1.94
Chloroform	2.82	3.65
Ethyl acetate	2.63	2.97
Ethanol	2.60	2.38

4.3.3 Impact of TI on DNA strand breakage in colon cells *in vitro*.

TI induced DNA SSB in both NCM460 and CaCo-2 cells after exposure to increasing concentrations of TI extract. All four TI extracts significantly ($p<0.001$) increased DNA SSB in colon cells at concentrations ≥0.5 mg/ml when compared to control. However, treatment with petroleum ether at a lower concentration of 0.25 mg/ml significantly ($p<0.05$) induced DNA SSBs only in CaCo-2 cells (Figure 4.5).

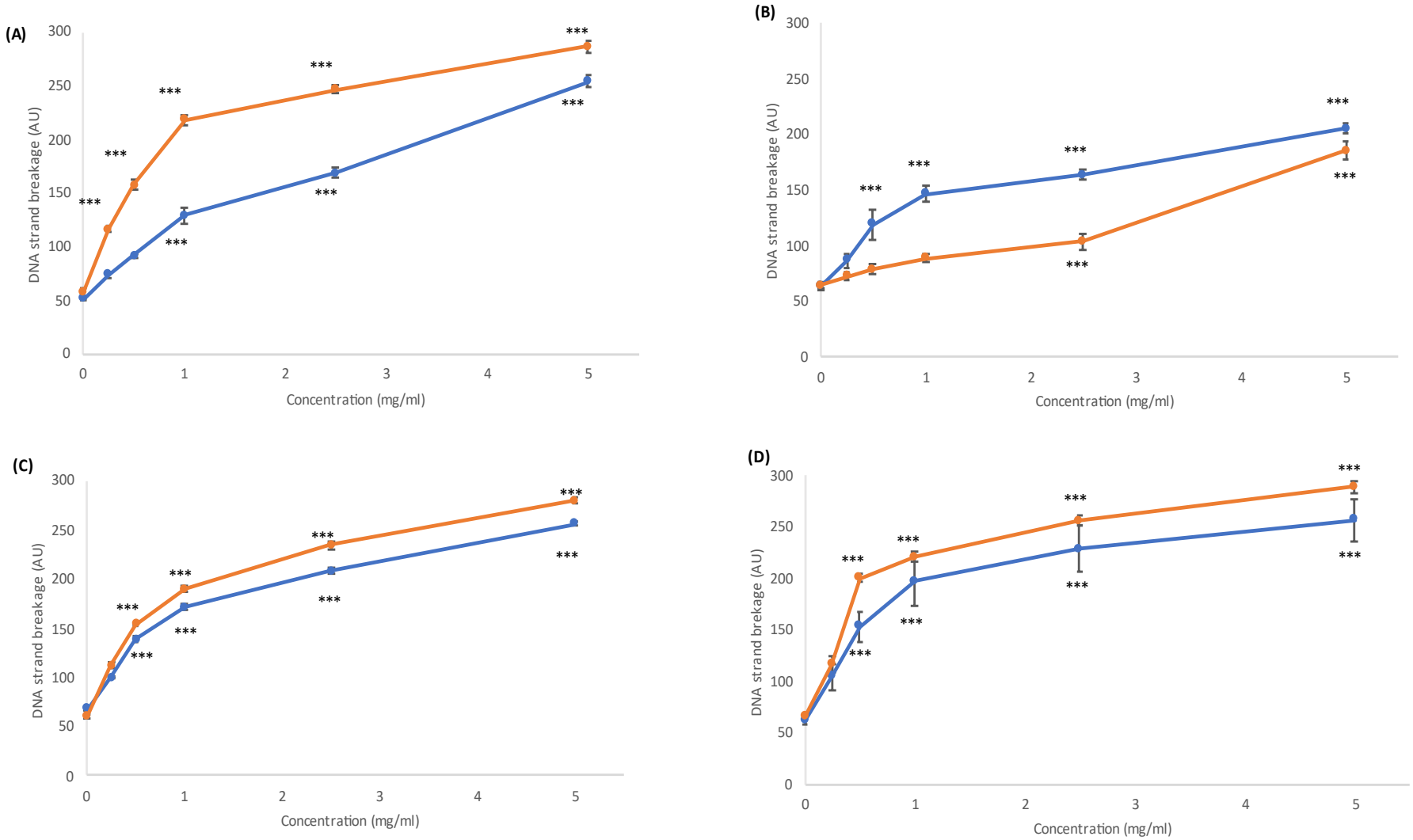


Figure 4.5**:** Effect of TI extract on CaCo-2 or NCM460 DNA strand breakage after 24 h treatment.

(A) petroleum ether extract; (B) chloroform extract; (C) ethyl acetate extract and (D) ethanol extract. Data are presented as mean ± SEM, blue lines are NCM460, and orange lines are CaCo-2 cells; n=3; ***p<0.001 as compared to DMSO control by One-way ANOVA followed by Bonferroni's multiple comparison test.

ANOVA was used to establish the difference in genomic stability in the 2 colon cell lines. DNA SSB was significantly ($p<0.001$) higher in CaCo-2 cells than in NCM460 cells when the cells were pre-treated with petroleum ether or ethyl acetate extracts. Conversely, treatment with chloroform extract caused a significantly ($p<0.001$) higher DNA SSB in NCM460 cells than in CaCo-2 cells. Ethanol extract affected DNA SSBs in both colon cell types similarly (Table 4.5).

Table 4.5: Statistical comparison of DNA SSB in CaCo-2 versus NCM460 cells after treatment with TI extracts.

	CaCo-2 vs NCM460 DNA SSBs					
TI Extract (mg/ml)	0	0.25	0.5	1.0	2.5	5.0
Petroleum ether	ns	<0.001	<0.001	<0.001	<0.001	<0.001
Chloroform	ns	ns	0.001	<0.001	<0.001	
Ethyl acetate	ns	ns	ns	0.001	<0.001	<0.001
Ethanol	ns	ns	ns	ns	ns	ns

ns indicates not significant ($p>0.05$), n=3, by ANOVA followed by Bonferroni's multiple comparison test.

Ethanol extract induced the highest genotoxicity in both CaCo-2 (LC_{50} = 0.39 mg/ml) and NCM460 cells (LC_{50} = 0.26 mg/ml). Conversely, petroleum ether extract (LC_{50} = 1.51 mg/ml) was the least genotoxic to the NCM460 cells while chloroform extract (LC_{50} = 1.27 mg/ml) was least genotoxic to CaCo-2 cells (Table 4.6).

Table 4.6: Comparison of LC_{50} for TI extracts on CaCo-2 and NCM460 cells DNA strand breakage.

	LC_{50} (mg/ml)	
Extract	NCM460	CaCo-2
Petroleum ether	1.51	0.58
Chloroform	0.40	1.27
Ethyl acetate	0.81	0.70
Ethanol	0.39	0.26

4.3.4 Impact of TI on DNA strand break repair (SBR) capacity in colon cells *in vitro.*

4.3.4.1 Establishing a dose-response for H_2O_2-induced DNA SSB in human colon cells.

Treatment of both colon cell types with increasing concentration of H_2O_2 caused a dose-dependent increase in DNA SSB. No difference was observed in DNA SSBs between NCM460 and CaCo-2 cells after treatment with H_2O_2 (Figure 4.6). A concentration of 30 µM was used to investigate the impact of TI on DNA SBR in both colon cell lines because at this concentration, both repair or further DNA SSB could be monitored.

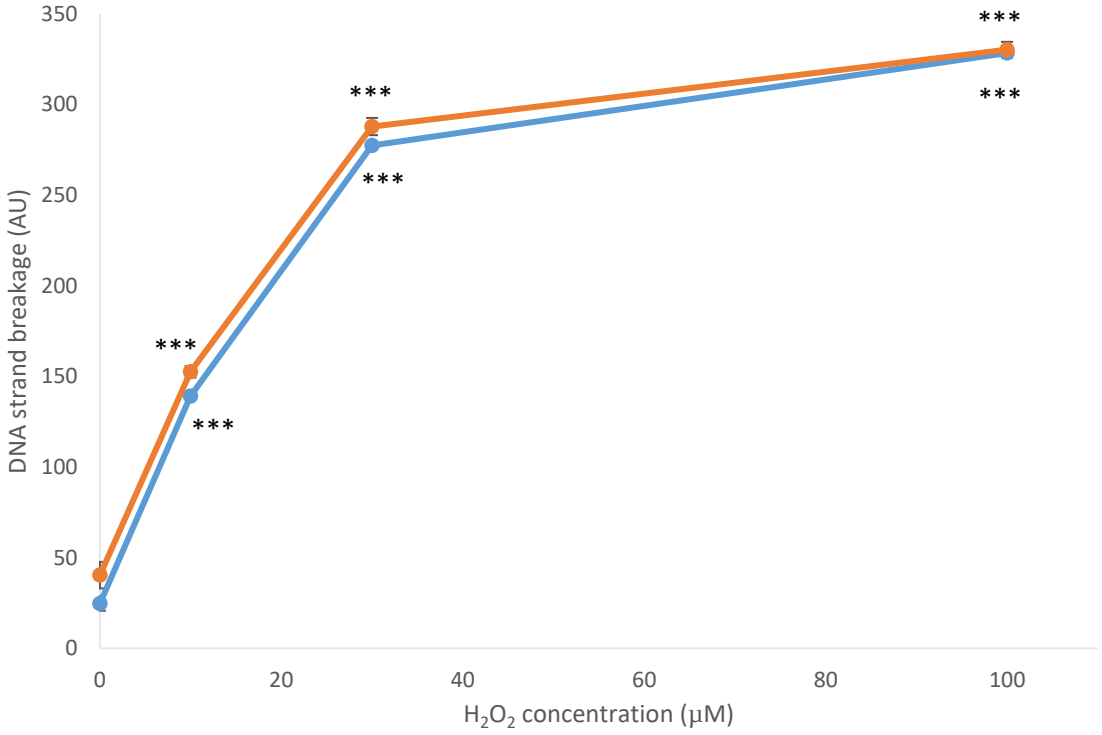


Figure 4.6: Effect of H_2O_2 on NCM460 or CaCo-2 cell DNA strand breakage after 5 min. Data are presented as mean ± SEM; blue line are NCM460; and orange line are CaCo-2 cells; n=3; ***p<0.001 as compared to control (DMSO) by ANOVA followed by Bonferroni's multiple comparison test.

4.3.4.2 Impact of TI on DNA SBR in human colon cells.

As expected, there was a significant increase in DNA SSB in control cells after exposure to H_2O_2. This was followed by a time-dependent decrease in DNA SSB after 30 and 60 min in both colon cell lines. NCM460 cells showed a higher DNA SBR in untreated cells (control) as compared to CaCo-2 cells.
A higher DNA SBR was observed in NCM460 cells treated with ethyl acetate extract as compared to the DMSO control (Figure 4.7).

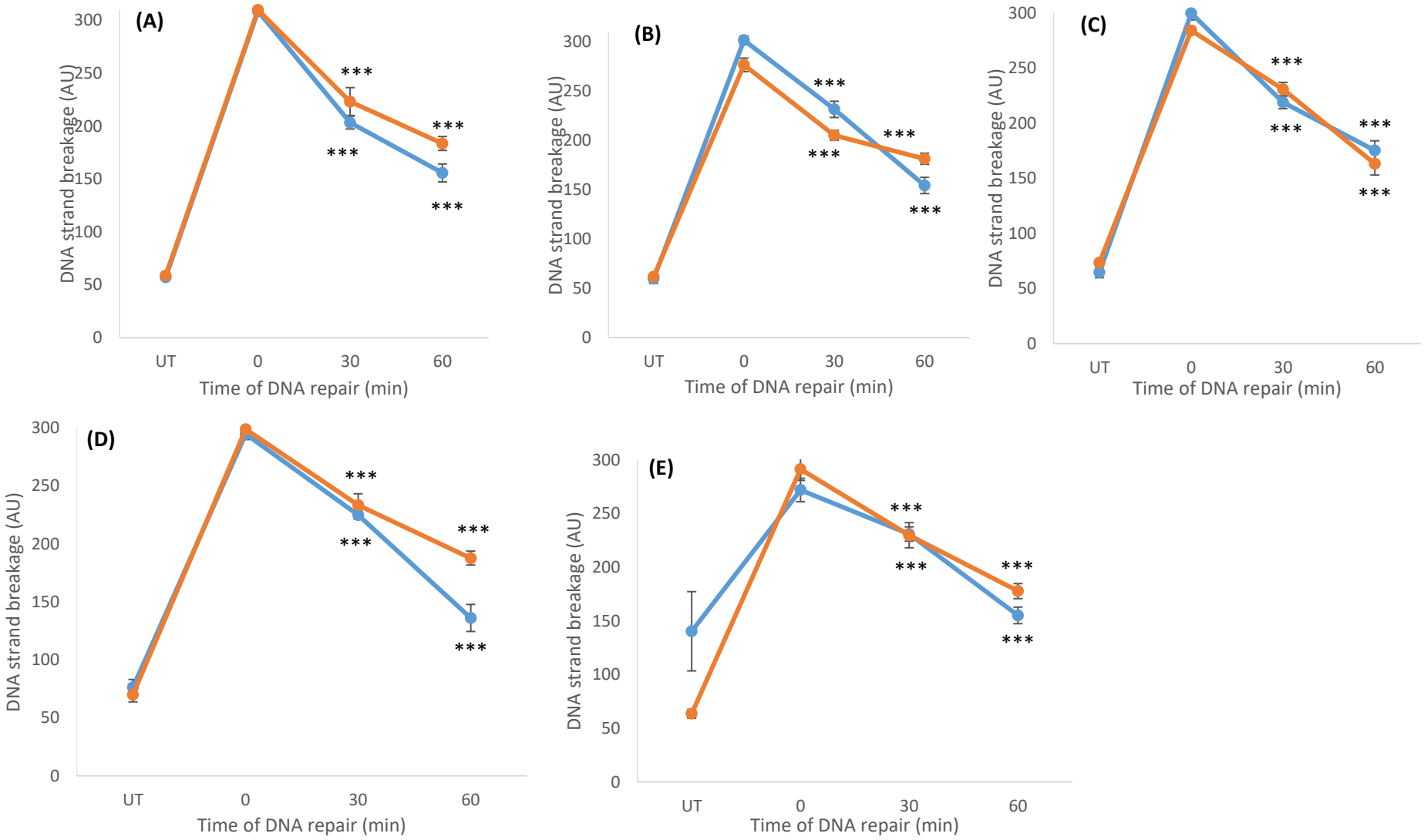


Figure 4.7: Effect of TI extract on NCM460 or CaCo-2 DNA SBR after exposure to H_2O_2. (A) DMSO control; (B) petroleum ether extract; (C) chloroform extract; (D) ethyl acetate extract and (E) ethanol extract. Data are presented as mean ± SEM; blue lines are NCM460 and orange lines as CaCo-2 cells; n=3; ***p<0.001 as compared to time 0 by ANOVA followed by Bonferroni's multiple comparison test.

ANOVA comparison of the difference in DNA SBR between CaCo-2 and NCM460 cells showed that treatment of cells with ethyl acetate extract showed a significant ($p<0.001$) induction in DNA SBR in NCM460 after 60 min when compared with CaCo-2 cells. No difference in DNA SBR was observed between NCM460 and CaCo-2 cells after treatment with petroleum ether, chloroform or ethanol extract (Table 4.7).

Table 4.7: Statistical comparison of DNA SBR in CaCo-2 versus NCM460 cells after treatment with TI extracts for 60 min.

Comparison of DNA SBR in CaCo-2 versus NCM460 cells				
TI Extract (mg/ml)	UT	0	30	60
Control	ns	ns	ns	0.05
Petroleum ether	ns	ns	ns	ns
Chloroform	ns	ns	ns	ns
Ethyl acetate	ns	ns	ns	0.001
Ethanol	<0.001	ns	ns	ns

ns indicates not significant ($p>0.05$), n=3, UT untreated with H_2O_2, by ANOVA followed by Bonferroni's multiple comparison test.

The rate of SBR in TI ethanol extract treated NCM460 cells was approximately 3x lower after 30 min and approximately 2x lower 60 min after exposure to H_2O_2 when compared with the NCM40 control cells. Similarly, DNA SBR in chloroform extract-treated CaCo-2 cells was approximately 2x lower after 30 min as compared to CaCo-2 control cells (Table 4.8).

Table 4.8: Effect of TI extract on the rate of DNA stand break repair in NCM460 and CaCo-2 cells after exposure to H_2O_2.

	Rate of DNA SBR (AU/min)			
	NCM460 cells (AU/min)		CaCo-2 cells (AU/min)	
Treatment/Time	30 min	60 min	30 min	60 min
Control	3.51	2.55	2.89	2.10
Pet ether	2.34	2.46	2.38	1.59
Chloroform	2.70	2.08	1.78	2.02
Ethyl acetate	2.33	2.64	2.18	1.85
Ethanol	1.37	1.95	2.05	1.89

4.3.4.3 Effect of TI phytochemical metabolites on DNA SBR in colon cells.

Based on the measured effects of the TI extracts on DNA SBR, treatment with ethyl acetate extract inhibited DNA SBR in CaCo-2 cells after 60 min as compared to SBR in NCM460 cells while no difference in SBR was observed between NCM460 and CaCo-2 cells after treatment with petroleum ether, chloroform or ethanol extract (Table 4.7). The possible associated phytochemicals impacting on DNA SBR were analysed by PLS-DA (Figure 4.8). After H_2O_2-induced DNA damage in the colon cells, inhibition in SBR capacity in CaCo-2 cells but not in NCM460 cells was associated with the presence of 4-hydroxy-3-methoxyphenylpropionic acid; hydroxytyrosol and indole-3-acetic acid which were present in only TI ethyl acetate extract but not in ethanol, chloroform or petroleum ether extract. List of all phytochemicals associated with inhibition of SBR in both CaCo-2 and NCM460 cells are shown in appendix 2 (Table 8.6).

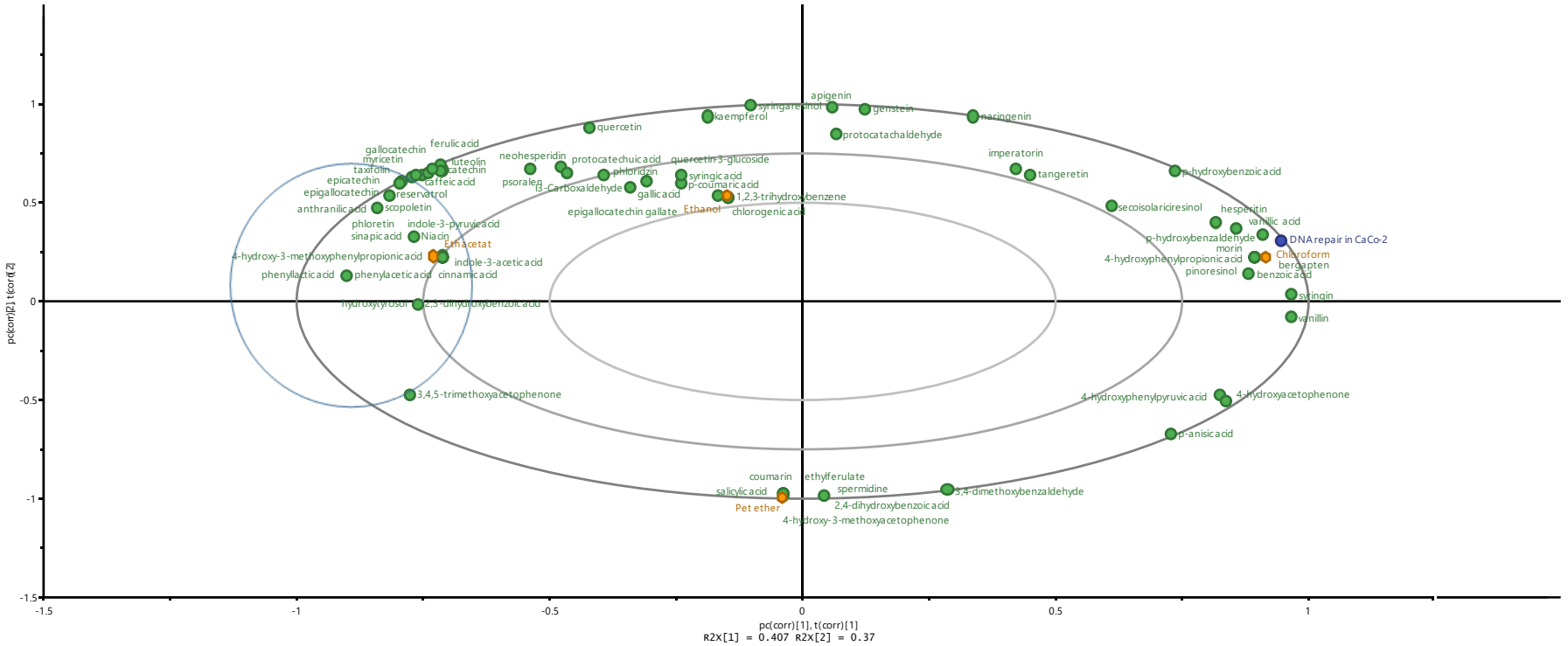


Figure 4.8: Phytochemical compounds isolated from TI using chloroform; ethanol; ethyl acetate and petroleum ether and their relationship with DNA SBR in CaCo-2 cells. The plot was colour coded as: deep blue for DNA SBR; green for phytochemical compounds; and orange for TI extracts.

4.3.5 Impact of TI on human colon cancer cell migration *in vitro.*

4.3.5.1 Establishing a dose-response for the effect of hydroxyurea on CaCo-2 cell viability and cell growth.

This study was carried out to determine an appropriate concentration of HU to inhibit CaCo-2 cell growth and not viability. Cells were treated with varying concentrations (0 – 10 mM) for 24 h and cell viability and growth were determined by MTT assay and cell count respectively. HU showed no toxicity on CaCo-2 cell viability at the concentrations tested (Figure 4.9A). However, treatment with HU at concentrations of ≥1 mM significantly ($p<0.05$) inhibited CaCo-2 cell growth compared to the control (Figure 4.9B). HU at a concentration of 5 mM was chosen to determine the impact of TI on CaCo-2 cell migration because it showed no effect on cell viability but caused an approximately 60% inhibitory effect on cell growth.

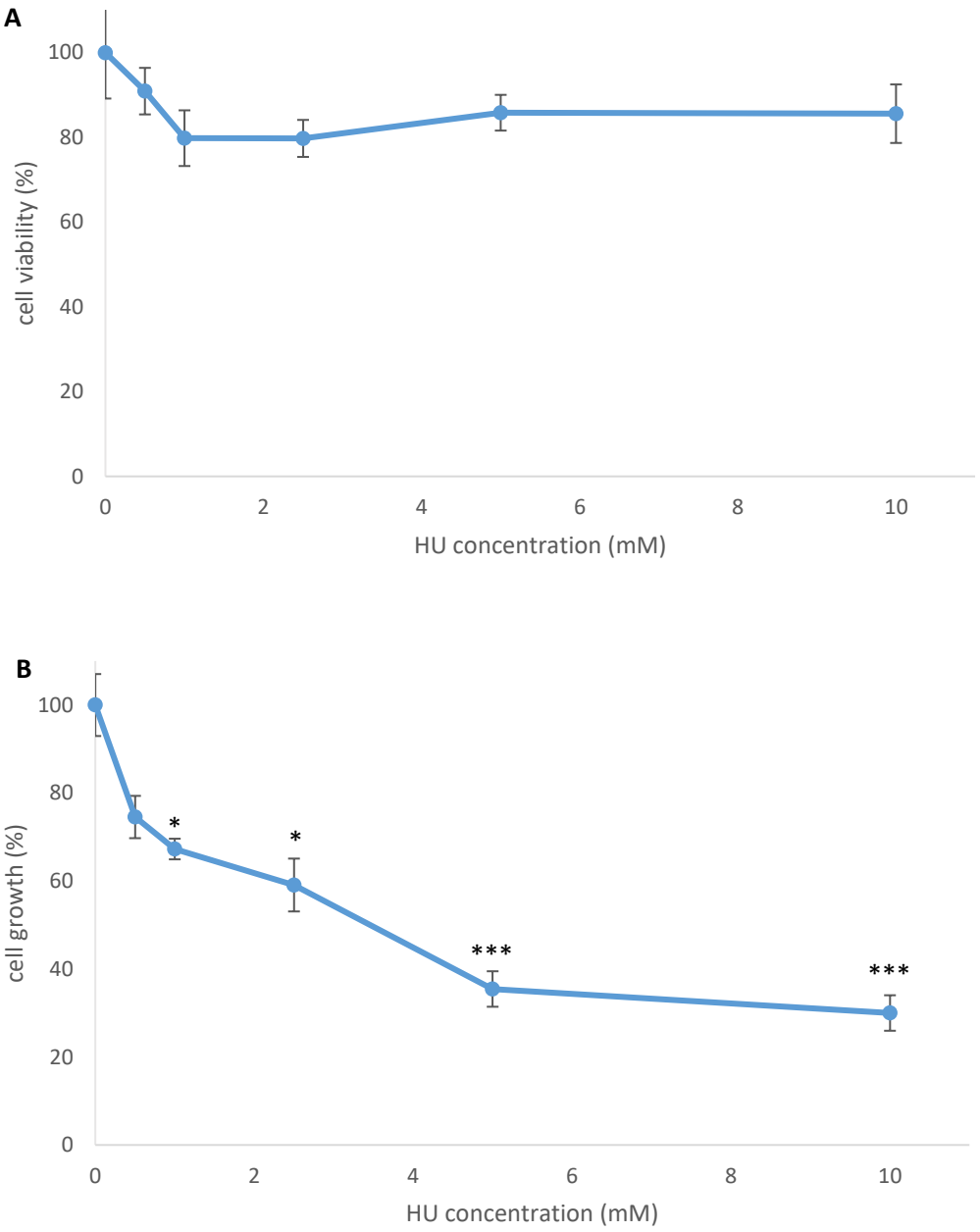


Figure 4.9: Effect of HU on CaCo-2 cell: (A) viability and (B) growth after 24 h treatment. Data are presented as mean ± SEM; n=3; *p<0.05 and ***p<0.001 as compared to control (0 mM) by ANOVA followed by Bonferroni's multiple comparison test.

4.3.5.2 Optimising seeding density and incubation time for CaCo-2 cell migration.

To determine the impact of TI on colon cancer cell migration, CaCo-2 cells were tested at densities between $1x10^2$ – $4x10^5$ cells/ring and incubated at 37 °C for 4 h. Cell densities of $1x10^2$ – $4x10^2$ cells/ring (Figure 4.10 a and b) were too low a density to observe cells on the surface. A density of $1x10^5$ cells/ring showed a higher number of cells but was not enough to cover the surface of the cloning ring (Figure 4.10c). A density of $4x10^5$ cells/ring was observed to have an optimum number of cells that covered the surface of the cloning ring and was selected to determine the optimal incubation time of CaCo-2 cells (Figure 4.10d).

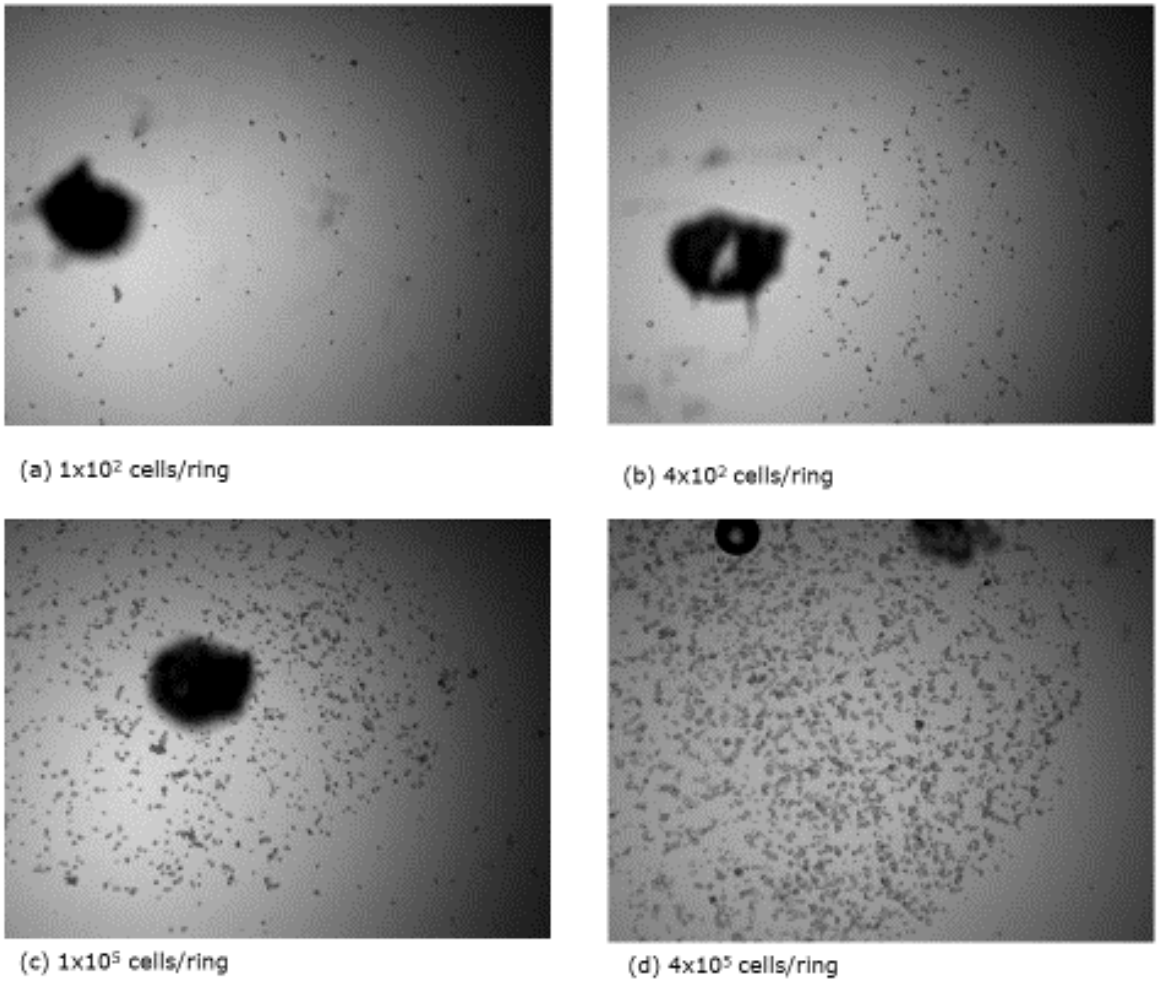


Figure 4.10: Optimising CaCo-2 cell density for measuring the impact of TI on cell migration.

Cells were seeded at different densities (a)$1X10^2$ cells/ring; (b) $4x10^2$ cells/ring; (c) $1x10^5$ cells/ring and (d) $4x10^5$ cells/ring.

CaCo-2 cells were seeded at a density of $4x10^5$ cells/ring and incubated at 37 °C and the cloning rings were removed after 4, 6, 8 or 24 h. Between 4 – 6 h of incubation, the cells were observed to attach to the tissue culture plate and started to flatten after removal of the rings. After 8 h of incubation in the cloning rings, the cells started to detach from the plates when the rings were removed and most of the cells had detached after 24 h (Figure 4.11). Cell seeding density of $4x10^5$ cells/ring and incubation time of 4 – 6 h were therefore chosen to assess the impact of TI on CaCo-2 cell migration, going forward.

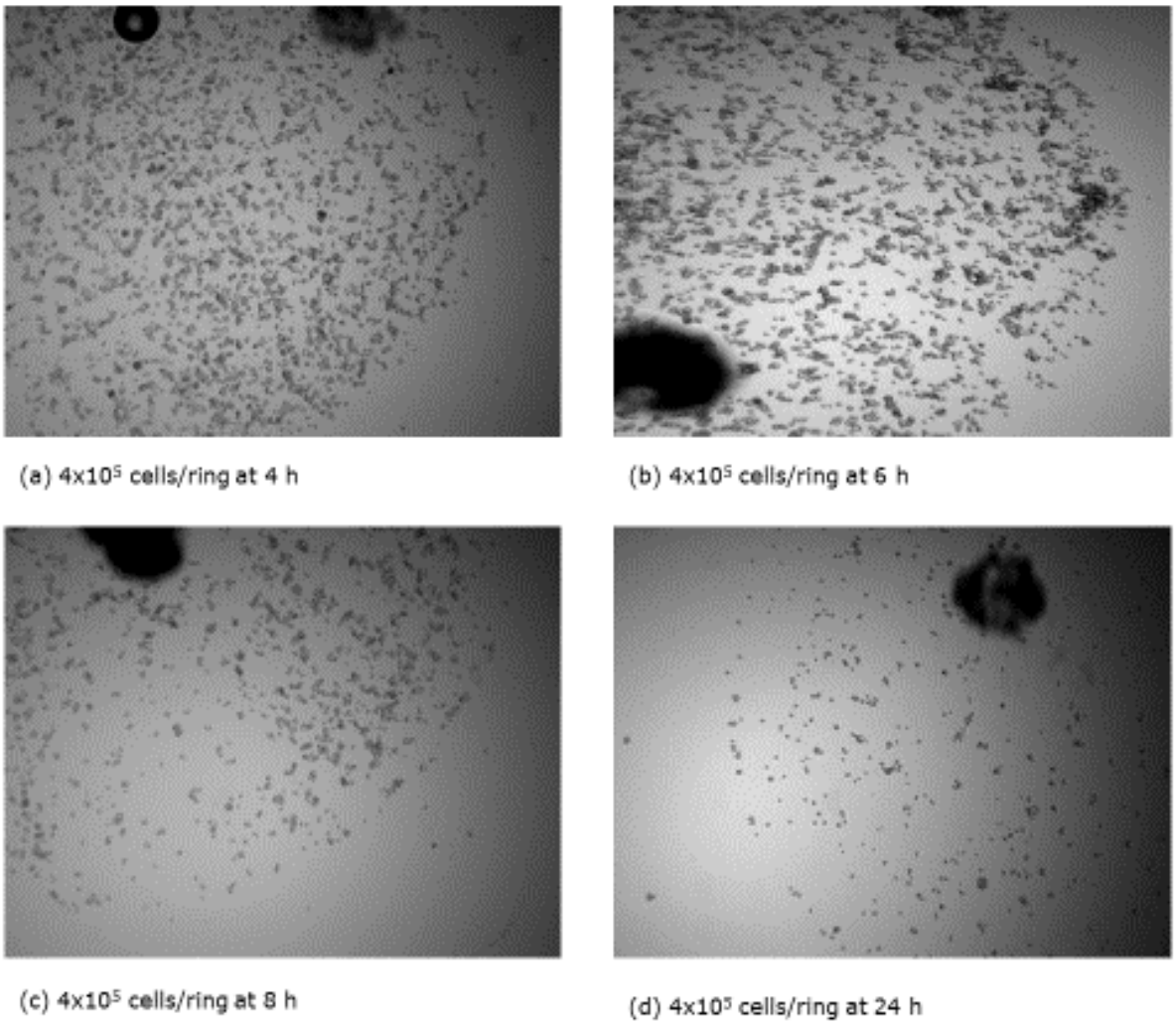
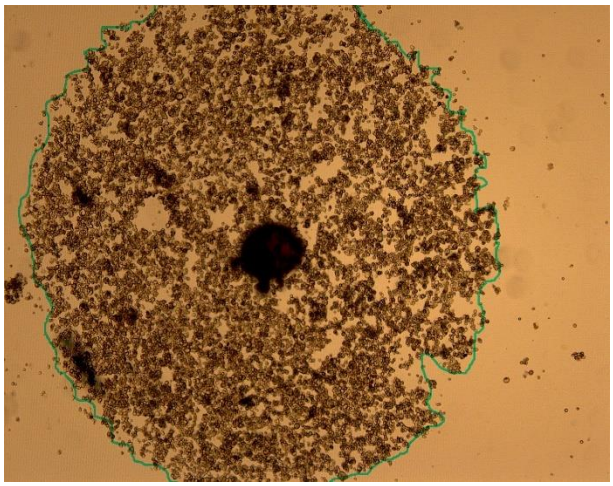


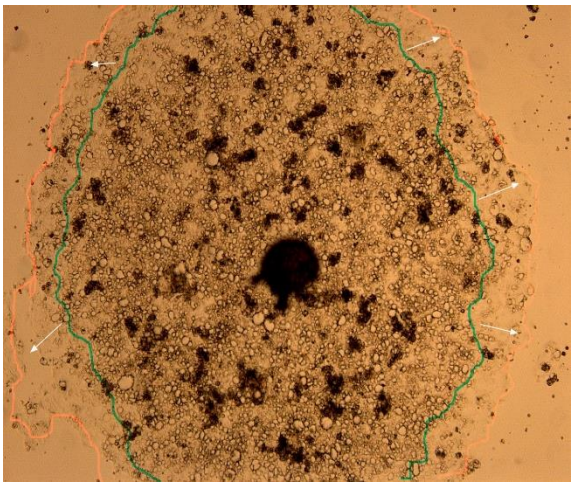
Figure 4.11: Optimising CaCo-2 cell incubation time for measuring the impact of TI on cell migration. Cells were incubated at different times (a) 4 h; (b) 6 h; (c) 8 h and (d) 24 h.

4.3.5.3 Impact of TI on CaCo-2 cell migration

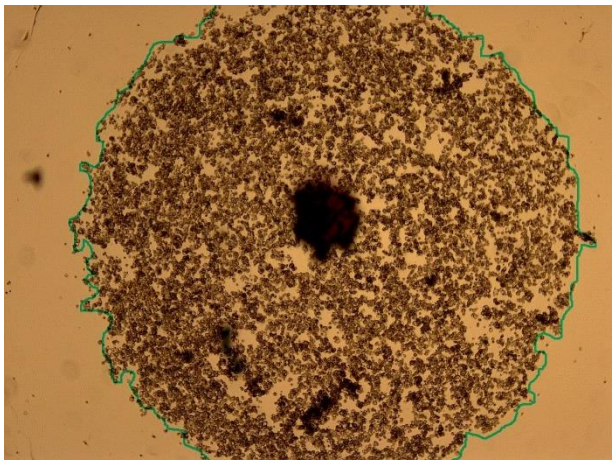
Figure 4.12 show the images of CaCo-2 cells seeded at a density of $4x10^5$ cells/ring and treated with control (DMSO or HU) or TI + HU for 0 or 2 days. The green lines show the edges of the plated cells at 0 h before treatment, and the white arrows show the leading edges of migration after treatment. After 2 days of treatment, CaCo-2 cells were observed to attach and migrate as observed in the control cells ($p<0.001$). A similar effect was seen in CaCo-2 cells pre-treated with petroleum ether or chloroform extract. Conversely, substantially reduced migration was seen in cells pre-treated with ethyl acetate or ethanol extracts (Figure 4.12).



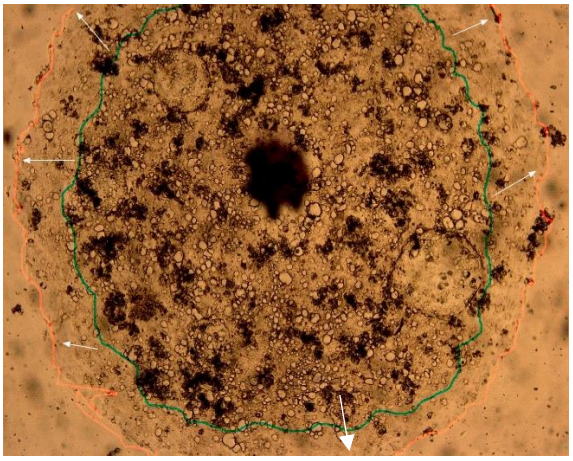
(a) DMSO control + HU at day 0



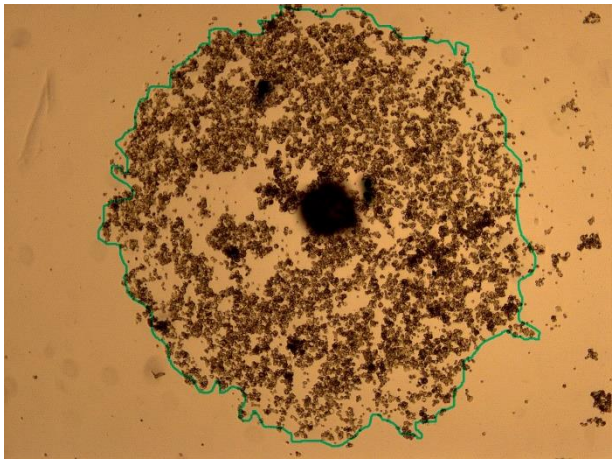
(b) DMSO control + HU at day 2



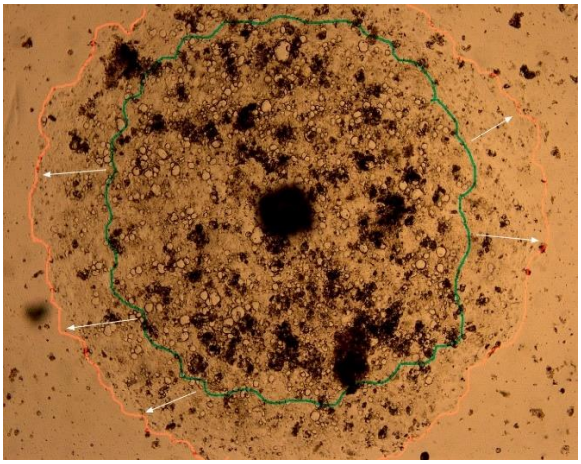
(c) Petroleum ether extract + HU at day 0



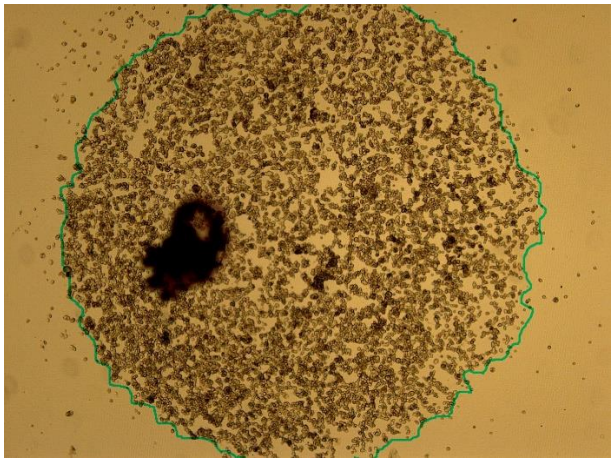
(d) Petroleum ether extract + HU at day 2



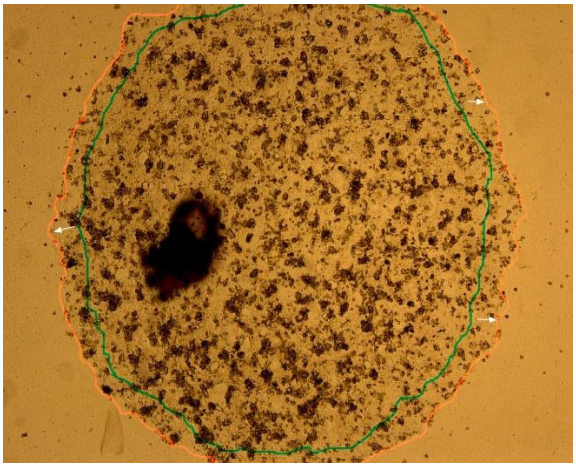
(e) Chloroform extract + HU at day 0



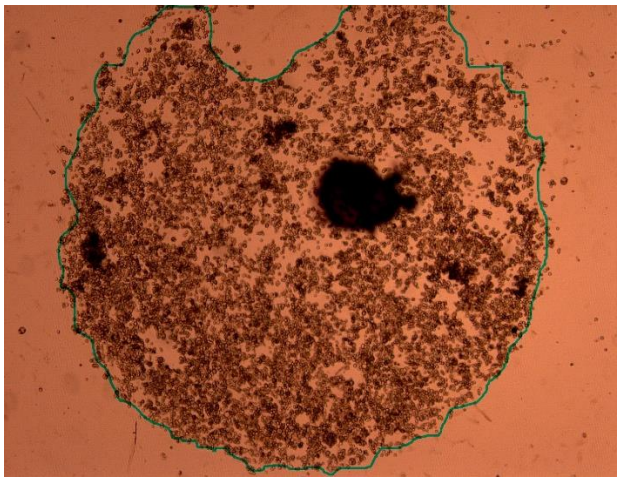
(f) Chloroform extract + HU at day 2



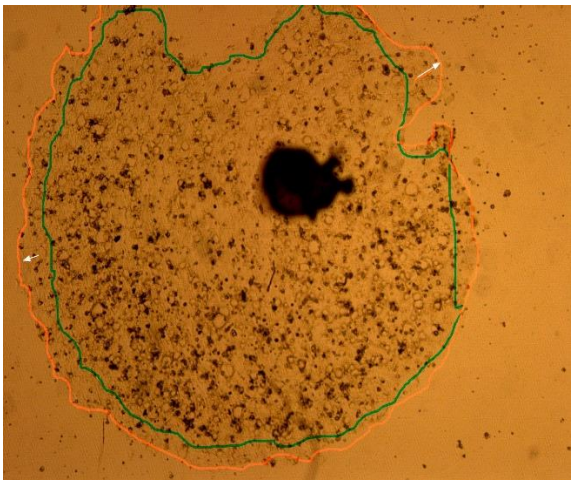
(g) Ethyl acetate extract + HU at day 0



(h) Ethyl acetate extract + HU at day 2



(i) Ethanol extract + HU at day 0



(j) Ethanol extract + HU at day 2

Figure 4.12: Circular monolayers of CaCo-2 cells treated with TI extract and HU. Green lines showing cells at day 0, orange lines showing the leading edges of cell migration and white arrows showing the leading edges of migration at day 2 of treatment.

In control cells (DMSO+HU), there was a time-dependent increase ($p<0.001$) in migration for up 130 % on day 7. A similar increasing effect was seen in cells pre-treated with petroleum ether or chloroform extract. Conversely, pre-treatment with ethyl acetate or ethanol extract significantly ($p<0.01$) inhibited cell migration by approximately 80 and 75 % respectively as compared to the control cells (Figure 4.13).

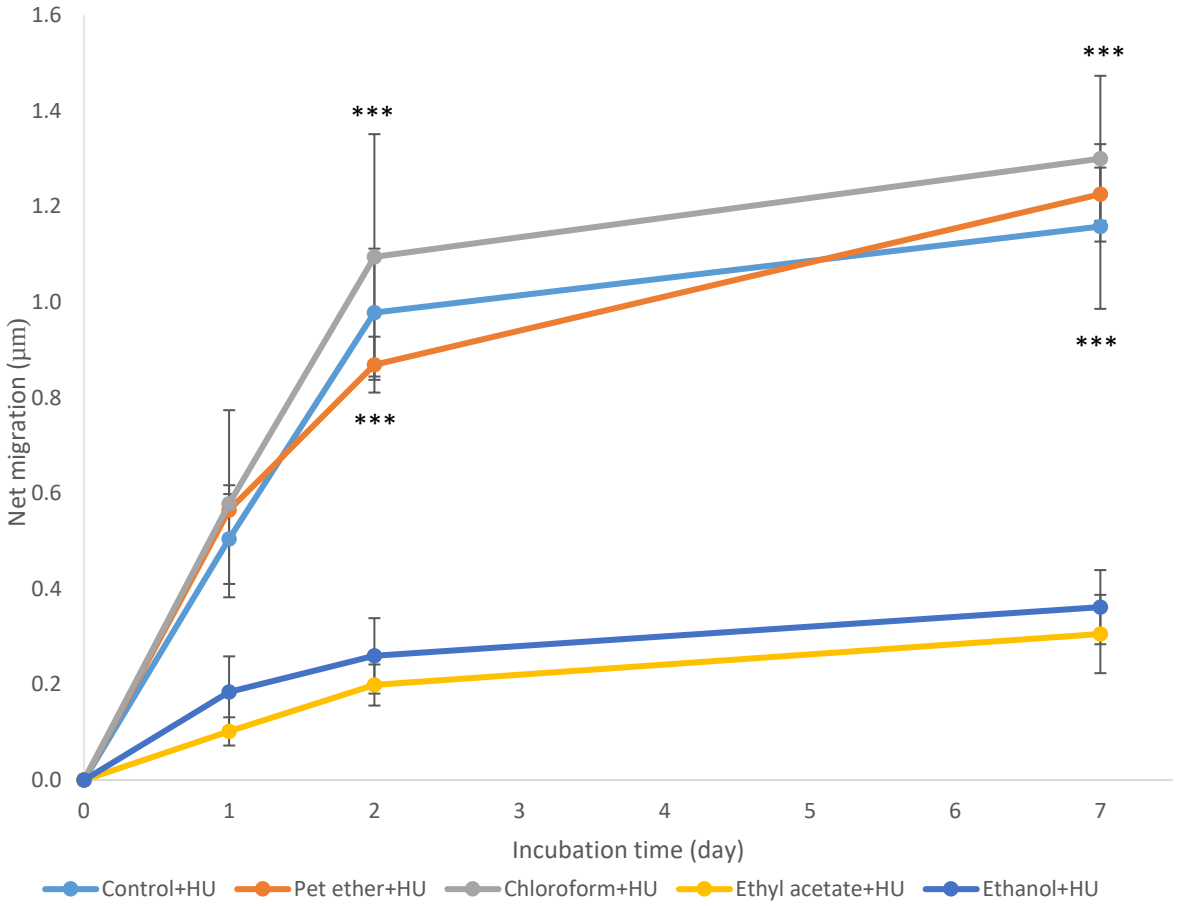


Figure 4.13**:** Effect of TI extracts on CaCo-2 cell migration.
Data are presented as mean ± SEM; n=3; **p<0.01 and ***p<0.001 as compared to time 0 by One-way ANOVA followed by Bonferroni's multiple comparison test.

Comparing the TI extracts, cells treated with ethyl acetate or ethanol extracts showed a significant inhibition in migration at 2 or 7 days as compared to those treated with chloroform or petroleum ether extracts (Table 4.9).

Table 4.9: One-way ANOVA comparison of effect of TI extracts on CaCo-2 cell migration.

Treatment	Time (day)			
	0	1	2	7
Ctrl vs pet	ns	ns	ns	ns
Ctrl vs chlo	ns	ns	ns	ns
Ctrl vs eth ace	ns	ns	0.002	0.001
Ctrl vs etOH	ns	ns	0.008	0.002
Pet vs chlo	ns	ns	ns	ns
Pet vs eth ace	ns	ns	0.011	<0.001
Pet vs etOH	ns	ns	0.018	<0.001
Chlo vs eth ace	ns	ns	0.001	<0.001
Chlo vs etOH	ns	ns	0.001	<0.001
Eth ace vs etOH	ns	ns	ns	ns

ns indicates not significant ($p>0.05$); ctrl is DMSO control; Pet is petroleum extract; Chlo is chloroform extract; Eth ace is ethyl acetate extract; and EtOH is ethanol extract; n=3; by ANOVA followed by Bonferroni's multiple comparison test.

4.3.5.4 Effect of TI phytochemical metabolites on CaCo-2 cell migration.

The measured effects of the TI extracts on migration showed that treatment with TI ethanol or ethyl acetate extract inhibited migration in CaCo-2 cells after 2 or 7 days as compared to the control cells while no difference in migration was observed in CaCo-2 cells after treatment with petroleum ether or chloroform extract (Table 4.10). The relationship between the possible phytochemicals associated with the inhibitory impact of TI extracts on cell migration was analysed by PLS-DA (Figure 4.14)

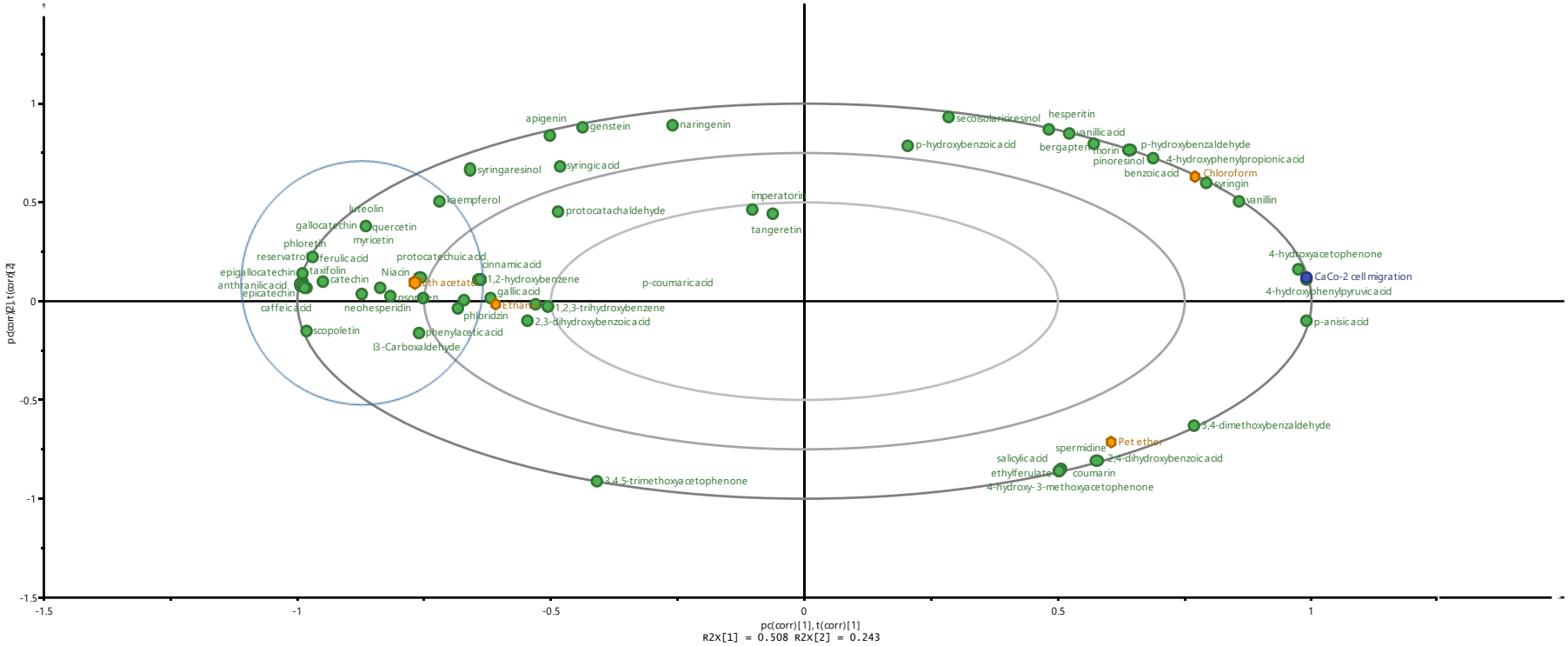


Figure 4.14: Phytochemical compounds isolated from TI using chloroform, ethanol, ethyl acetate and petroleum ether and their relationship with CaCo-2 cell migration. The plot was colour coded as: deep blue for cell migration; green for phytochemical compounds; and orange for TI extracts.

The inhibitory effect of TI ethanol or ethyl acetate extracts on CaCao-2 cell migration was associated with phytochemicals that were exclusively present in these extracts but not in TI chloroform or petroleum ether extracts. The associated phytochemicals are anthranilic acid, caffeic acid, epicatechin, epigallocatechin, gallocatechin, kaempferol, luteolin, myricetin, neohesperidin, niacin, phenylacetic acid, phloretin, psolaren and resveratrol (Table 4.10).

Table 4.10: Relationship between phytochemical compounds isolated from TI ethanol and ethyl acetate extracts and the impact on migration in colon cells.

TI	Phytochemical metabolites showing the greatest impact on CaCo-2 cell migration
Ethyl acetate extract	1,2-dihydroxybenzene; anthranilic acid; caffeic acid; catechin; cinnamic acid; epicatechin; epigallocatechin; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; niacin; neohesperidin; phenylacetic acid; phloretin; phloridzin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin and taxifolin.
Ethanol extract	anthranilic acid; caffeic acid; catechin; epicatechin; epigallocatechin; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; niacin; neohesperidin; phenylacetic acid; phloretin; phloridzin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin and taxifolin.

4.4 DISCUSSION

Globally, at least 10 million people are diagnosed with cancer every year (Bray et al., 2018; Prakash et al., 2013). According to the American Cancer Society, cancer causes 2–3% of deaths annually throughout the world which accounts for about 10 million deaths worldwide (International Agency for Research on Cancer, 2018; Prakash et al., 2013). Due to the global burden of the condition, numerous strategies have been used in cancer therapy and management which include chemotherapy, radiotherapy and surgery (Prakash et al., 2013; Qi et al., 2010). These treatment methods are usually linked with severe side effects with cancerous cells gradually developing resistance against treatment (Qi et al., 2010; Wang et al., 2012). Hence, cancer researchers continue to seek new and safer approaches to improve the treatment of cancer which include the use of NPs (Safarzadeh et al., 2014). For instance, plant alkaloids such paclitaxel and vinblastine have been synthesised in orthodox medicine for the treatment of various cancers which include brain, breast, cervical, lung and pancreatic cancers (Cragg & Newman, 2013).

To study the development of cancer and effectiveness of treatment *in vivo*, samples are usually obtained from animal models or directly from cancer patients (Lindhorst & Hummon, 2020). The commonly used samples obtained from patients for the study of cancer include formalin-fixed paraffin embedded tissues, tissues from primary tumour and stool samples (Alves Martins et al., 2019; Lindhorst & Hummon, 2020; Wang et al., 2017). Patient-derived samples give the most accurate representation of human cancer since they are obtained directly from the actual site of cancer, but they are difficult to maintain outside the body because they may not be grown under the exact conditions found in the patient (Alves Martins et al., 2019). Therefore, cultured cell models have been used to simplify tissue handling and offer greater reproducibility for cancer studies (Wang et al., 2017). Cell lines are the most common models used for cancer research and have been in use consistently since the development of the HeLa cell line from cervical cancer in 1951 (Gey, 1952; Wang et al., 2017). There are no data showing the effect of TI extract on colon cancer and therefore, in this study, the use of TI as a potential anticancer agent was investigated in human colon cells *in vitro* to inform if TI acts differently against cancerous (CaCo-2) and

normal colon (NCM460) cells. The study used the four TI extracts namely: chloroform, ethanol, ethyl acetate or petroleum ether and investigated their effect on primary biomarkers of cancer, for instance: cell viability, cell growth, DNA damage, DNA repair and cell migration.

In this study, both CaCo-2 and NCM460 cells were treated with varying concentrations (0 – 5 mg/ml) of the four TI extracts for 24 h and the impact on viability and growth were measured. All four TI extracts showed inversely proportional relationship on colon cell viability and growth. The four TI extracts showed no significant effect on viability and growth of the colon cells at concentrations ≤0.5 mg/ml. It was found that the CaCo-2 colon cancer cells showed more susceptibility to the TI extracts than the normal colon cells. This was evident as seen as an approximately 2-fold reduction in the viability of CaCo-2 cells (IC_{50} values) as compared to the NCM460 cells. However, there was no such obvious difference in effect on the growth of the colon cells after treatment with the TI extracts. Among the four TI extracts, the chloroform and petroleum ether extracts showed higher toxicity on the viability of both colon cells than ethyl acetate and ethanol extracts.

To the best of my knowledge, there are no studies comparing directly the effect of TI extracts on either CaCo-2 or NCM460 cells *in vitro,* hence, previous studies on other NPs and natural phytochemicals are used to support these findings. Ponou et al. (2010) investigated the cytotoxic effects of bioactive compounds from TI by administering 0 – 200 µM of arjungenin, arjunic acid, betulinic acid, ivorenosides (A, B or C), oleanolic acid or sericoside to human breast (MDA-MB-231), prostate (PC3), colon (HCT116), and brain (T98G) cancer cells for 24 h. They found that ivorenosides (A, B or C) or sericoside at 200 µM were shown to reduce the proliferative activity of the cell lines by 50 – 80 % compared to the respective controls. They concluded that ivorenoside A was the most toxic metabolite against MDA-MB-231 and HCT116 cell lines with IC_{50} values of 3.96 and 3.43 µM respectively. The current study did not measure these other metabolites. Another study also treated Caco-2 cells with 0 – 200 µM of myricetin, quercetin or rutin for 24 h followed by treatment with 50 µM H_2O_2 for 30 min and cell viability was not significantly affected by treatment with 10, 50 or 200 µM of the flavonoids (Aherne & O'brien, 1999). However, in this study, TI has been shown to contain phytochemicals including myricetin and quercetin

which might have contributed to the dose-dependent decrease in the colon cell viability and growth, but the metabolites were not tested individually to confirm this prediction. Additionally, administering increasing concentrations (1 – 10 μM) of falcarinol (a polyacetylene isolated from carrots) to CaCo-2 cells for 72 h has also been shown to increase cell proliferation by approximately 30 – 80 % compared to the control (Young et al., 2007).

The development of cancer starts with genomic instability, which directs the cells to divide and grow continuously and ultimately become cancerous (Prakash et al., 2013). Therefore, the four TI extracts were used to determine their influence on DNA SSB and SBR in normal colon cells versus their cancerous counterparts.

In this study, TI induced DNA SSB in both NCM460 and CaCo-2 cells after exposure to increasing concentrations of extract. All four TI extracts increased DNA SSB up to 100 % in the colon cells at concentrations ≥0.5 mg/ml when compared to the controls. Similar to the effect of TI extract on cell viability and growth, genomic stability in CaCo-2 cells was more susceptible to TI ethanol, ethyl acetate or petroleum ether extract than in NCM460 cells. Petroleum ether extract caused approximately 3-fold increase in DNA SSB in CaCo-2 cells compared to NCM460 cells. Additionally, among the four TI extracts, ethanol extract caused the highest genotoxicity in the cancerous cells (LC_{50} = 0.39 mg/ml) and in normal colon cells (LC_{50} = 0.26 mg/ml). Conversely, TI chloroform extract showed an approximate 3-fold increase in DNA SSB in normal colon cells compared with the cancerous colon cells.

In similar studies investigating the genotoxicity of natural products, treatment of CaCo-2 cells with falcarinol at concentrations >10 μM for 72 h was shown to significantly ($p<0.001$) increase DNA SSB by >90 % in the colon cells (Young et al., 2007). Duthie et al. (1997) also exposed CaCo-2 cells to various concentrations (0–2500 μM) of flavonoids (including myricetin, quercetin and silymarin) for 18 h which showed significant ($p<0.01$) increase in DNA SSB by approximately 20, 40 and 100 % for silymarin, quercetin and myricetin respectively as compared to the controls. Quercetin at concentrations above 10 μM was shown to protect against H_2O_2-induced DNA damage. These compounds, except silymarin, are measured in the TI extracts used in the current studies. However, there are no data showing the impact of TI extracts on genomic

stability in colon cells. Therefore, this work is highly novel and showed the effect of TI on DNA SSB particularly comparing normal and transformed colon cells. Hence, the ability of TI ethanol, ethyl acetate or petroleum ether extract to cause higher DNA damage in cancer cells than in normal cells is an advantage for potential search for anti-cancer drugs.

Normal cells have checkpoints in the cell proliferation cycle that safeguard genomic integrity (Li et al., 2003). These checkpoint mechanisms help to regulate genetic material through a series of activities which may include transduction of information, sensing of DNA damage and ultimately triggering effector responses to control DNA damage (Zhou & Elledge, 2000). When conditions are unfavourable for growth or there is irreparable DNA damage in the cells, apoptosis is triggered to remove the damaged cells (Li et al., 2003; Zhou & Elledge, 2000). In cancer cells however, these cellular control mechanisms are inhibited resulting in accumulation of damaged genetic material and consequently, DNA instructing continuous division and growth of abnormal cells (Zhou & Elledge, 2000). Most therapeutic agents used in the treatment of cancer eliminate cancerous cells by directly or indirectly inducing checkpoint-mediated mechanisms and causing non-selective DNA damage which accounts for their toxicity in both cancerous and normal cells (Li et al., 2003; Rich et al., 2000; Zhou & Elledge, 2000). Such limited selective toxicity to cancer cells and non-specific damage to DNA largely accounts for the severe adverse effects mostly seen in cancer therapy (Li et al., 2003; Rich et al., 2000).

Although direct damage to DNA is an essential element of cytotoxicity, the relative toxicity of potential anti-tumour drugs suggest that the magnitude of DNA damage is not an indicator of overall cellular toxicity (Antonini et al., 2008). The extent of DNA repair in cells after damage will affect overall genotoxicity (Hansen & Kelley, 2000). Bestwick et al. (2011), proposed that inhibition of DNA repair capacity in response to bisnaphthalimidopropyl polyamines is also an indicator of cytotoxicity as it has the potential to boost the effects of other DNA-damaging drugs and increase overall cytotoxicity. Therefore, this study determined the influence of TI extracts on DNA repair as a hallmark of cancer in the colon cells.

In the present study, a dose-response for H_2O_2-induced DNA SSB in the human colon cells was established to determine an optimum dose of H_2O_2 that could cause DNA SSB to monitor further damage or SBR. Hence, treatment of both colon cell types with increasing concentration of H_2O_2 caused a dose-dependent increase in DNA SSB. No difference was observed in DNA SSB between NCM460 and CaCo-2 cells after treatment with H_2O_2. An optimum concentration of 30 µM H_2O_2 was established and used to investigate the impact of TI on colon cell DNA SBR or further DNA SSB.

In a previous study, a similar concentration of H_2O_2 (25 µM) was used to determine the impact of *Aronia melanocarpa*, *Chaenomeles superba* and *Cornus mas* extracts on DNA SBR in CaCo-2 cells (Efenberger-Szmechtyk et al., 2020). In disagreement with these findings, a higher concentration of H_2O_2 (150 µM) was used to monitor the effect of bisnaphthalimidopropyl polyamines on DNA stability in CaCo-2 cells (Bestwick et al., 2011). A study by Duthie et al. (2008) also reported using a lower concentration of H_2O_2 (10 µM) to determine the effect of folate intake on DNA SBR in NCM460 cells (Duthie et al., 2008). According to Collins et al. (1995), different cell types or sub-populations respond differently to H_2O_2-induced DNA damage due to variations in cellular Fe^{2+} or NAD(P)H which influences the activities of various cellular antioxidant enzymes like GPx and catalase and hence, the different abilities of the cells to resist oxidative stress (Collins et al., 1995). Duthie and Collins (1997) also reported that cells respond differently to H_2O_2 due to different cell culture conditions for instance cell culture medium and whether cells were exposed on flask or within Eppendorf tubes for treatment with H_2O_2.

In the current study, a non-cyto- and non-genotoxic concentration of TI (0.25 mg/ml) was used to measure the DNA SBR activity in the colon cells after inducing DNA SSB with 30 µM H_2O_2. As expected, there was a significant increase in DNA SSB in control cells after exposure to H_2O_2 which was followed by a time-dependent decrease in DNA SSBs after 30 and 60 min in both colon cell lines. NCM460 cells showed a higher endogenous or background DNA SBR in the untreated cells (control) as compared to CaCo-2 cells. The rate of DNA repair in TI ethanol extract treated NCM460 cells was lowered by approximately 3-fold after 30 min and by approximately 2-fold after 60 min of exposure to H_2O_2 when

compared with the control cells. Similarly, DNA SBR in chloroform extract-treated CaCo-2 cells was also lowered by approximately 2-fold after 30 min as compared to control CaCo-2 cells. Treatment of cells with ethyl acetate extract showed a significant ($p<0.001$) induction in DNA repair in NCM460 after 60 min when compared with CaCo-2 cells.

As certain extracts showed inhibition on DNA SBR in the colon cells, PLS-DA was used to try to assess the effects of the TI phytochemicals associated with the inhibitory effects. The measured effects of the TI extracts on DNA SBR, treatment with ethyl acetate extract inhibited DNA SBR in CaCo-2 cells after 60 min as compared to DNA SBR in NCM460 cells while no difference in DNA SBR was observed between NCM460 and CaCo-2 cells after treatment with petroleum ether, chloroform or ethanol extract. After oxidative damage (exposure to H_2O_2), inhibition in DNA SBR in CaCo-2 cells was possibly associated with 4-hydroxy-3-methoxyphenylpropionic acid; hydroxytyrosol and indole-3-acetic acid as they were present exclusively in TI ethyl acetate extract but not in ethanol, chloroform or petroleum ether extract. The TI phytochemicals could possibly account for the selective genotoxic effect in cancer cells (CaCo-2 cells) but not in the normal counterpart (NCM460 cells).

There are no available data on the effect of TI extracts on DNA SBR activity and hence, other natural phytochemicals and NP were used to support the current study. De Melo et al. (2004) treated neutrophils with 1 mM indole-3-acetic acid for 12 h and observed cell death which resulted from the induction of loss in cell membrane integrity and DNA fragmentation (de Melo et al., 2004). Folkes and Wardman (2001) proposed that the effect of indole-3-acetic acid to cause cytotoxicity may be due to the ability to form 3-methylene-2-oxindole, which conjugates with protein thiols and DNA bases (Folkes & Wardman, 2001). Thus, indole-3-acetic acid, which is present in TI ethyl acetate extract, could serve as a potential anti-tumour agent as it is well tolerated in normal tissues but toxic to tumour cells (Folkes et al., 1999; Folkes & Wardman, 2001).

In other studies, Efenberger-Szmechtyk et al. (2020) determined the effect of *Aronia melanocarpa*, *Chaenomeles superba* and *Cornus mas* extracts on DNA SBR in CaCo-2 cells by treating the cells with extract concentrations of 0 – 10 % (v/v) for 1 h. They found that the extracts induced DNA damage in a dose-

dependent manner while non-toxic concentrations (0.04 – 0.08 %) significantly ($p<0.05$) induced DNA SBR in Caco-2 cells by approximately 40 % after 60 min of exposure to H_2O_2. Similarly, treatment of CaCo-2 cells with 50 µg/ml of *Viburnum opulus* fruit extract for 60 – 120 min showed >90 % induction in DNA repair after 120 min of exposure to H_2O_2 (Zakłos-Szyda et al., 2019).

Induction of DNA repair by polyphenols is facilitated through promotion of the activities of DNA repair enzymes (Tan et al., 2009). According to Hengel et al. (2017), normal cells use the mechanism of DNA repair to maintain their genomic integrity and minimise aging and reduce the risk of cancer (Hengel et al., 2017). However, cancer cells acquire this characteristic of normal cells and resist DNA damage from radiation and chemotherapeutic agents (Du & Tang, 2014; Hengel et al., 2017). Therefore, this study found that the ability of TI extract to selectively inhibit DNA repair and cause lethality in colon cancer cells but not in normal colon cells can be instrumental as a promising anti-cancer agent.

Cell invasiveness/ migration is the single most important feature that differentiates malignant and benign cells (Eccles et al., 2005). Contrary to benign lesions, cancerous/ malignant cells develop mechanisms that enable them to migrate from the original point of development and form new colonies in different sites of an organism (Berx & van Roy, 2009). As such, several *in vitro* methods have been developed to study the process of cell migration (Eccles et al., 2005). These include the scratch assay, which involves scratching part of the cellular layer and measuring the distance covered by the cells into the wounded region (Li et al., 2006). Other researchers measure the number of cells that migrated into the scratched region or some researchers use the transwell migration (Boyden chambers) assay (Hamuro et al., 2002; Hervé et al., 2005). In addition, radial migration is also a commonly used method for studying the mechanism of wound healing and cell migration where cells are set up in a circular ring and their outward migration is measured with reference to a central point (Eccles et al., 2005; Gadad et al., 2013).

This study used radial migration to determine the impact of TI extracts on cell migration using CaCo-2 cell line as the model for cancerous cell. CaCo-2 cells only were used because they are malignantly transformed and acquired the ability to migrate to form new colonies while NCM460 are non-malignant and

hence, only CaCo-2 cells were used for studying the effect of TI extract on colon cell migration. In this study, CaCao-2 cells were optimised at a density of $4x10^5$ cells/ring and at optimum attachment time of 4 – 6 h to study the impact of TI on cell migration. In previous published studies, CaCo-2 cells were seeded at $8x10^4$ cells/well and incubated for 3 days to study wound healing after inhibition of protease-activated receptor-2 (Fernando et al., 2018). Additionally, colon cancer cell lines (CaCo-2, SW480 and SW620) were seeded at $1x10^5$ cells/well and incubated for 2 days to study how micro-RNA-566 mediates cell migration (Zhang et al., 2019). Gadad et al. (2013) also found the optimum density for human microvascular endothelial cells of adult dermis (HMVECad) to be $2x10^5$ cells/ring with optimum incubation time of 4 h. These studies show how experimental parameters such as cell density and incubation time are affected by the cell type.

Moreover, since this study was aimed at measuring cell migration and not just normal cell growth, a growth inhibitor was required to separate the influence of cell growth on the migration assay. Hence, CaCo-2 cells were treated with TI extracts in the presence of a mitotic inhibitor hydroxyurea (HU). HU at a concentration of 5 mM was found to show no significant effect on CaCo-2 viability but to significantly ($p<0.001$) inhibit growth. CaCao-2 cells were seeded at a density of $4x10^5$ cells/ring and after allowing them to attach for 4 – 6 h, the cells were treated with TI extracts with HU and the radial migration was measured at 1, 2 or 7 days. In control cells (DMSO or HU), there was a time-dependent increase ($p<0.001$) in migration for up to 7 days as shown by the leading cellular edges of migration (Figure 4.13). Treatment of CaCo-2 cells with TI extracts showed differential effects on cell migration. The study found that treatment of cells with ethyl acetate or ethanol extracts showed >70 % inhibition in migration at 2 - 7 days as compared to the controls. However, treatment with TI chloroform or petroleum ether extract showed no effect on cell migration. This is the first study to comprehensively investigate the ability of complex extract metabolites (rather than single metabolites) on cell migration.

Hence, a graph of phytochemicals versus cell migration was plotted to determine the phytochemicals having the greatest impact on migration. As pre-treatment of cells with TI ethanol or ethyl acetate extract resulted in significant ($p<0.001$)

inhibition of cell migration, phytochemicals found exclusively in these two TI extracts were possibly the contributing metabolites to the anti-cancer potential of TI. The metabolites include benzenes (especially 1,2-dihydroxybenzene), benzoic acids (especially anthranilic acid), cinnamic acids (especially caffeic acid and cinnamic acid) and flavonoids (especially epicatechin, epigallocatechin, gallocatechin, kaempferol, luteolin, myricetin, neohesperidin, niacin, phenylacetic acid, phloretin, psolaren and resveratrol).

In previous studies and in agreement with these findings, several studies have shown the effects of some of these metabolites on cell migration. For instance, treatment of radioresistant lung cancer (A549 or A549-IR) cells with 100 μM of a flavonoid; myricetin; for 1 or 2 days significantly ($p<0.001$) reduced the migration of the cells (Kang et al., 2020). According to Chen et al. (2017), 30 μM of taxifolin (a flavonoid present in TI) significantly ($p<0.001$) decreased the migration of human osteosarcoma cell lines (U2OS and Saos-2) after 2 days of treatment (Chen et al., 2018). Platten et al. (2001) also found that treating human malignant glioma (LN-18 and T98G) cells with 300 μM of benzoic acid or N-[3,4-dimethoxycinnamoyl]-anthranilic acid (metabolites present in TI extracts); for 4 days showed inhibition in cell proliferation and migration by approximately 60 % in both cell lines as compared to the respective controls (Platten et al., 2001). Ling et al. (2015) also found that treating human colon (Lovo) cells with 5 μM of cinnamic acid for 1 – 2 days significantly ($p<0.05$) reduced the migration of the cells by approximately 78 % as compared to the control (Ling et al., 2015).

In disagreement with these findings, Viana et al. (2020) found that exposure of fibroblasts to 30 μM of cinnamic acids (especially p-coumaric acid or trans-cinnamic acid) for 18 h showed that cell migration was significantly ($p<0.05$) induced by 17 and 33 % by p-coumaric acid and trans-cinnamic acid respectively as compared to the controls (Viana et al., 2020).

In summary, specific extracts of TI have shown different effects on several hallmarks of cancer. All four TI extracts from chloroform, ethanol, ethyl acetate or petroleum ether showed dose-dependent cyto- and genotoxicity as evident from the effects on colon cell viability, growth and DNA damage. Additionally, a non-cyto- and genotoxic dose of TI ethyl acetate extract selectively inhibited

DNA repair in colon cancer cell but not in normal colon cells. Moreover, TI ethanol and ethyl acetate extracts also inhibited colon cancer cell migration. Therefore, the anti-cancer potential shown by TI was associated with certain benzenes, benzoic acids, cinnamic acids and flavonoids in the extracts.

4.5 CONCLUSIONS

TI extracts were equally cyto- and genotoxic to both normal and cancerous colon cells at higher concentrations. However, cancerous colon cells were more susceptible to the TI extracts at lower concentrations. The anti-cancer potential shown by TI extracts as evidenced by induction of DNA damage, inhibition of DNA repair and inhibition of cell migration was accounted for by the presence of certain benzenes, benzoic acids, cinnamic acids and flavonoids in the TI extracts.

CHAPTER FIVE

EFFECTS OF SEASON, STORAGE AND EXTRACTION TECHNIQUE ON THE PHYTOCHEMICAL PROFILE OF TI.

5.1 INTRODUCTION

Due to their potential use in the treatment of various disease conditions and as dietary supplements, analysis of secondary metabolites from plants is of great interest to the food and pharmaceutical industries (Kinghorn et al., 2011). In addition, the analysis of the molecular structures of secondary plant metabolites allows chemists to synthesize them at a lower production cost than when isolating them from natural sources (Ji et al., 2009). The structural elucidation of natural phytochemicals also aids chemists to synthesise and modify the phytochemicals to enhance their efficiency, absorption, solubility, or stability in the human body (Buss et al., 1995; Ji et al., 2009). For instance, the structural elucidation of salicylic acid led to the synthesis of acetylsalicylic acid, named as aspirin, which has been used as a painkiller (Buss et al., 1995).

Seasonality noticeably impacts on the life cycle, distribution and composition of phytochemical in plants (Kumar et al., 2017). Changes in seasons, which are characterised by changes in light intensity, temperature, rain and wind patterns, affect plant morphology, flowering, fruiting, phytochemical components and the ability to compete with other species for survival (Holopainen & Gershenzon, 2010; Kumar et al., 2017). Being relatively immobile organisms, plants have developed alternative defence mechanisms to overcome stress conditions resulting from the changes in weather, herbivory and microbial attack (Holopainen & Gershenzon, 2010). Production of an enormous variety of secondary metabolites, including anthocyanins and cinnamic acids, is a major adaptation used by plants to overcome these stressful conditions (Holopainen & Gershenzon, 2010; Kumar et al., 2017). The synthesis of secondary metabolites is closely regulated and restricted to specific plant tissues and developmental stages and is produced in response to stimuli (Osbourn et al., 2003; Wink, 2003). Several studies have reported changes in secondary metabolites at the genetic or protein level as a consequence of stress conditions in plants (Dangl & Jones, 2001; Lawal et al., 2010; Szathmary et al., 2001). For instance, decreased irrigation induced an increase in total phenolics in red beet by 82 % while lettuce showed an increase in total phenolics by 98 % as compared to adequate water to the plants (Sahin et al., 2016; Stagnari et al., 2014). Soil pollution with heavy metals such as cadmium, chromium and lead, have also

been shown to increase total phenolics (by 18.15 and 6.94 %) and flavonoid content (by 12.84 and 7.44 % respectively) in *Ficus carica* and *Shinus molle* when compared to samples from less polluted soils (Radwan et al., 2018). *Aloe vera* collected from different locations of India with different altitudes, temperatures and rainfall patterns showed different amounts of alkaloids, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids (Kumar et al., 2017).

However, there are fewer data on the influence of key environmental factors such as change in season on the phytochemical metabolites of TI. Therefore, this research focused on the effects of the dry or rainy season on the phytochemicals present in TI. TI samples were obtained from Asakakra Kwahu in the Eastern region of Southern Ghana. The climate of Ghana is tropical, and characterised by rainy and dry seasons (Logah et al., 2013). The northern part of the country (made up of Northern, Upper East and Upper West) has only one rainy season which occurs from April to September (Figure 5.1) (Abbam et al., 2018). On the other hand, the southern part is made of Ashanti, Brong Ahafo, Central, Eastern, Greater Accra, Volta and Western regions. The southern part, where the study sample was obtained, has two rainy seasons which occur from April to July and from September to November. The national mean annual rainfall is 1100 – 1900 mm. The dry season spans November to April (Abbam et al., 2018; Asante & Amuakwa-Mensah, 2015; Logah et al., 2013). There is approximately 12 h of daylight daily, and the temperature in the country fluctuates with season, with mean temperature of generally between 21 ºC and 35 ºC (Dickson et al., 1988).

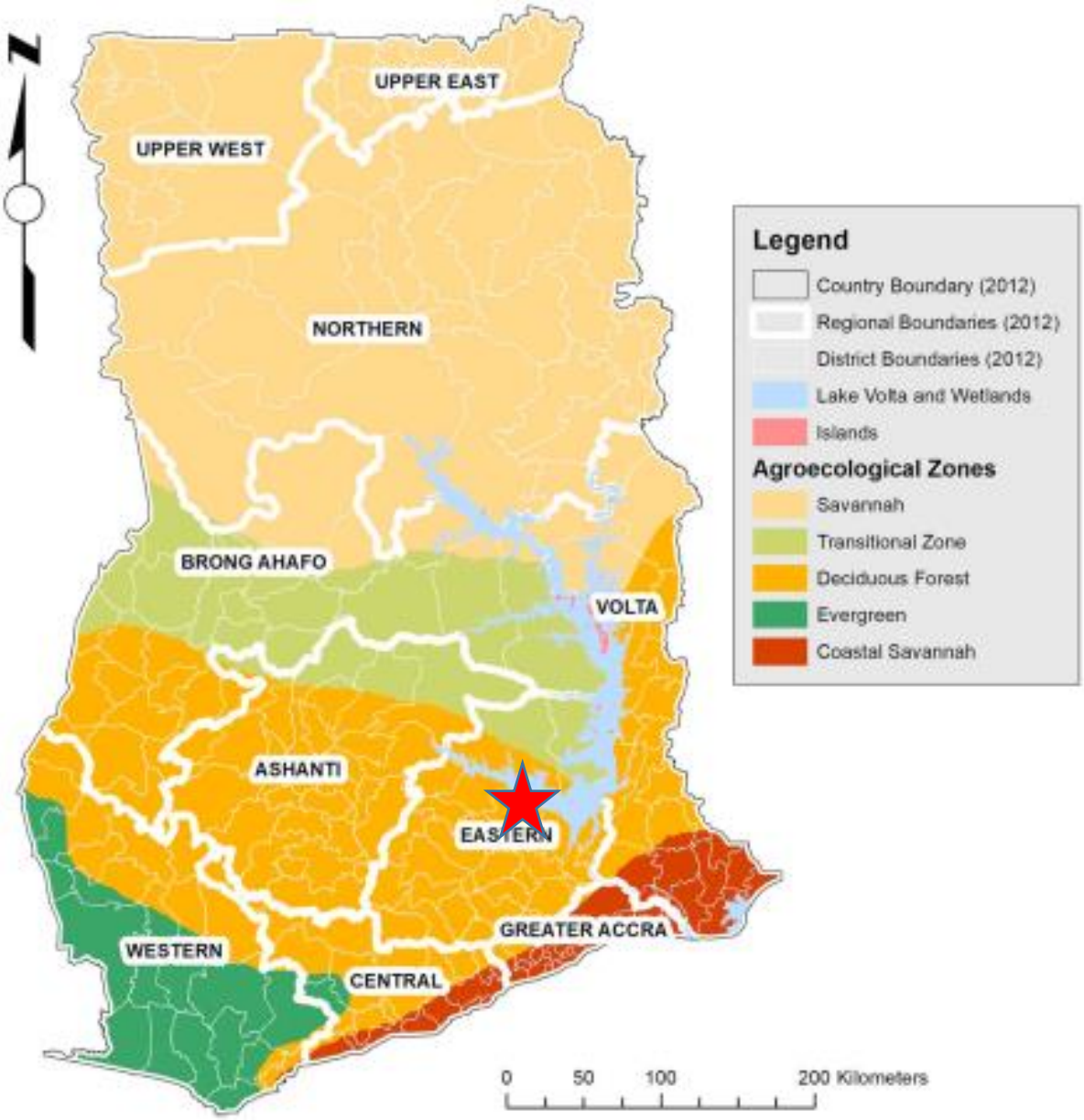


Figure 5.1: Regions of Ghana showing the ecological zones. Red star indicates the Eastern region where the TI sample was obtained for this study. Taken from Abbam et al. (2018).

Herbalists usually store medicinal plant samples for days to several months in the chain of production of their products, particularly herbal bitters (Chan et al., 2009). However, many of the plant metabolites are unstable and easily degraded, or are modified to other metabolites during storage (Javadi et al., 2015). For instance, flavonones can be modified into anthocyanins in raspberry (Xu et al., 2019). Moreover, depleted levels of phytochemical metabolites such as α-tocopherol, benzoic acid, catechin, cyclohexen-1-carboxylic acid, lycopene, myoinositol and stigmasterol were recorded *in Cosmos caudatus* stored at room temperature for 12 h as compared to samples stored for less than 12 h (Javadi

et al., 2015). Studies to determine the impact of storage on plant samples provide important information for herbalists in designing sustainable plant harvesting, processing and storage techniques for medicine production (Chan et al., 2009). The studies also provide knowledge on the "shelf-life" of plants used in traditional medicine (Suvarnakuta et al., 2011).

TI has been used in traditional medicine for the treatment of diuresis, general body pains, haemorrhoids, malaria, wounds and yellow fever (Akinyemi et al., 2006; Etukudo, 2003; Sitapha et al., 2013). To the best of my knowledge, there are no data showing the effect of storage on TI phytochemical compounds. Therefore, this research also compared phytochemical metabolites from freshly harvested TI sample with TI sample stored for 4 years.

From my observation, traditional medical practitioners in Ghana usually employ the use of various solvents for extraction of plant products for therapeutic purposes. Sometimes, the herbalists recommend the use of proportional mixing of solvents (such as alcohol, soda, vinegar and water). In addition, they frequently use and recommend soaking plant samples, such as flowers, leaves, bark or roots in water at room temperature overnight, or boiling in water for several hours. However, there are no data comparing the phytochemical profiles from traditional and more experimental chemical methods of extraction of TI. There are also no data showing herbalists preferential use of cold or hot extraction procedures on metabolites extracted from TI. Therefore, this research identified phytochemical compounds isolated by the method of extraction commonly used by traditional medical practitioners in Ghana (cold water or hot water extraction) with chemical extraction (i.e. sequential Soxhlet extraction). The research also compared the two commonly used traditional extraction methods: hot and cold-water extraction.

5.1.1 Aim:

To investigate the effects of season, storage, and water and chemical extraction methods on the phytochemical profile of TI.

5.1.2 Specific objectives:

- To compare the identity and amount of phytochemicals extracted from TI samples obtained in the dry season against samples obtained in the rainy season in Ghana.
- To investigate if there are differences in the identities and amount of phytochemicals extracted from fresh TI sample versus TI sample stored for 4 years.
- To evaluate the phytochemical profile of TI samples obtained from water versus chemical extraction.
- To compare the identity and amount of phytochemicals extracted from TI samples obtained using traditional methods of extraction by using hot water versus cold water for extraction.

5.2 METHODS

5.2.1 Effect of season on the phytochemical profile of TI.

Fresh TI samples were obtained and prepared either in September 2018 or February 2018 in the rainy and dry seasons respectively as described previously (2.2.1 and 2.2.2). For chemical extraction, each sample was extracted sequentially with petroleum ether, chloroform, ethyl acetate and ethanol as described previously (2.2.3) and the phytochemical compounds obtained from the samples were compared.

5.2.2 Effect of storage on the phytochemical profile of TI.

TI sample was obtained in the rainy season in September 2014, prepared and stored at room temperature for 4 years as described previously (2.2.1 and 2.2.2). Fresh TI sample was also obtained and prepared in the rainy season in September 2018. For chemical extraction, each sample was extracted sequentially with petroleum ether, chloroform, ethyl acetate and ethanol as described previously (2.2.3) and the phytochemical compounds obtained from the samples were compared.

5.2.3 TI phytochemical profile obtained from water extraction.

As described previously (5.2.1) TI sample obtained in the dry season (February 2018) was used in the main work throughout. This sample was used to compare metabolite profile for chemical extraction versus water extraction.

5.2.3.1 Hot water isolation of phytochemicals from TI.

TI sample (25 g) was weighed into a conical flask and 250 ml of distilled water at ambient temperature was added with gentle swirling of the flask for 2 min to ensure that the sample was soaked properly without forming bubbles. The sample was heated on a hot plate (FB 15001, Fisher Scientific, UK) at 100 ^{0}C for 2 h. The extracted sample in the water solution was allowed to cool to room temperature and then filtered with a vacuum pump through a 70 mm filter paper (FB 59017, Fisher Scientific, UK). The filtrate was freeze dried at -20 ^{0}C for 2 days to obtain dried powdered TI hot water extract for further analysis. This extraction procedure was carried out in triplicate and the extract stored at -20 ^{0}C until required for analysis.

5.2.3.2 Cold water isolation of phytochemicals from TI.

TI sample (25 g) was weighed into a conical flask and 250 ml of distilled water was added with gentle swirling of the flask for 2 min to ensure that the sample was soaked properly without forming bubbles. The conical flask containing the sample was covered with aluminium foil and allowed to stand for 72 hours and extraction was carried out at ambient temperature. The content of the flask was filtered with a vacuum pump through a 70 mm filter paper. The filtrate was freeze dried at -20 ^{0}C for 2 days to obtain dried powdered TI cold water extract. This extraction procedure was carried out in triplicate and the extract stored at -20 ^{0}C until required for analysis.

5.2.4 Identification and analysis of phytochemicals from TI samples.

The phytochemicals extracted from samples in sections 5.2.1 - 5.2.3 were identified as described previously in section 2.2.4. Venn diagrams were used to assess the relationship between the metabolites from the different treatments. The amount of the phytochemicals present in the samples were then compared using ANOVA, with $p<0.05$ considered as significant. The average amount of phytochemicals from TI was calculated from soxhlet extraction using petroleum ether, chloroform, ethyl acetate or ethanol, which was termed as chemical extraction, and compared with the average amount of phytochemicals recovered from water (cold and hot water) extractions. TI phytochemical compounds extracted using cold versus hot water were also compared.

5.3 RESULTS

5.3.1 Effect of season on the phytochemical profile of TI.

5.3.1.1 Effect of season on TI phytochemical profile extracted using petroleum ether.

Using petroleum ether for isolation, more metabolites were isolated from TI sample obtained in the rainy season compared with TI sample obtained in the dry season. A total number of 36 phytochemicals were identified across samples obtained in both the dry and rainy seasons. Phytochemicals identified from TI obtained in the dry season were taken as the control for comparisons relative to sample prepared in the rainy season. Certain acetophenones (especially 3,4,5-trimethoxyacetophenone; 4-hydroxyacetophenone), amines (especially spermidine), benzoic acids (especially 2,3-dihydroxybenzoic acid and 2,4-dihydroxybenzoic acid), coumarins (especially coumarin), flavonoids (especially phloridzin) and phenols (especially ethylferulate) were absent from the TI sample obtained in the rainy season. On the other hand, amines (especially dopamine); benzoic acids (especially syringic acid); cinnamic acids (especially cinnamic acid); benzaldehydes (especially p-hydroxybenzaldehyde); flavonoids (especially biochanin A, didymin, formononetin, hesperitin, imperatorin and morin); lignans (especially pinoresinol and secoisolariciresinol); phenols (2-hydroxybenzyl alcohol) or phenylpropionic acids (especially 4-hydroxy-3-methoxyphenylpropionic acid) were present in the TI sample obtained in the rainy season (Figure 5.2). Comparison of the metabolites present in both samples is shown in Table 5.1.

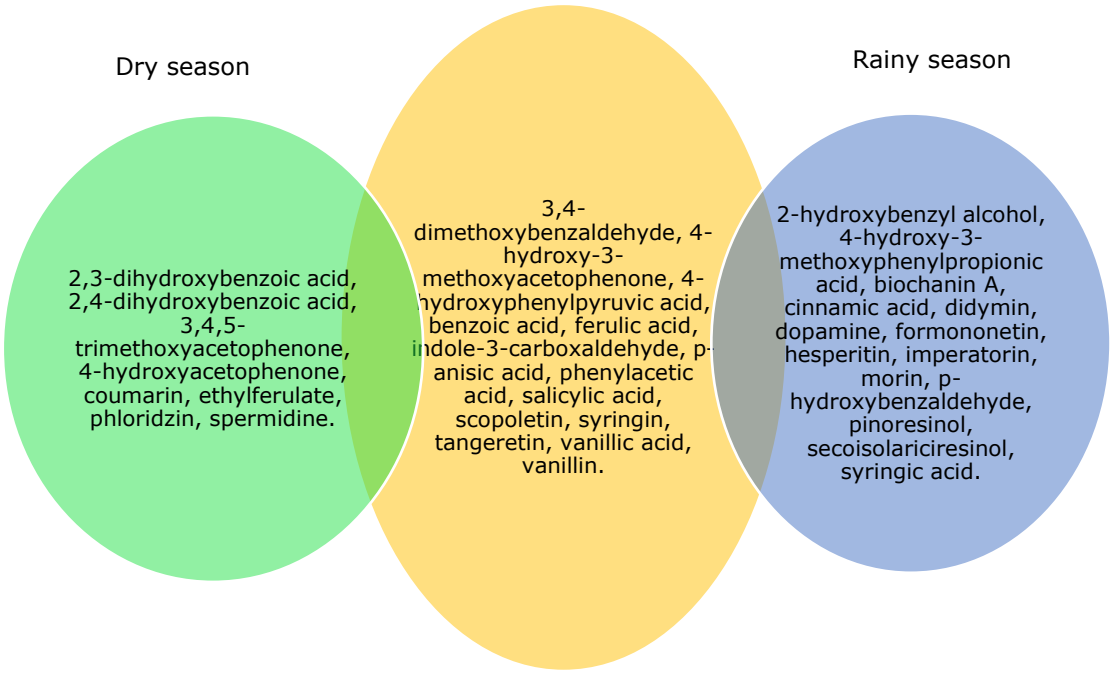


Figure 5.2: Relationship between the phytochemicals identified from TI obtained only in the dry season (green); both samples (gold); and only in the rainy season (blue) using petroleum ether.

Salicylic acid was the most abundant phytochemical, and phloridzin was the least abundant from TI sample obtained in the dry season extracted using petroleum ether. While 4-hydroxyphenylpyruvic acid was the most abundant phytochemical, syringic acid was the least measured extracted from TI sample obtained in the rainy season.

Significantly ($p<0.001$) lower amounts 4-hydroxy-3-methoxyacetophenone; ferulic acid; salicylic acid and scopoletin were measured in the TI sample obtained in the rainy season as compared to TI sample obtained in the dry season. Conversely, 4-hydroxyphenylpyruvic acid; phenylacetic acid and syringin amounts were significantly ($p<0.05$) higher in TI sample obtained in the rainy season as compared to TI sample obtained in the dry season (Table 5.1).

Table 5.1: Comparison of phytochemicals identified from TI samples obtained in the dry or rainy season and extracted using petroleum ether.

Phytochemical	Dry season	Rainy season
3,4-dimethoxybenzaldehyde	4.9 ± 1.1	9.3 ± 1.3
4-hydroxy-3-methoxyacetophenone	36.4 ± 6.2	0.2 ± 0.1***
4-hydroxyphenylpyruvic acid	540 ± 60.2	2796 ± 306.8***
Benzoic acid	291.6 ± 29.3	165.6 ± 31.1
Ferulic acid	15 ± 3.6	3.1 ± 0.7**
Indole-3-carboxaldehyde	2.2 ± 0.2	2.6 ± 0.4
P-anisic acid	31.1 ± 4.8	26.3 ± 5.1
Phenylacetic acid	12.2 ± 4.3	49.6 ± 6.5*
Salicylic acid	25280 ± 3026.7	71.2 ± 14.7***
Scopoletin	13.1 ± 3.3	1.5 ± 0.1***
Syringin	21.2 ± 4.2	55.2 ± 6.5*
Tangeretin	6.5 ± 2.1	7.6 ± 2.2
Vanillic acid	48.4 ± 6.9	39.8 ± 11.4
Vanillin	132.8 ± 23.7	210.4 ± 23.8

Data are presented as amount ± standard deviation (SD, pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to TI sample obtained in the dry season by ANOVA followed by Bonferroni's post hoc test.

5.3.1.2 Effect of season on TI phytochemical profile extracted using chloroform.

More metabolites were isolated from TI sample obtained in the dry season than from TI sample obtained in the rainy season for the extraction with chloroform which yielded 46 phytochemicals across both samples. Comparisons between the samples were made relative to the TI sample obtained in the dry season. Acetophenones (especially 4-hydroxy-3-methoxyacetophenone), amines (especially spermidine), benzaldehydes (especially 3,4-dimethoxybenzaldehyde and protocatachaldehyde), benzoic acids (especially gallic acid and protocatechuic acid), flavonoids (especially kaempferol, quercetin and taxifolin) or phenylpropionic acids (especially 4-hydroxyphenylpropionic acid) were not

present in the TI sample obtained in the rainy season. Contrary, amines (especially dopamine); cinnamic acids (especially cinnamic acid); flavonoids (especially didymin, formononetin, neohesperidin and phloretin); indoles (especially indole); phenols (especially 2-hydroxybenzyl alcohol) or phenylpropionic acids (especially 4-hydroxy-3-methoxyphenylpropionic acid) were found exclusively in the TI sample obtained in the rainy season (Figure 5.2). Comparison of the metabolites present in both samples is shown in Table 5.2.

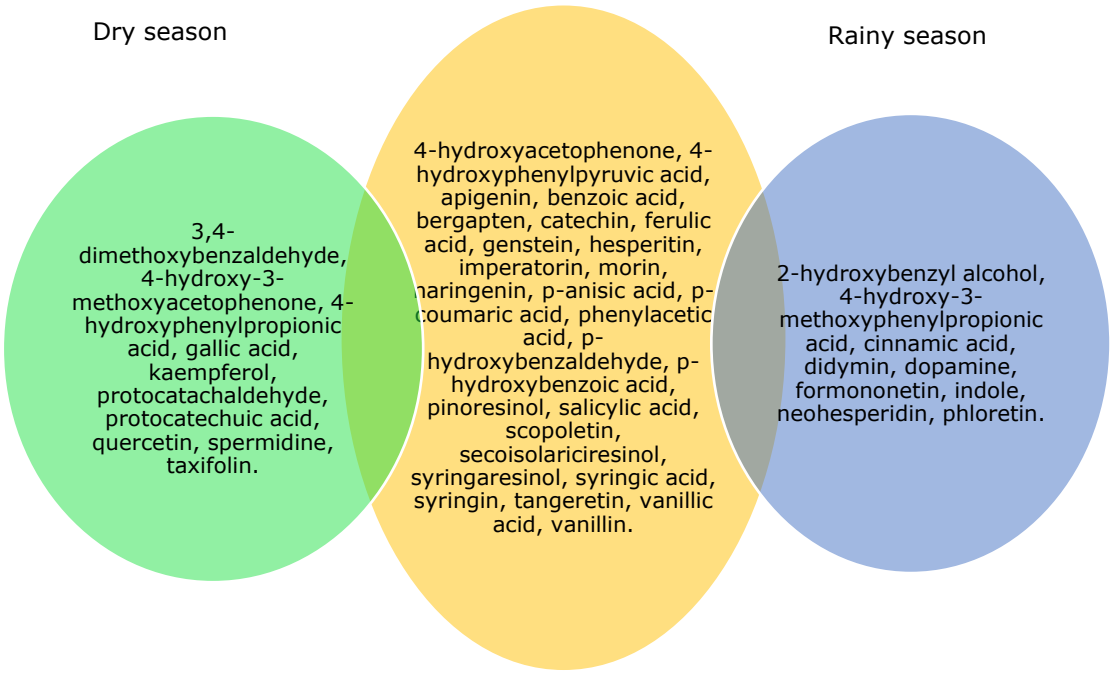


Figure 5.3: Relationship between the phytochemicals identified from TI obtained only in the dry season (green); both samples (gold); and only in the rainy season (blue) using chloroform.

With chloroform extraction, 4-hydroxyphenylpyruvic acid was the most abundant metabolite in TI sample obtained in the dry season, while benzoic acid was the most abundant metabolite in TI sample obtained in the rainy season and bergapten was the least abundant phytochemical in both samples.

Significantly ($p<0.05$) lower amounts of p-hydroxybenzoic acid; secoisolariciresinol; vanillic acid and vanillin were measured in TI sample obtained in the rainy season as compared to TI sample obtained in the dry season. Conversely, significantly ($p<0.05$) higher amounts of hesperitin; indole-3-carboxaldehyde; morin; phenylacetic acid and scopoletin were measured in TI sample obtained in the rainy season as compared to TI sample obtained in the dry season (Table 5.2).

Table 5.2: Comparison of phytochemicals identified from TI sample obtained in the dry or rainy season and extracted using chloroform.

Phytochemical	Dry season	Rainy season
4-hydroxyacetophenone	12.2 ± 2.8	4.1 ± 1.1
4-hydroxyphenylpyruvic acid	708.2 ± 65.5	408.3 ± 40.7
Apigenin	12.7 ± 2.6	9.6 ± 2.9
Benzoic acid	620.1 ± 79.7	468.3 ± 72.2
Bergapten	1.1 ± 0.2	0.8 ± 0.1
Catechin	25.9 ± 3.4	32.5 ± 5.7
Ferulic acid	92.4 ± 8.3	54.4 ± 7.9
Genstein	16.1 ± 2.1	13.2 ± 3.2
Hesperitin	17.7 ± 3.5	82.6 ± 13.4***
Imperatorin	3.5 ± 0.9	3.7 ± 0.7
Indole-3-carboxaldehyde	2.3 ± 0.4	9.6 ± 1.8*
Morin	100.4 ± 15.7	208.5 ± 34.2*
Naringenin	66.4 ± 11.2	77.2 ± 13.6
P-anisic acid	30.1 ± 4.6	18.6 ± 2.3
P-coumaric acid	11.2 ± 2.2	11.4 ± 2.5
Phenylacetic acid	2.4 ± 0.7	34.4 ± 4.9**

P-hydroxybenzaldehyde	34.4 ± 4.7	24.7 ± 5.1
P-hydroxybenzoic acid	55.6 ± 5.8	1.2 ± 0.4***
Pinoresinol	45.6 ± 5.6	29.9 ± 3.7
Salicylic acid	39.1 ±3.1	57.6 ± 8.2
Scopoletin	6.3 ± 1.2	41.2 ± 4.4*
Secoisolariciresinol	468.2 ± 38.4	145.6 ± 23.3*
Syringaresinol	444.0 ± 50.2	440.0 ± 46.7
Syringic acid	62.4 ± 12.1	37.6 ± 2.5
Syringin	77.6 ± 14.2	57.2 ± 9.6
Tangeretin	12.8 ± 3.1	9.0 ± 2.7
Vanillic acid	348.8 ± 47.8	96.4 ± 15.3**
Vanillin	359.6 ± 48.6	179.2 ± 23.3*

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to TI sample obtained in the dry season by ANOVA followed by Bonferroni's post hoc test.

5.3.1.3 Effect of season on TI phytochemical profile extracted using ethyl acetate.

Using ethyl acetate for isolation, more metabolites were isolated from TI sample obtained in the dry season than from TI sample obtained in the rainy season. A combined total of 66 phytochemicals were identified across both samples obtained in the dry and rainy seasons. In comparing the samples relative the sample obtained in the dry season, several acetophenones (especially 3,4,5-trimethoxyacetophenone and 4-hydroxy-3-methoxyacetophenone), benzaldehydes (especially protocatachaldehyde), benzenes (especially 1,2-hydroxybenzene), benzoic acids (especially 2,3-dihydroxybenzoic acid; 2,5-dihydroxybenzoic acid; 2,6-dihydroxybenzoic acid; 3,4-dimethoxybenzoic acid and anthranilic acid), cinnamic acids (especially cinnamic acid and sinapic acid), flavonoids (especially bergapten; epicatechin; epigallocatechin; kaempferol; morin; niacin; psoralen and scopoletin), indoles (especially indole-3-acetic acid),

lignans (especially syringaresinol), phenols (especially hydroxytyrosol), phenylacetic acid or phenyllactic acid were absent from the TI sample obtained in the rainy season. On the other hand, amine (especially dopamine), benzoic acid (especially p-anisic acid), benzaldehyde (especially p-hydroxybenzaldehyde), flavonoids (especially didymin; formononetin; hesperidin; imperatorin and quercetin-3-glucoside), or phenylpyruvic acids (especially 4-hydroxyphenylpyruvic acid) were found exclusively in the TI sample obtained in the rainy season (Figure 5.3). Comparison of the metabolites present in both samples is shown in Table 5.3.

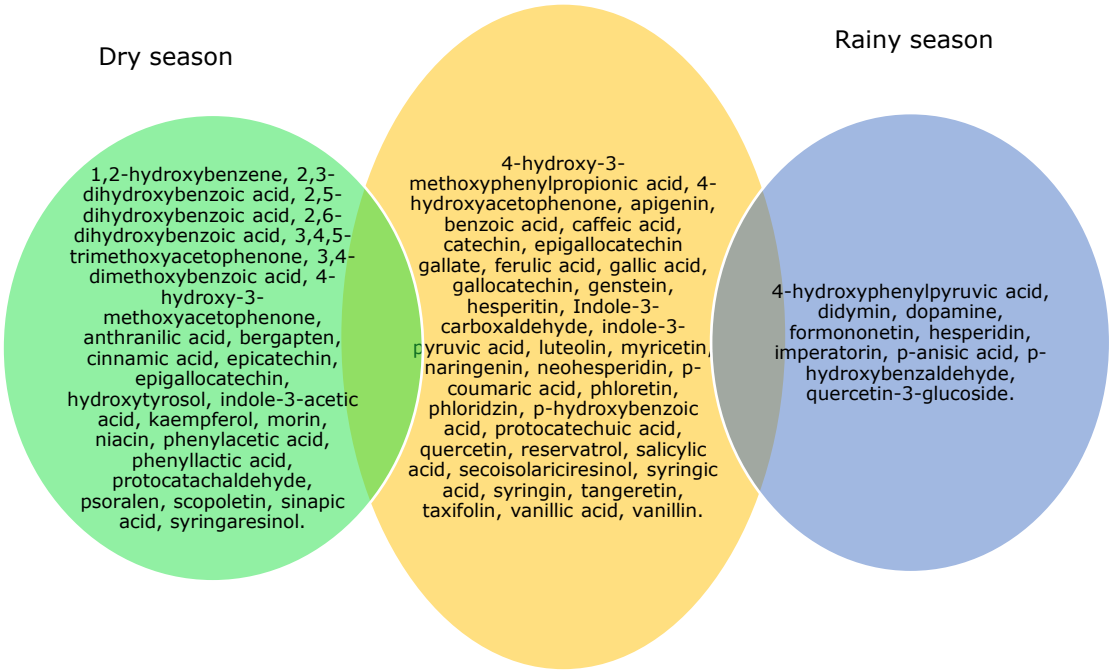


Figure 5.4: Relationship between the phytochemicals identified from TI obtained only in the dry season (green); both samples (gold); and only in the rainy season (blue) using ethyl acetate.

Catechin was the predominant phytochemical present in both samples. Syringin was the least abundant metabolite in TI sample collected in the dry season while 4-hydroxy-3-methoxyphenylpropionic acid was the least abundant metabolite in TI sample obtained in the rainy season. TI sample obtained in the rainy season showed significantly ($p<0.05$) lower amount of caffeic acid, ferulic acid, gallocatechin, resveratrol, secoisolariciresinol and syringic acid as compared to TI sample obtained in the dry season. On the other hand, the sample obtained in the rainy season showed significantly ($p<0.05$) higher amount of epigallocatechin gallate, hesperitin, indole-3-pyruvic acid, naringenin, phloridzin, syringin, tangeretin, vanillic acid and vanillin as compared to TI sample obtained in the dry season (Table 5.3).

Table 5.3: Comparison of phytochemicals identified from TI sample obtained in the dry or rainy season and extracted using ethyl acetate.

Phytochemical	Dry season	Rainy season
4-hydroxy-3-methoxyphenylpropionic acid	2.8 ± 0.3	1.6 ± 0.3
4-hydroxyacetophenone	5.3 ±1.2	8.1 ± 1.8
Apigenin	12.2 ± 2.3	14.9 ± 3.4
Benzoic acid	281.6 ±37.5	218.0 ± 34.6
Caffeic acid	391.6 ± 42.8	23.5 ± 4.1***
Catechin	18800 ± 4286.1	16280 ± 3621.3
Epigallocatechin gallate	34.3 ± 3.9	165.2 ± 31.4***
Ferulic acid	516.0 ±52.7	52.4 ± 8.4***
Gallic acid	3200 ± 512.3	3880 ± 486.8
Gallocatechin	5720 ± 652.5	684 ± 55.8***
Genstein	14.4 ± 4.1	16.9 ± 3.8
Hesperitin	3.3 ± 0.4	55.6 ± 5.1***
Indole-3-carboxaldehyde	7.0 ± 1.3	11.2 ± 2.4
Indole-3-pyruvic acid	201.2 ± 31.7	624 ± 121.7***
Luteolin	31.2 ± 2.5	33.2 ± 4.8
Myricetin	138.8 ± 32.3	94.8 ±19.8

Naringenin	42.4 ± 6.7	114.8 ± 16.8**
Neohesperidin	16.9 ± 2.5	25.9 ± 4.6
P-coumaric acid	58.8 ± 6.8	54.8 ±7.1
Phloretin	40.4 ±5.2	64 ± 7.3
Phloridzin	8.1 ± 1.2	75.2 ± 12.5***
P-hydroxybenzoic acid	6.2 ± 1.8	22.1 ± 3.2
Protocatechuic acid	249.2 ± 25.6	134 ± 18.7
Quercetin	68.8 ± 9.2	63.2 ± 8.4
Resveratrol	23.8 ± 5.4	5.1 ± 1.3*
Salicylic acid	76.4 ± 16.8	46.4 ± 13.3
Secoisolariciresinol	221.6 ± 39.7	112.8 ± 23.8*
Syringic acid	100.4 ± 21.5	17.1 ± 3.3***
Syringin	1.0 ± 0.1	17.2 ±3.4***
Tangeretin	5.9 ± 1.3	15.1 ± 2.4*
Taxifolin	112 ± 25.6	135.6 ± 24.3
Vanillic acid	84.8 ± 16.2	44.0 ± 8.1*
Vanillin	7.7 ± 1.4	48.2 ± 8.3*

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to TI sample obtained in the dry season by ANOVA followed by Bonferroni's post hoc test.

5.3.1.4 Effect of season on TI phytochemical profile extracted using ethanol.

With ethanol, 57 phytochemicals were identified from both samples obtained in the dry and rainy seasons. More metabolites were isolated from TI sample obtained in the dry season than from TI sample obtained in the rainy season (Figure 5.5). Comparisons were carried out relative to the sample obtained in the dry season. Acetophenones (especially 3,4,5-trimethoxyacetophenone; 4-hydroxy-3-methoxyacetophenone and 4-hydroxyacetophenone), benzaldehydes (protocatachaldehyde), benzene (especially 1,2,3-trihydroxybenzene), benzoic acids (especially anthranilic acid and syringic acid), flavonoids (especially epigallocatechin; hesperidin; kaempferol; niacin; resveratrol and scopoletin), lignans (especially syringaresinol), phenylacetic acid and phenyllactic acid were not present in TI sample obtained in the rainy season. Conversely, amine (particularly dopamine), benzoic acids (particularly p-anisic acid), flavonoids (particularly didymin; formononetin and morin), indole (particularly indole-3-pyruvic acid) or phenylpyruvic acids (particularly 4-hydroxyphenylpyruvic acid) were found exclusively in TI sample obtained in the rainy season (Figure 5.5). Comparison of the metabolites present in both samples is shown in Table 5.4.

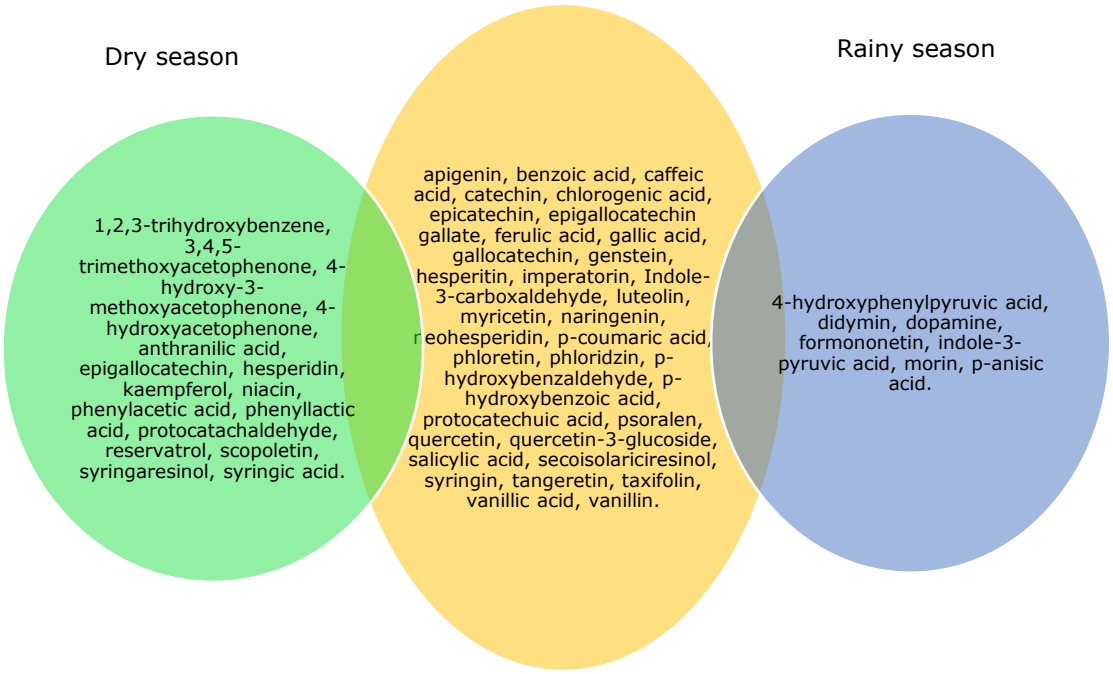


Figure 5.5: Relationship between the phytochemicals identified from TI obtained only in the dry season (green); both samples (gold); and only in the rainy season (blue) using ethanol.

TI extracted with ethanol showed that catechin was the predominant phytochemical identified from both samples. Phenyllactic acid was the least abundant metabolite in TI sample obtained in the dry season while psoralen was the least abundant phytochemical in TI sample obtained in the rainy season. Significantly ($p<0.05$) decreased amount of caffeic acid, epicatechin, epigallocatechin gallate, ferulic acid, gallic acid, gallocatechin, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, quercetin and vanillic acid were measured in TI sample obtained in the rainy season as compared to TI sample obtained in the dry season. Contrary, TI sample obtained in the rainy season showed significantly ($p<0.05$) higher amount of benzoic acid, chlorogenic acid, hesperitin and neohesperidin as compared to TI sample obtained in the dry season (Table 5.4).

Table 5.4: Comparison of phytochemicals identified from TI sample obtained from dry or rainy season and extracted using ethanol.

Phytochemical	Dry season	Rainy season
Apigenin	13.4 ± 3.2	12.4 ± 3.4
Benzoic acid	249.2 ± 31.6	476 ± 49.8*
Caffeic acid	303.6 ± 43.4	19.3 ± 4.2***
Catechin	17920 ± 3532.3	12480 ± 2642.4
Chlorogenic acid	10.1 ± 2.2	27.2 ± 3.4*
Epicatechin	2408 ± 147.7	56.2 ± 14.2***
Epigallocatechin gallate	1128 ± 122.4	114.4 ± 21.6***
Ferulic acid	400.9 ± 76.5	22.2 ± 3.2***
Gallic acid	15360 ± 4102.3	2684 ± 245.3***
Gallocatechin	4960 ± 672.5	456 ±59.8***
Genstein	15.4 ± 2.8	15.0 ± 3.2
Hesperitin	2.8 ± 0.3	57.6 ± 4.4***
Imperatorin	5.6 ± 1.5	4.8 ± 1.4
Indole-3-carboxaldehyde	17.2 ± 4.2	5.8 ± 1.3
Luteolin	28.1 ± 5.2	31.8 ± 4.5
Myricetin	139.2 ± 24.4	94.4 ± 21.2
Naringenin	61.6 ± 17.9	99.2 ± 15.3**
Neohesperidin	32.4 ± 5.7	124 ± 23.5***
P-coumaric acid	384.8 ± 34.8	38.8 ± 5.5***
Phloretin	44.4 ± 5.3	34.1 ± 5.1
Phloridzin	34.0 ± 4.5	58.4 ± 6.2
P-hydroxybenzaldehyde	6.6 ± 1.3	3.7 ± 0.9
P-hydroxybenzoic acid	40.8 ± 3.6	7.8 ± 2.2*
Protocatechuic acid	532 ± 54.8	119.2 ± 32.4**
Psoralen	3.2 ± 0.7	2.6 ± 0.4

Quercetin	90.4 ± 8.7	52.8 ± 6.3*
Quercetin-3-glucoside	30.1 ± 3.8	51.2 ± 4.4
Salicylic acid	36.1 ± 4.8	50.8 ± 4.9
Secoisolariciresinol	82.8 ± 5.7	82.4 ± 8.2
Syringin	10.4 ± 2.1	4.8 ± 1.2
Tangeretin	16.5 ± 3.4	13.1 ± 2.8
Taxifolin	100 ± 19.7	111.2 ± 24.4
Vanillic acid	100.4 ± 17.3	29.2 ± 7.2***
Vanillin	43.2 ± 6.1	42.3 ± 5.6

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to TI sample obtained in the dry season by ANOVA followed by Bonferroni's post hoc test.

5.3.2 Effect of 4-year storage at room temperature on the phytochemical profile of TI

5.3.2.1 Effect of storage on TI phytochemical profile extracted using petroleum ether.

Extraction of stored or fresh TI samples with petroleum ether yielded 37 phytochemicals. More metabolites were isolated from fresh TI sample than stored TI sample. Samples were compared relative to the fresh TI sample. Some amines (especially spermidine and spermine), benzoic acids (especially gallic acid) or flavonoids (especially bergapten; catechin; ethylferulate; gallocatechin; myricetin and quercetin) were present exclusively in stored TI sample. However, acetophenones (especially 4-hydroxy-3-methoxyacetophenone), benzaldehydes (especially 3,4-dimethoxybenzaldehyde and p-hydroxybenzaldehyde), benzoic acids (especially salicylic acid; p-anisic acid and syringic acid), cinnamic acids (especially cinnamic acid and ferulic acid), flavonoids (especially biochanin A; didymin; formononetin; hesperitin; imperatorin; morin and scopoletin), lignans (especially indole-3-carboxaldehyde; pinoresinol and secoisolariciresinol), phenols (especially 2-hydroxybenzyl alcohol), phenylacetic acid or phenylpropionic acids (especially 4-hydroxy-3-methoxyphenylpropionic acid) were not present in the stored sample as compared to the fresh TI sample

(Figure 5.6). Comparison of the metabolites present in both samples is shown in Table 5.5.

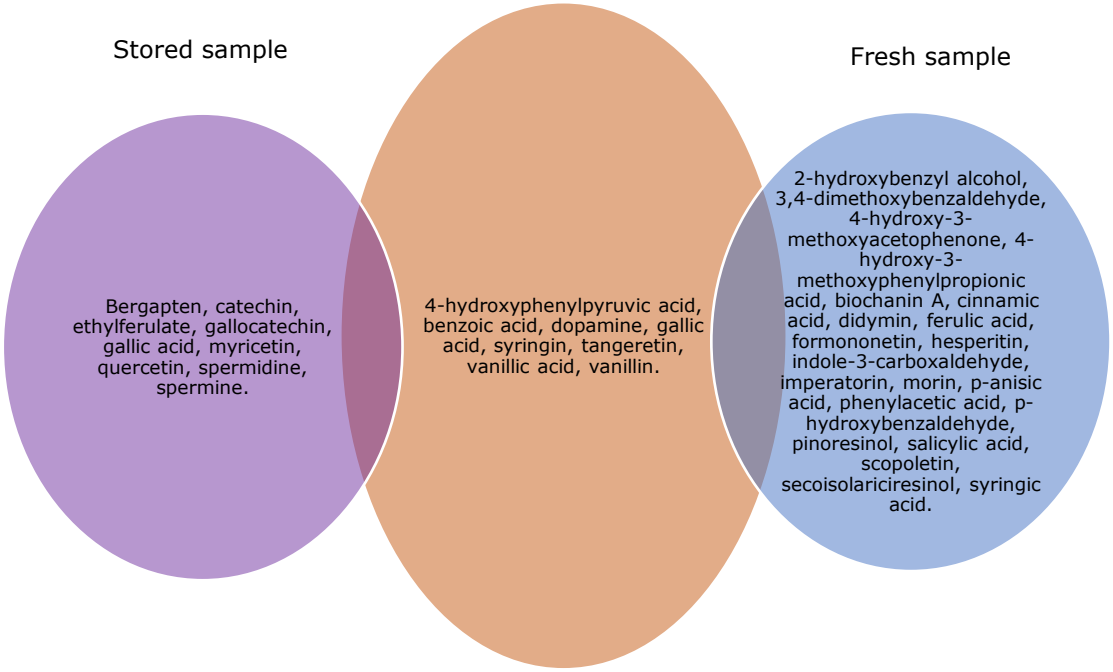


Figure 5.6: Relationship between phytochemicals identified from stored (purple); both samples (orange) or fresh TI sample using petroleum ether.

4-hydroxyphenylpyruvic acid was the predominant phytochemical identified from both stored and fresh TI samples while bergapten was the least abundant metabolite in stored sample and syringic acid was the least abundant metabolite in fresh TI sample. Significantly ($p<0.001$) lower amount of 4-hydroxyphenylpyruvic acid and syringin were measured in stored sample as compared to fresh sample (Table 5.5).

Table 5.5: Comparison of phytochemicals identified from fresh or stored TI sample using petroleum ether.

Phytochemical	Fresh sample	Stored sample
4-hydroxyphenylpyruvic acid	2796 ± 699	664 ± 166***
Benzoic acid	165.6 ± 41.4	130.4 ± 32.6
Dopamine	3.1 ± 0.7	3.7 ± 0.9
Syringin	55.2 ± 13.8	9.1 ± 2.2***
Tangeretin	7.6 ± 1.9	8.0 ± 2.0
vanillic acid	39.8 ± 9.9	33.6 ± 8.4
Vanillin	210.4 ± 52.6	147.6 ± 36.9

Data are presented as amount ± SD (pg/mg); n=3; and ***$p<0.001$ as compared to fresh TI sample by ANOVA followed by Bonferroni's post hoc test.

5.3.2.2 Effect of storage on TI phytochemical profile extracted using chloroform.

For chloroform extraction of fresh and stored TI samples, 52 phytochemicals were identified, with more metabolites isolated from stored TI sample than fresh TI sample. Comparison of the samples was carried out relative to the fresh TI sample. Certain acetophenones (especially 4-hydroxy-3-methoxyacetophenone), amines (especially spermine), benzaldehydes (especially 3,4-dimethoxybenzaldehyde), benzoic acids (especially 3,4-dimethoxybenzoic acid and gallic acid), flavonoids (especially ethylferulate; kaempferol; myricetin and taxifolin), indoles (especially indole-3-carboxylic acid), phenols (especially caffeine) or phenylpropionic acids (especially 3,4-dihydroxyphenylpropionic acid and 4-hydroxyphenylpropionic acid) were found only in stored TI sample while phytochemicals such as benzaldehydes (especially protocatachaldehyde), benzoic

acids (especially p-anisic acid and protocatechuic acid), cinnamic acid (especially cinnamic acid) flavonoids (especially didymin; formononetin; imperatorin; neohesperidin and quercetin), phenols (especially 2-hydroxybenzyl alcohol) or phenylacetic acid were not found in the sample as compared to the fresh TI sample (Figure 5.7). The common metabolites present in both samples are compared in Table 5.6.

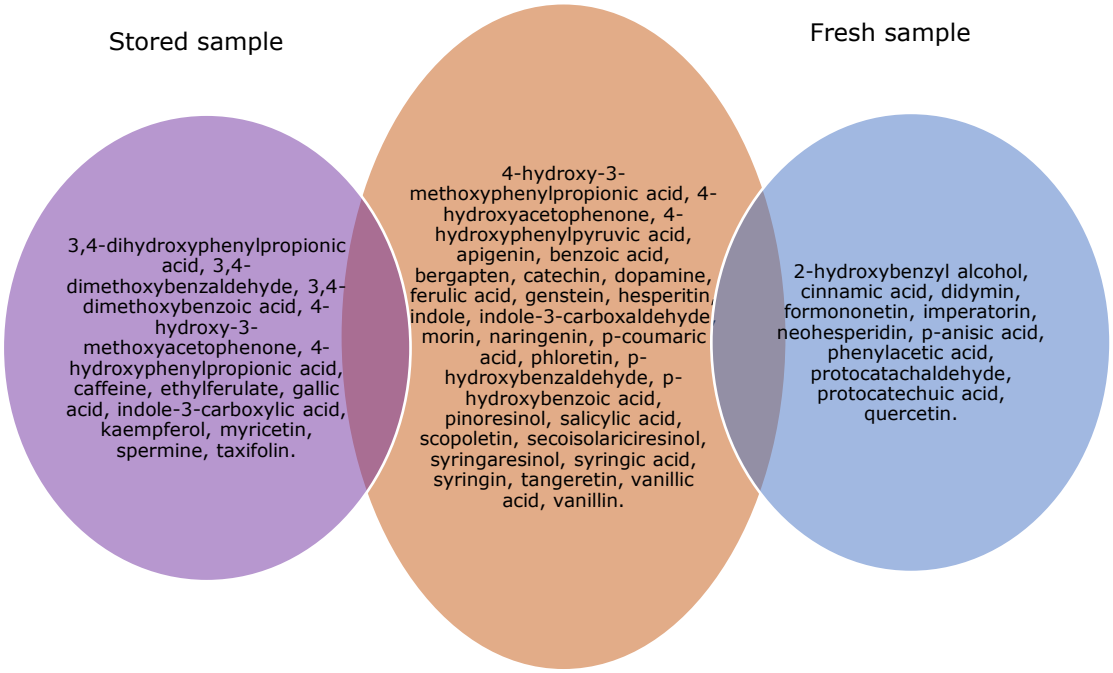


Figure 5.7: Relationship between phytochemicals identified from stored (purple); both samples (orange) or fresh TI sample using chloroform.

4-hydroxyphenylpropionic acid was the predominant phytochemical identified from the stored TI sample and benzoic acid was the most predominant in fresh sample while bergapten was the least abundant metabolite in both samples when the extraction was carried out using chloroform.

Stored sample showed a significantly ($p<0.05$) higher amount of 4-hydroxyacetophenone; benzoic acid; ferulic acid; p- coumaric acid; phloretin; p-hydroxybenzaldehyde; p-hydroxybenzoic acid; pinoresinol; salicylic acid; secoisolariciresinol; syringic acid; syringin; vanillic acid and vanillin as compared to fresh sample. Conversely, stored sample showed a significantly ($p<0.05$) lower amount of hesperitin and morin as compared to fresh sample (Table 5.6).

Table 5.6: Comparison of phytochemicals identified from stored or fresh TI sample using chloroform.

Phytochemical	Fresh sample	Stored sample
4-hydroxy-3-methoxyphenylpropionic acid	17.5 ± 4.3	14.6 ± 3.6
4-hydroxyacetophenone	4.1 ± 1.0	64.0 ± 16.0***
4-hydroxyphenylpyruvic acid	408 ± 102	504 ± 126
Apigenin	9.6 ± 2.4	14.8 ± 3.7
Benzoic acid	468 ± 117	1204 ± 301**
Bergapten	0.8 ± 0.2	0.8 ± 0.2
Catechin	32.5 ± 8.1	37.7 ± 9.4
Dopamine	2.9 ± 0.7	3.7 ± 0.9
Ferulic acid	54.4 ± 13.6	400 ± 96.4***
Genstein	13 ± 3.2	18 ± 4.5
Hesperitin	82 ± 18.9	21.9 ± 5.4**
Indole	11.4 ± 2.8	6.2 ± 1.5
Indole-3-carboxaldehyde	9.6 ± 2.4	5.2 ± 1.3
Morin	208 ± 43.8	98 ± 19.5*
Naringenin	77.2 ± 14.8	106 ± 22.8
P-coumaric acid	11.4 ± 2.8	97.6 ± 21.7***

Phloretin	3.7 ± 0.9	15.0 ± 3.5**
P-hydroxybenzaldehyde	24.7 ± 6.1	121.6 ± 30.4***
P-hydroxybenzoic acid	1.2 ± 0.3	149.6 ± 37.4***
Pinoresinol	29.9 ± 7.4	60.4 ± 15.1*
Salicylic acid	57.6 ± 7.4	142.8 ± 35.7*
Scopoletin	41.2 ± 9.8	2.7 ± 0.6
Secoisolariciresinol	145.6 ± 36.4	572 ± 143**
Syringaresinol	440 ±110	496 ± 124
Syringic acid	37.6 ± 9.4	234 ± 58.5***
Syringin	57.2 ± 9.4	271.2 ± 52.8***
Tangeretin	9.0 ± 2.2	9.4 ± 2.3
Vanillic acid	96.4 ± 24.1	1200 ± 300***
Vanillin	179.2 ± 44.8	1276 ± 319***

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to fresh TI sample by ANOVA followed by Bonferroni's post hoc test.

5.3.2.3 Effect of storage on TI phytochemical profile extracted using ethyl acetate.

50 phytochemicals were identified across both samples; with more metabolites isolated from fresh TI sample than stored TI sample (Figure 5.8). Relative to the fresh TI sample, phytochemical compounds such as acetophenones (especially 4-hydroxy-3-methoxyacetophenone), amines (especially spermine), benzaldehydes (especially 3,4-dimethoxybenzaldehyde), benzoic acids (especially 3,4-dimethoxybenzoic acid and gallic acid), flavonoids (especially ethylferulate; kaempferol; myricetin and taxifolin), indoles (especially indole-3-carboxylic acid), phenols (especially caffeine) or phenylpropionic acids (especially 3,4-dihydroxyphenylpropionic acid and 4-hydroxyphenylpropionic acid) were found only in stored sample. Storage of TI sample resulted in certain phytochemicals not identified in the sample as compared to the fresh TI sample. These were benzaldehydes (especially protocatachaldehyde), benzoic acids (especially p-anisic acid and protocatechuic acid), cinnamic acids (especially cinnamic acid), flavonoids (especially didymin; formononetin; imperatorin; neohesperidin and

quercetin), phenols (especially 2-hydroxybenzyl alcohol) or phenylacetic acid (Figure 5.8). The common metabolites present in both samples are compared in Table 5.7.

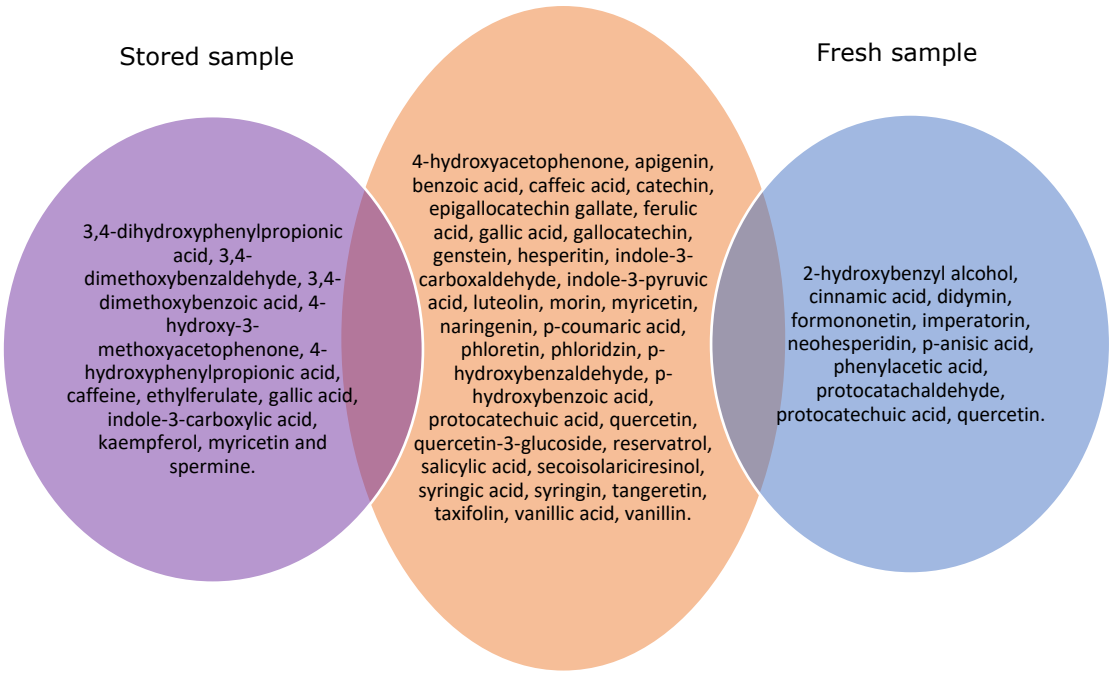


Figure 5.8: Relationship between the phytochemicals identified from old (purple); both samples (orange) or fresh (blue) TI sample using ethyl acetate.

Gallic acid was the predominant phytochemical identified from stored TI sample and catechin from fresh TI sample. Indole-3-carboxaldehyde was the least abundant phytochemical in stored TI sample while 4-hydroxy-3-methoxyphenylpropionic acid was the least abundant identified in fresh TI sample using ethyl acetate for the extraction.

Stored TI sample showed significantly ($p<0.05$) higher amounts of 4-hydroxyacetophenone; ferulic acid; gallic acid; p-coumaric acid; phloridzin; p-hydroxybenzaldehyde; p-hydroxybenzoic acid; protocatechuic acid; quercetin; secoisolariciresinol; syringic acid; syringin; vanillic acid and vanillin as compared to fresh TI sample. On the other hand, stored TI sample showed significantly ($p<0.05$) lower amount of benzoic acid, epigallocatechin gallate, hesperitin and indole-3-carboxaldehyde as compared to fresh sample (Table 5.7).

Table 5.7: Comparison of phytochemicals identified from stored or fresh TI sample using ethyl acetate.

Phytochemical	Fresh sample	Stored sample
4-hydroxyacetophenone	8.1 ± 1.8	36.9 ± 5.1*
Apigenin	14.9 ± 3.0	15.4 ± 3.1
Benzoic acid	218 ± 16.7	74.8 ± 9.9*
Caffeic acid	23.5 ± 5.2	42.8 ± 8.7
Catechin	16280 ± 1843.5	14000 ± 1261.2
Epigallocatechin gallate	165.2 ± 21.7	86.8 ± 16.4*
Ferulic acid	52.4 ± 13.6	135.2 ± 15.9*
Gallic acid	3880 ± 560.2	14720 ± 1525.6*
Gallocatechin	684 ± 54.5	800 ± 67.9
Genstein	16.9 ± 2.3	20.9 ± 3.6
Hesperitin	55.6 ± 5.4	15.4 ± 3.3*
Indole-3-carboxaldehyde	11.2 ± 1.4	2.6 ± 0.3*
Indole-3-pyruvic acid	624 ± 39.6	724 ± 43.8
Luteolin	33.2 ± 5.4	59.2 ± 8.3
Morin	82 ± 7.4	51.2 ± 7.1
Myricetin	94.8 ± 6.5	104.4 ± 8.6
Naringenin	114.8 ± 11.5	122.4 ± 10.3
P-coumaric acid	54.8 ± 6.5	143.2 ± 21.3*
Phloretin	64 ± 6.1	65.6 ± 5.5
Phloridzin	75.2 ± 12.5	158.8 ± 24.2*
P-hydroxybenzaldehyde	8.9 ± 1.4	37.4 ± 8.1*
P-hydroxybenzoic acid	22.1 ± 4.2	157.6 ± 16.9***
Protocatechuic acid	134 ± 17.4	1900 ± 354.6***
Quercetin	63.2 ± 10.3	125.2 ± 16.7*
Quercetin-3-glucoside	65.6 ± 8.5	94.2 ± 12.6

Resveratrol	5.1 ± 1.7	8.4 ± 1.4
Salicylic acid	46.4 ± 5.6	54.4 ± 8.3
Secoisolariciresinol	112.8 ± 16.2	261.2 ± 34.5*
Syringic acid	17.1 ± 2.9	110.4 ± 23.5***
Syringin	17.2 ± 3.4	110.1 ± 16.8***
Tangeretin	15.1 ± 2.5	6.9 ± 1.2
Taxifolin	135.6 ± 18.7	211.2 ± 28.4
Vanillic acid	44.3 ± 5.6	428 ± 40.1***
Vanillin	48.8 ± 7.3	416 ± 42.7***

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to fresh TI sample by ANOVA followed by Bonferroni's post hoc test.

5.3.2.4 Effect of storage on TI phytochemical profile extracted using ethanol.

A combined total of 50 phytochemicals were isolated across both stored and fresh TI samples using ethanol. As before, more metabolites were present in the fresh sample than in the stored sample. Comparison of the samples relative to the fresh sample showed that benzaldehydes especially 3,4-dimethoxybenzaldehyde and protocatachaldehyde), benzoic acids (especially kynurenic acid and syringic acid), flavonoids (especially kaempferol and reservatrol) or lignans (especially pinoresinol and syringaresinol) were present only in stored TI sample. Conversely, metabolites such as amines (particularly dopamine), benzoic acids (particularly chlorogenic acid and p-anisic acid) or flavonoids (particularly didymin; epicatechin; formononetin; hesperidin; imperatorin; morin; and neohesperidin) were not present in the stored sample (Figure 5.9). The common metabolites in both samples are compared in Table 5.8.

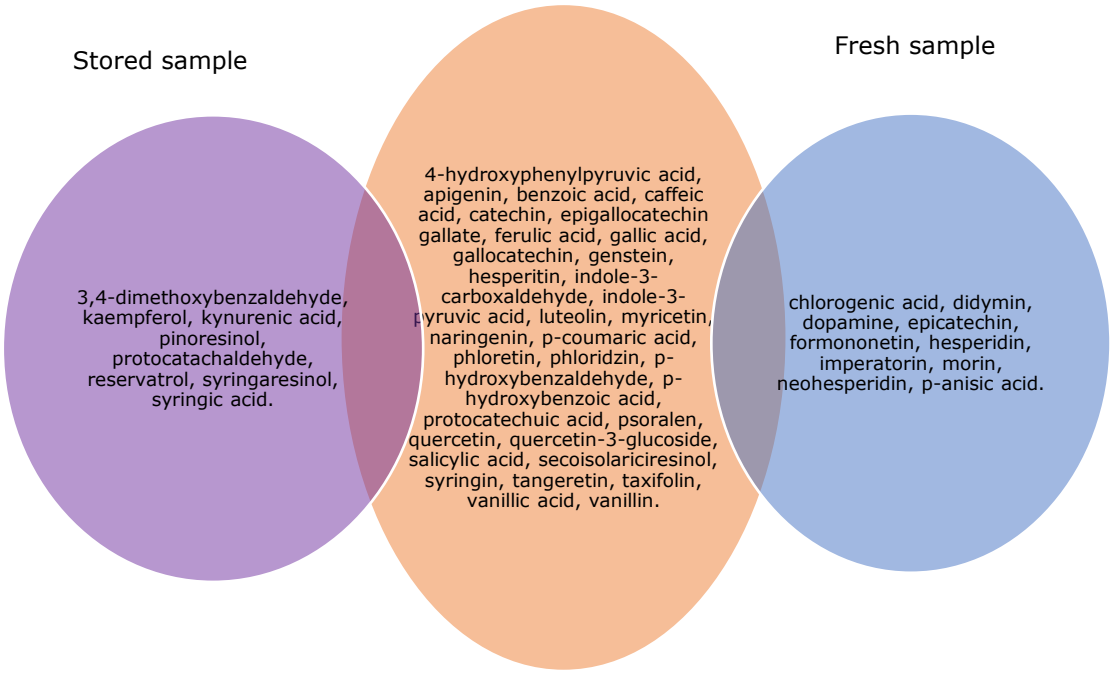


Figure 5.9: Relationship between the phytochemicals identified from stored (purple); both samples (orange) or fresh (blue) TI sample using ethanol.

Gallic acid was the predominant phytochemical measured from stored TI sample and catechin from fresh TI sample while psoralen was the least abundant phytochemical measured in both samples.

Significantly ($p<0.05$) higher amounts of 4-hydroxyphenylpyruvic acid; gallic acid; indole-3-pyruvic acid; p-hydroxybenzoic acid; protocatechuic acid; quercetin; secoisolariciresinol; syringin; vanillic acid and vanillin were measured in stored TI sample as compared to fresh TI sample. Conversely, significantly ($p<0.05$) lower amounts of benzoic acid; caffeic acid; catechin; epigallocatechin gallate; gallocatechin; hesperitin; phloridzin; quercetin-3-glucoside and salicylic acid were measured in stored TI sample as compared to fresh TI sample (Table 5.8).

Table 5.8: Comparison of phytochemicals identified from stored or fresh TI sample using ethanol.

Phytochemical	Fresh sample	Stored sample
4-hydroxyphenylpyruvic acid	608 ±152	1560 ± 390***
Apigenin	12.4 ± 3.1	15.2 ± 3.8
Benzoic acid	476 ± 119	209.2 ± 52.3***
Caffeic acid	19.3 ± 4.8	8.4 ± 2.1**
Catechin	12480 ± 3120	5640 ± 1410***
Epigallocatechin gallate	114.4 ± 28.6	42 ± 10.5***
Ferulic acid	22.2 ± 5.5	32.8 ± 8.2
Gallic acid	2684 ± 671	6080 ± 1520***
Gallocatechin	456 ± 114	273.2 ± 68.3**
Genstein	15.0 ± 3.7	17.0 ± 4.2
Hesperitin	57.6 ± 14.4	8.2 ± 2.0***
Indole-3-carboxaldehyde	5.8 ± 1.4	6.0 ± 1.5
Indole-3-pyruvic acid	844 ± 211	1400 ± 350*
Luteolin	31.8 ± 7.9	50 ± 12.5
Myricetin	94.4 ± 23.6	101.2 ± 25.3
Naringenin	99.2 ± 24.8	105.6 ± 26.4
P-coumaric acid	38.8 ± 9.7	48.8 ± 12.2
Phloretin	34.1 ± 8.5	26.2 ± 6.5
Phloridzin	58.4 ± 14.6	19.7 ± 4.9***
P-hydroxybenzaldehyde	3.8 ± 0.9	8.0 ± 2.0
P-hydroxybenzoic acid	7.8 ± 1.9	40.8 ± 9.6***
Protocatechuic acid	119.2 ± 29.8	656 ± 164***
Psoralen	2.6 ± 0.6	3.6 ± 0.9
Quercetin	52.8 ± 13.2	90.4 ± 22.6*
Quercetin-3-glucoside	51.2 ± 12.7	25.8 ± 6.4**
Salicylic acid	50.8 ± 12.3	21.6 ± 5.4**

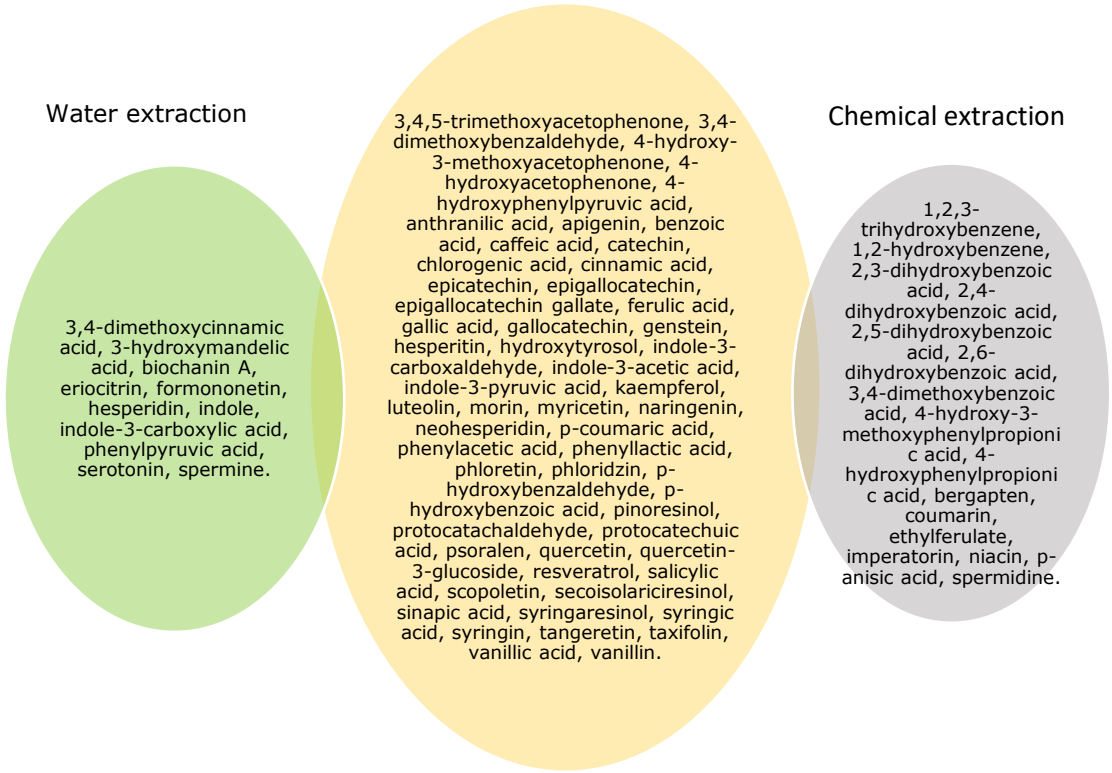
Secoisolariciresinol	82.4 ± 20.6	249.6 ± 62.4***
Syringin	4.8 ± 1.2	32.1 ± 8.0***
Tangeretin	13.1 ± 3.2	10.1 ± 2.5
Taxifolin	111.2 ± 27.8	207.2 ± 51.8
Vanillic acid	29.2 ± 7.3	134.4 ± 33.6***
Vanillin	42 ± 10.5	138 ± 34.5***

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to fresh TI sample by ANOVA followed by Bonferroni's post hoc test.

5.3.3 TI phytochemical profile obtained from water extraction.

5.3.3.1 TI phytochemical profile obtained from water versus chemical extraction.

A combined total of 82 phytochemicals were identified from both chemical and water extraction of TI sample obtained from dry season. More metabolites were isolated by using chemical extraction as compared to water extraction. Comparisons between the samples were carried out relative to chemical extraction. Phytochemicals including amines (particularly serotonin and spermine), cinnamic acids (particularly 3,4-dimethoxycinnamic acid), flavonoids (particularly biochanin A; eriocitrin; formononetin and hesperidin), indoles (particularly indole and indole-3-carboxylic acid), mandelic acids (particularly 3-hydroxymandelic acid) or phenylpyruvic acid (particularly phenylpyruvic acid) were found only using water extraction. Conversely, phytochemicals such as amines (particularly spermidine), benzoic acids (2,3-dihydroxybenzoic acid; 2,4-dihydroxybenzoic acid; 2,5-dihydroxybenzoic acid; 2,6-dihydroxybenzoic acid; 3,4-dimethoxybenzoic acid and p-anisic acid), benzenes (particularly 1,2,3-trihydroxybenzene and 1,2-hydroxybenzene), coumarins (particularly coumarin), flavonoids (particularly bergapten; ethylferulate; imperatorin and niacin) or phenylpropionic acids (particularly 4-hydroxy-3-methoxyphenylpropionic acid and 4-hydroxyphenylpropionic acid) were not present when using water extraction for TI sample (Figure 5.10). Statistical comparison of the common metabolites found using both extraction methods is shown in Table 5.9.



5.10: Distribution of phytochemicals between water (green); both extractions (gold) or chemical extraction (grey) from TI sample.

Using both water extraction and chemical extraction, catechin was the most abundant phytochemical present while psoralen was the least abundant phytochemical.

3,4-dimethoxybenzaldehyde; 4-hydroxyphenylpyruvic acid; apigenin; benzoic acid; catechin; chlorogenic acid; epigallocatechin gallate; gallocatechin; genstein; hydroxytyrosol; indole-3-acetic acid; indole-3-carboxaldehyde; indole-3-pyruvic acid; kaempferol; luteolin; myricetin; naringenin; phloretin; phloridzin; p-hydroxybenzoic acid; pinoresinol; protocatachaldehyde; protocatechuic acid; quercetin; quercetin-3-glucoside; reservatrol; scopoletin; sinapic acid; syringaresinol and taxifolin amounts were significantly ($p<0.05$) higher for water extraction as compared to chemical extraction. On the other hand, significantly ($p<0.05$) lower amounts of phenyllactic acid and salicylic acid were measured using water extraction as compared to chemical extraction (Table 5.9).

Table 5.9: Comparison of phytochemicals identified from TI sample using water or chemical extraction.

Phytochemical	Chemical extraction	Water extraction
3,4,5-trimethoxyacetophenone	1.6 ± 0.8	4.2 ± 1.1
3,4-dimethoxybenzaldehyde	1.6 ± 0.4	13.3 ± 3.8*
4-hydroxy-3-methoxyacetophenone	12.4 ± 2.3	20.0 ± 4.5
4-hydroxyacetophenone	8.0 ± 2.1	44.2 ± 14.2
4-hydroxyphenylpyruvic acid	312 ± 92.6	882 ± 118.9**
Anthranilic acid	3.5 ± 1.2	1.8 ± 0.6
Apigenin	9.6 ± 2.8	37.2 ± 16.8*
Benzoic acid	360.6 ± 89.2	793.8 ± 164.3*
Caffeic acid	173.8 ± 69.6	211.8 ± 51.2
Catechin	9186.4 ± 1246	40220 ± 12130***
Chlorogenic acid	2.5 ± 0.8	11.1 ± 2.7*
Cinnamic acid	1.7 ± 0.5	3.2 ± 1.0
Epicatechin	1403 ± 450.4	2264 ± 560.6
Epigallocatechin	1076 ± 463.6	1968 ± 590.4
Epigallocatechin gallate	290.5 ±50.4	5240 ± 1201***
Ferulic acid	255.8 ± 46.8	210.6 ± 57.9
Gallic acid	4649.4 ±998.8	6520 ± 2591
Gallocatechin	2670 ± 989.6	8360 ± 2131***
Genstein	11.4 ± 2.8	41.1 ± 11.3*
Hesperitin	5.7 ± 2.3	8.4 ± 3.9
Hydroxytyrosol	39.8 ± 13.5	86.4 ± 18.6*
Indole-3-acetic acid	5.2 ± 1.2	31.3 ± 5.8**
Indole-3-carboxaldehyde	7.2 ± 1.5	51.6 ± 4.7**
Indole-3-pyruvic acid	50.3 ± 9.3	514 ± 92.7***
Kaempferol	29.5 ± 8.7	137 ± 18.9***

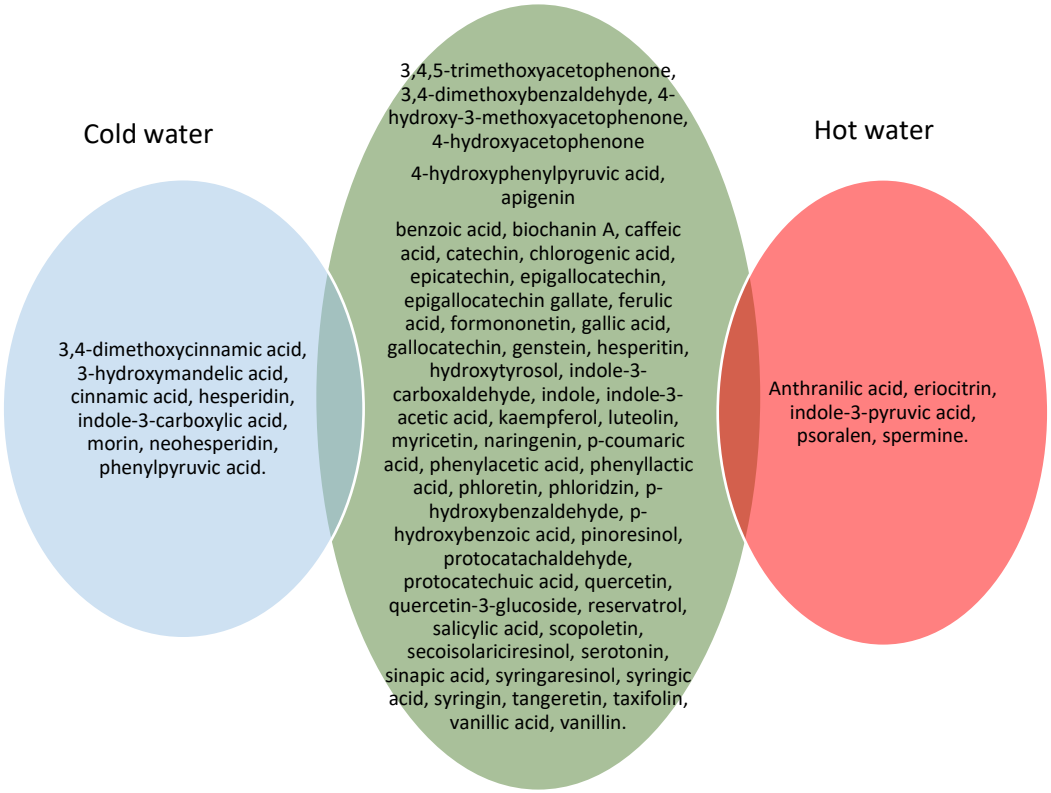
Luteolin	14.8 ±5.5	69.6 ± 12.8***
Morin	25.1 ± 4.6	36.8 ± 6.8
Myricetin	69.5 ± 18.4	198.2 ± 46.4**
Naringenin	42.6 ± 8.2	232.4 ± 51.5***
Neohesperidin	12.3 ± 4.3	14.8 ± 4.1
P-coumaric acid	113.7 ± 17.4	167.6 ± 19.6
Phenylacetic acid	16.2 ± 4.5	30.2 ± 5.9
Phenyllactic acid	10.1 ± 2.8	2.9 ± 0.9**
Phloretin	21.2 ± 3.9	322.6 ± 53.4***
Phloridzin	10.9 ± 3.5	116.4 ± 23.6***
P-hydroxybenzaldehyde	10.2 ± 3.3	16.9 ± 4.4
P-hydroxybenzoic acid	25.6 ± 4.1	91 ± 17.3***
Pinoresinol	11.4 ± 2.2	35.1 ± 5.1**
Protocatachaldehyde	51.5 ± 17.9	240.8 ± 41.5***
Protocatechuic acid	200.1 ± 55.7	692 ± 112.4***
Psoralen	1.1 ± 0.4	0.8 ± 0.2
Quercetin	47.7 ± 8.2	216.4 ± 46.7***
Quercetin-3-glucoside	7.5 ± 2.2	133.2 ± 34.6***
Reservatrol	8.9 ± 1.8	46.4 ± 8.3**
Salicylic acid	6357.9 ± 1087.8	44.3 ± 5.8***
Scopoletin	20.2 ± 3.7	78 ± 20.3**
Secoisolariciresinol	193.1 ± 38.6	199.4 ± 40.5
Sinapic acid	2.8 ± 0.9	7.6 ± 2.3*
Syringaresinol	405 ± 100.2	1114 ± 345.7***
Syringic acid	49.1 ± 14.3	55.2 ± 14.9
Syringin	27.5 ± 4.8	25.8 ± 5.2
Tangeretin	10.4 ± 3.2	7.62 ± 2.1
Taxifolin	55.1 ± 15.3	329.2 ± 57.4***

Vanillic acid	145.6 ± 48.4	182 ± 45.1
Vanillin	135.8 ± 40.8	103.4 ± 36.7

Data are presented as amount ± SD (pg/mg); n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to chemical extraction by ANOVA followed by Bonferroni's post hoc test.

5.3.3.2 TI phytochemical profile obtained from cold water versus hot water.

For water extraction, 66 phytochemicals were identified using both cold and hot water. More phytochemicals were found using cold water than hot water isolation. The comparison of the metabolites of water extractions was carried out relative to the hot water. Metabolites such as cinnamic acids (especially 3,4-dimethoxycinnamic acid and cinnamic acid), mandelic acids (especially 3-hydroxymandelic acid), flavonoids (especially hesperidin; morin and neohesperidin), indoles (especially indole-3-carboxylic acid) or phenylpyruvic acids (especially phenylpyruvic acid) were found only in cold water extracts. Conversely, amines (particularly spermine), benzoic acids (especially anthranilic acid), flavonoids (especially eriocitrin and psoralen) or indoles (especially indole-3-pyruvic acid) were absent in the cold-water extracts of TI (Figure 5.11). Table 5.10 showed comparison of the common phytochemicals present using both extraction methods.



5.11: Distribution of phytochemicals between cold (blue); both (green) or hot (pink) water extraction from TI sample.

Catechin was the most abundant phytochemical detected using both cold and hot water extractions while formononetin was the least abundant phytochemical measured using cold water extraction and sinapic acid was the least abundant phytochemical measured using hot water extraction.

A significantly ($p<0.05$) higher amounts of 3,4,5-trimethoxyacetophenone; 4-hydroxy-3-methoxyacetophenone; 4-hydroxyacetophenone; benzoic acid; caffeic acid; catechin; ferulic acid; gallic acid; hydroxytyrosol; indole; indole-3-acetic acid; indole-3-carboxaldehyde; kaempferol; naringenin; p-coumaric acid; phenylacetic acid; phloretin; p-hydroxybenzaldehyde; p-hydroxybenzoic acid; protocatachaldehyde; protocatechuic acid; quercetin; quercetin-3-glucoside; scopoletin; sinapic acid; syringic acid; syringin; taxifolin; vanillic acid and vanillin were identified using cold water as compared to hot water extraction. In contrast, a significantly ($p<0.05$) lower amount of 4-hydroxyphenylpyruvic acid and tangeretin was identified using cold water as compared to hot water extraction (Table 5.10).

Table 5.10: Comparison of the phytochemicals identified from TI samples using cold or hot water extraction.

Phytochemical	Hot water	Cold water
3,4,5-trimethoxyacetophenone	1.8 ± 0.4	6.5 ± 1.8***
3,4-dimethoxybenzaldehyde	10.0 ± 2.2	16.7 ± 4.1
4-hydroxy-3-methoxyacetophenone	6.8 ± 1.7	33.3 ± 8.2***
4-hydroxyacetophenone	8.0 ± 2.0	80.4 ± 20.1***
4-hydroxyphenylpyruvic acid	1100 ± 275	664 ± 166**
Apigenin	25.2 ± 6.3	49.2 ± 12.3
Benzoic acid	107.6 ± 26.9	1480 ± 370***
Biochanin A	16.3 ± 4.0	18.9 ± 4.7
Caffeic acid	95.2 ± 23.8	328.4 ± 82.1***
Catechin	27640 ± 6910	52800 ± 13200**
Chlorogenic acid	10.4 ± 2.6	11.9 ± 2.9
Epicatechin	2840 ± 710	1688 ± 422
Epigallocatechin	2128 ± 532	1808 ± 452
Epigallocatechin gallate	5320 ± 1330	5160 ± 1290
Ferulic acid	131.6 ± 32.9	289.6 ± 72.4***
Formononetin	3.1 ± 0.7	3.2 ± 0.8
Gallic acid	4040 ± 1010	9000 ± 2250**
Gallocatechin	7680 ± 1920	9040 ± 2260
Genstein	28.6 ± 7.1	53.6 ± 13.4
Hesperitin	5.9 ± 1.9	10.9 ± 2.7
Hydroxytyrosol	64.4 ± 16.1	108.4 ± 27.1**
Indole	24.2 ± 16.1	110.4 ± 27.6***
Indole-3-acetic acid	6.2 1.5	56.4 ± 14.1***
Indole-3-carboxaldehyde	19.6 ± 4.9	83.6 ± 20.9***
Kaempferol	89.2 ± 22.3	184.8 ± 46.2*

Luteolin	59.6 14.9	79.6 ± 19.9
Myricetin	210 ± 52.5	186.4 ± 46.6
Naringenin	179.6 ± 44.9	285.2 ± 71.3*
P-coumaric acid	108.8 ± 27.2	226.4 ± 56.5**
Phenylacetic acid	14.9 ± 3.7	45.6 ± 11.4***
Phenyllactic acid	1.7 ± 0.4	4.2 ± 1.0
Phloretin	225.2 ± 56.3	420 ± 105*
Phloridzin	91.2 ± 22.8	141.6 ± 35.4
P-hydroxybenzaldehyde	5.8 ± 1.4	28 ± 6.9***
P-hydroxybenzoic acid	44.8 ± 11.2	137.2 ± 34.3***
Pinoresinol	35.0 ± 8.7	35.1 ± 8.7
Protocatachaldehyde	133.2 ± 33.3	348.4 ± 87.1***
Protocatechuic acid	524 ± 131	860 ± 215*
Quercetin	170 ± 42.5	262.8 ± 65.7*
Quercetin-3-glucoside	83.6 ± 20.9	182.8 ± 45.7**
Reservatrol	41.6 ± 10.4	51.2 ± 12.8
Salicylic acid	32.3 ± 8.0	56.4 ± 14.1
Scopoletin	42 ± 10.5	114 ± 28.5***
Secoisolariciresinol	151.6 ± 37.9	247.2 ± 61.8
Serotonin	9.1 ± 2.2	5.0 ± 1.2
Sinapic acid	1.0 ± 0.2	14.2 ± 3.5***
Syringaresinol	1312 ± 329	916 ± 229
Syringic acid	36.9 ± 9.2	73.6 ± 18.4*
Syringin	10.8 ± 2.4	40.8 ± 9.8***
Tangeretin	10.7 ± 2.6	4.4 ± 1.1*
Taxifolin	226.4 ± 56.6	432 ± 108**
Vanillic acid	109.2 ± 27.2	254.8 ± 63.7**
Vanillin	51.2 ± 12.8	155.6 ± 38.8***

Data are presented as amount ± SD (pg/mg), n=3; *p<0.05; **p<0.01 and ***p<0.001 as compared to hot water extraction by ANOVA followed by Bonferroni's post hoc test.

5.4 DISCUSSION

Plants are essentially immobile in nature and cannot escape unfavourable environmental factors. However, they produce secondary metabolites through various physiological and biochemical processes that improve their growth and survival in response to changes in the environment (Kliebenstein, 2013; Wani et al., 2016). The type and amount of secondary metabolites produced by a plant are species-dependent, are influenced by the developmental stage, and by environmental conditions during growth (Ncube et al., 2012). The physiological processes involved in the synthesis of secondary metabolites result in alterations in gene expression, regulation of protein activity, ion homeostasis and endogenous levels of secondary metabolites (Wani et al., 2016). For centuries, humans have exploited such physiological changes in plants for drug discovery or by directly using the plants as medicines (Wurtzel & Kutchan, 2016). The use of plant-based products for medicines or nutritional purposes has increased substantially in the last decades, such that at least 65 % of people worldwide use herbal medicines as treatment alternatives for many diseases because of their ready availability and accessibility (Amponsah et al., 2013; Awodele et al., 2013). This study focused on the assessment of the influence of change in season (dry and rainy) and storage has on the secondary metabolites of TI and also investigated the impact of solvent (water versus chemical) extraction on the phytochemical profile.

The four solvents, namely: chloroform, ethanol, ethyl acetate and petroleum ether, used in the sequential Soxhlet extraction previously in chapter 2 were used to extract and compare the effect of change in season and storage on the type and amount of phytochemicals from TI. In this study, analysis of the influence of change in season on TI secondary metabolites showed that 82 phytochemical metabolites were identified from TI samples obtained in the dry season and the rainy season. The highest number of secondary metabolites were detected by using ethyl acetate (66 secondary metabolites) while the least number (36 secondary metabolites) were detected by using petroleum ether as the solvent. Additionally, TI petroleum ether extract showed more phytochemicals from the TI sample obtained in the rainy season as compared to the TI sample obtained in the dry season. In contrast, less phytochemicals were extracted from the sample when compared with the TI sample obtained in the

dry season when chloroform, ethanol or ethyl acetate was used for the isolation of phytochemicals. This showed that TI, generally produced more secondary metabolites under stressful condition in the dry season than under favourable conditions in the rainy season except with petroleum ether extract.

Moreover, acetophenones (particularly 3,4,5-trimethoxyacetophenone), benzaldehydes (particularly protocatachaldehyde), benzoic acids (particularly 2,3-dihydroxybenzoic acid; 2,5-dihydroxybenzoic acid; 2,6-dihydroxybenzoic acid; 3,4-dimethoxybenzoic acid and anthranilic acid), benzenes (particularly 1,2-hydroxybenzene), cinnamic acids (particularly sinapic acid), flavonoids (particularly bergapten; kaempferol; niacin and psoralen), indoles (particularly indole-3-acetic acid), lignans (particularly syringaresinol), phenols (particularly hydroxytyrosol), phenylacetic acids (particularly phenylacetic acids) and phenyllactic acids (particularly phenyllactic acid) were present exclusively in the TI sample obtained in the dry season. For the secondary metabolites present in both samples, the TI sample obtained in the dry season showed significantly ($p<0.05$) higher amounts acetophenones (particularly 4-hydroxy-3-methoxyacetophenone); benzoic acids (particularly gallic acid, p-hydroxybenzoic acid, protocatechuic acid and salicylic acid); cinnamic acids (particularly caffeic acid, ferulic acid and p-coumaric acid); flavonoids (particularly epicatechin, gallocatechin and quercetin) and lignans (particularly secoisolariciresinol) as compared to the sample obtained in the rainy season.

During the dry season, there is a decrease in water and nutrient supply to plants (Niinemets, 2016). Nutritional stress may result in the accumulation of osmo-protectants to stabilise proteins structure and maintain membrane integrity and scavenge ROS with possibility biomass and secondary metabolites production (Niinemets, 2016; Rejeb et al., 2014). For instance: nitrogen, potassium, phosphate and sulphur-induced stresses stimulated the biosynthesis of phenylpropanoids and phenolics in numerous plant species (Akula & Ravishankar, 2011; Dixon & Paiva, 1995). Phenolics are shown to be involved in functions in plants such as reproduction, growth and tolerance of stress (Chaparro et al., 2013; Giménez et al., 2014). Plants that produce phenolics with allelopathic activity may compete and suppress the growth of surrounding plants (Lincoln & Zeiger, 2006). Phenolic compounds have redox activity that make them to act as

antioxidants and thereby detoxify ROS (Huang & Bie, 2010). Phenolics also play other essential functions in plants such as indicators of stress, nutrient uptake, photosynthesis, and protein synthesis (Achakzai et al., 2009; Fayez & Bazaid, 2014). A wide variety of compounds constitute phenolics which include: arylpyrones and styrylpyrones, coumarins, flavonoids, lignins, lignans and tannins (Fang et al., 2011)

Plant hormones such as auxins, salicylic acid, cytokinin, ethylene, gibberellic acid and jasmonic acid help to modulate developmental processes in plants and determine plant responses to environmental stresses (Fayez & Bazaid, 2014; Wani et al., 2016; Wolters & Jürgens, 2009). In agreement to previous studies, this study measured higher amounts of salicylic acid in the TI sample obtained in the stressful dry season as compared to the TI sample obtained in the rainy season. Observation of the structures of bioactive compounds of TI obtained in the dry season also showed the presence of terpenoid saponins such as ivorenoside A, B, C and sericoside (Ponou et al., 2010). Terpenes also facilitate the plant stress response because these are toxic to insects and mammals (Tomar et al., 2014). High levels of toxic substances can occur in medicinal plants when they are collected from areas prone to pollution, for example, areas close to industries, roadways, metal mining sites or oil refineries (Vasudevan et al., 2009). A study by Radwan et al. (2018) showed that pollution results in elevation in total flavonoids and total phenolics when compared with similar samples of plants from less polluted areas and this suggested that elevations in these secondary metabolites can be considered as a stress defence mechanism in plants. The characteristic water deficit of the dry season is also associated with induction of the synthesis of flavonoids, anthocyanins and phenolic acids in fruits and vegetables such as grape, lettuce, pomegranate and red beet (Xu et al., 2019). Absence of appropriate amounts of water or higher transpiration rate also results in drought stress in plants and changes secondary metabolites production (Wani et al., 2016; Wolters & Jürgens, 2009). Heat shock and water stress, both characteristic of the dry season, have also been shown to promote the synthesis of polyamines and phenylamides in bean and tobacco (Edreva et al., 1998; Edreva et al., 1995). In contrast, Mena et al. (2013) observed that deficit in irrigation reduced total anthocyanins and total phenolics in pomegranate as compared to the control group.

On the other hand, the rainy season is characterised by cloudy weather and relatively lower temperatures as compared to the sunny and high temperatures of up to 35 - 40 °C in the dry season (Asante & Amuakwa-Mensah, 2015). In this study, amines (especially dopamine), benzaldehydes (especially p-hydroxybenzaldehyde), flavonoids (especially biochanin A; didymin and formononetin) and phenols (especially 2-hydroxybenzyl alcohol) were present exclusively in the TI sample obtained in the rainy season. The TI sample obtained in the rainy season also showed significantly ($p<0.05$) higher amounts of benzoic acids (especially benzoic acid); cinnamic acids (especially chlorogenic acid); flavonoids (especially hesperitin, naringenin, morin, neohesperiden, phloredzin, scopoletin, syringin and tangeretin), indoles (especially indole-3-pyruvic acid) and lignans (especially indole-3-carboxaldehyde) as compared to the TI sample obtained in the dry season.

In agreement with this study, Pérez-Ilzarbe et al. (1997) found that cold weather was linked with the production of high levels of chlorogenic acid. Plants growing under low temperatures show significant adjustments in various physiological and biochemical processes that enable them to survive under low temperature stress, and this causes inhibition in the synthesis and storage of secondary metabolites (Verma & Shukla, 2015). Moreover, water uptake, dehydration and metabolism in plants are reduced at low temperatures (Chinnusamy et al., 2007). The light intensity and exposure period also have significant influence on the production and storage of secondary metabolites (Ahmad et al., 2018). The survival of plants centres greatly on their ability to carry out photosynthesis, carbon fixation and biomass accumulation, and thus, they have developed very sensitive abilities to detect different light spectra (Kazan & Manners, 2011). For instance, levels of endogenous coumarins have been shown to significantly decrease in different plant parts due to shorter light period (de Castro et al., 2007). In this study, TI obtained in the rainy season showed the presence of phenolic compounds such as progallin A; 3,3'-di-O-methylellagic acid; 2-hydroxy-3,7,8-trimethoxychromeno[5,4,3-cde]chromene-5,10-dione and these compounds have been associated with antioxidant and anti-inflammatory activities (Adiko et al., 2013). The study further assessed the effect of storage on TI phytochemical profile.

Medicinal plant gatherers or suppliers usually wait to collect enough plant stock before making a supply or making their way to the market for sale (Mander, 1998). In the chain of production of medicinal products, herbalists usually store medicinal plant samples, for instance days to several months before usage (Chan et al., 2009; Suvarnakuta et al., 2011). Many of the bioactive compounds from these medicinal products may degrade during storage. Where possible, plant samples should be extracted and analysed shortly after collection because secondary metabolites can decompose even if samples are frozen under liquid nitrogen (Cronin et al., 1995). This study further investigated the impact of storage on the phytochemicals from freshly obtained TI sample and 4 years stored TI sample. The TI samples were both obtained within a similar season (rainy season) to avoid variation in secondary metabolites that could occur due to change in season. Herbalist will normally not store TI sample for this long before using it for medicines, but for research purposes and for 'proof of principle'; that phytochemical contained in stem barks are tightly bound, this study was extended to 4 years.

The phytochemical profile showed that 66 phytochemical metabolites were identified from both fresh and stored TI samples. Approximately 12 % of the secondary metabolites were lost from the stored sample relative to the fresh TI sample. These include 2-hydroxybenzyl alcohol, biochanin A, chlorogenic acid, formononetin, hesperidin, imperatorin, neohesperidin and p-anisic acid. However, storage of TI resulted in the appearance of 4-hydroxyphenylpropionic acid, caffeine, eriocitrin, indole-3-carboxylic acid and spermine in the TI sample. This may be due to degraded products of the phytochemicals that were found in the fresh TI sample but absent in the stored TI sample. For the secondary metabolites found in both samples, the stored sample showed significantly ($p<0.05$) higher amounts of acetophenones (particularly 4-hydroxyacetophenone); benzoic acids (particularly gallic acid, p-hydroxybenzoic acid, protocatechuic acid, syringic acid and vanillic acid); benzaldehydes (particularly p-hydroxybenzaldehyde and vanillin); cinnamic acids (particularly ferulic acid and p-coumaric acid); flavonoids (particularly quercetin) and lignans (particularly pinoresinol and secoisolariciresinol) as compared to the fresh TI sample.

Conversely, significantly ($p<0.05$) lower amounts of benzoic acids (particularly benzoic acid and salicylic acid); cinnamic acids (particularly caffeic acid); flavonoids (particularly catechin, epicatechin, epigallocatechin, epigallocatechin gallate, hesperitin, morin, phloredzin, quercetin-3-glucoside); indoles (particularly indole-3-pyruvic acid); lignans (particularly indole-3-carboxaldehyde) and phenylpyruvic acids (particularly 4-phenylhydroxypyruvic acid) were measured in the stored sample as compared to the fresh sample.

To the best of my knowledge, there are no data comparing fresh TI and stored sample and hence, other research work comparing fresh and stored plant samples have been used to support this study. According to Zhao et al. (2021), storage of *Lilium* bulbs for 30 days resulted in a decrease in free amino acids, total polysaccharides and reducing sugars by approximately 39, 63 and 18 % respectively. Javadi et al. (2015) also reported that storage of *Cosmos caudatus* at room temperature for 12 h resulted in depletion of phenolic compounds (such as α-tocopherol, benzoic acid, catechin, cyclohexen-1-carboxylic acid, lycopene, myoinositol and stigmasterol) which were detected in samples stored for less than 12 h. It was concluded that the phenolic compounds were degraded into free sugars such as α-D-galactopyranose, sucrose and turanose, which was identified after 12 h of storage. Profiling of secondary metabolites from banana stored at room temperature showed depleted levels of dopamine after harvest (Yuan et al., 2017). The disappearance of dopamine from fresh samples was followed by the appearance of salsolinol, which has been reported as a metabolite from dopamine and acetaldehyde or ethanol, which is also generated in postharvest bananas (de Castro et al., 2007; Yuan et al., 2017). The depletion in phenolic compounds in stored samples is attributed to dehydration of plants which occur postharvest (Zamboni et al., 2010). In the presence of oxygen, polyphenol oxidase converts phenolic compounds to quinones (Segovia-Bravo et al., 2009). The activity of polyphenol oxidase in *Lilium* bulbs has been shown to increase by approximately 100 % after 30 days of storage (Zhao et al., 2021). Traditional healers commonly prefer fresh plant material because of some doubts around the degree of biological activity of stored plants (Stafford et al., 2005).

Contrary to the negative considerations about storage of plant material, modification of secondary metabolites during storage is not always detrimental

but the activity or levels of certain compounds increase with storage making the product of better value (Laher et al., 2013). For instance, this study found that higher levels of gallic acid (a natural antioxidant), and caffeine (a central nervous system stimulant) were measured in the stored TI sample compared with fresh TI sample. In agreement to these findings, Monribot-Villanueva et al. (2019) also found that gallic acid levels were elevated in some varieties of mango fruits after 6 days of storage (Monribot-Villanueva et al., 2019).

Griggs et al. (2001) reported that according to traditional medical practitioners who were interviewed, the shelf-life of stored plant sample did not vary with plant species (Griggs et al., 2001). The results obtained from this current study and findings from Laher et al. (2013) disagree with this claim. Results on secondary metabolites differed between species and different plant species showed different activity to various assays (Laher et al., 2013). The biological activity of medicinal plants is attributed to their chemical constituents (Stafford et al., 2005). For example, 3 years stored samples of *Ocimum basilicum* and *Senna petersiana* leaves showed higher antioxidant activity than the fresh sample whereas fresh *Hypoxis hemerocallidea* leaves showed higher antioxidant activity than stored leaves (Laher et al., 2013).

It is believed that plant parts such as bark, roots or underground storage organs (such as bulbs, corms, rhizomes and tubers) have a longer storage life as compared to fruits or leaves (Stafford et al., 2005). It is further speculated that because bark, roots and underground structures are storage sites for secondary metabolites, compounds they store are more stable than in fruits or leaves (Stafford et al., 2005). Furthermore, these organs are better suited to protect the stored compounds from degradation as compared to fruits or leaves, due to their lower surface area to volume ratio (Laher et al., 2013). This confirms the findings of this study that showed the bark of TI that was stored for 4 years retained most of its secondary metabolites similar to fresh TI stem bark. The stored TI bark still retained about 88 % of its secondary metabolites after 4 years of storage but fresh TI sample contained higher amounts of the secondary metabolites.

Successful isolation of biologically active compounds from a plant material is principally dependent on the type of solvent used in the extraction procedure

(Eloff 1998). Different solvent extracts of plant samples have been shown to contain different phytochemical composition and hence, show different biological activity (Laher et al., 2013; Ponou et al., 2010). For example, water and ethanol extracts of several plants have been shown to have the highest antibacterial and antioxidant activity than other extracts such as chloroform or dichloromethane extract (Laher et al., 2013; Stafford et al., 2005). In scientific research, samples are frequently extracted with organic solvents like acetone, chloroform, dichloromethane, ethyl acetate, ethanol, petroleum ether (Ben-Azu et al., 2016; Laher et al., 2013; Ponou et al., 2010). However, traditional medicines are not generally extracted with these solvents but are rather mostly extracted with water or alcohol and this can result in differences in biological activity between extracts from traditional and chemical extractions (Griggs et al., 2001). Therefore, this research compared phytochemical compounds isolated between traditional method of extraction with water (hot and cold) compared with chemical extraction.

Certain phytochemicals amongst amines, cinnamic acids, flavonoids, indoles, mandelic acids or phenylpyruvic acid were present exclusively using water extraction. Water extraction also showed significantly ($p<0.05$) higher amounts of certain benzaldehydes, benzoic acids, cinnamic acids, flavonoids, indoles, lignans, phenols or phenylpyruvic acids as compared to the chemical extraction.

Conversely, some phytochemicals from amines, benzoic acids, benzenes, coumarins, flavonoids or phenylpropionic acids were not detected when using water extraction for TI sample. Significantly ($p<0.05$) lower amounts of phenyllactic acid and salicylic acid were measured using water extraction as compared to chemical extraction. The water extraction yielded higher amounts of phytochemicals than the chemical extraction.

A good solvent for extraction should yield high quantities of target compounds, should allow ease of subsequent handling of the extracts and should not allow the compounds to dissociate or complex (Eloff, 1998). Thus, traditional extraction with cold and hot water showed higher amounts of phytochemical compounds measured while the sequential chemical extraction with chloroform, ethanol, ethyl acetate and petroleum ether showed a wider profile of phytochemicals extracted. In agreement with these findings, Cronin et al. (1995)

and Laher et al. (2013) have shown that storage and extraction methods of samples significantly affect yields of biologically active compounds (Cronin et al., 1995; Laher et al., 2013).

Furthermore, the composition and yield of phytochemicals obtained from the extractions are also dependent on the temperature of extraction, extraction time and polarity of solvent (Ncube et al., 2008; Tiwari et al., 2011). This research also compared the two most commonly used traditional extraction methods: hot and cold-water extraction. A higher number of secondary metabolites were detected using cold extraction when compared to hot water extraction. For instance, significantly ($p<0.05$) higher amounts of certain acetophenones, benzaldehydes, benzoic acids, cinnamic acids, flavonoids, indoles, lignans or phenols were identified using cold water as compared to hot water extraction. Contrary, a significantly ($p<0.05$) lower amounts of 4-hydroxyphenylpyruvic acid and tangeretin was identified using cold water as compared to hot water extraction. The decrease in number of secondary metabolites detected with hot water extraction and sequential Soxhlet extraction as compared to cold water extraction maybe due to the loss of thermo-unstable compounds.

5.5 CONCLUSIONS

Higher phytochemical metabolites levels were measured for TI sample collected in the dry season compared to sample obtained in the rainy season due to harsher environmental conditions in the dry season which may have induced higher synthesis and storage of secondary protective metabolites. Though secondary metabolites levels decreased with storage, TI that was stored for 4 years retained the vast majority of its secondary metabolites similar to fresh TI possibly due to the ability of the stem bark to provide a greater protection of the secondary metabolites. Traditional extraction with water was also shown to extract a higher quantity of majority of the metabolites while the chemical extraction showed a wider phytochemical profile qualitatively. Cold water extraction also showed higher levels of secondary metabolites than hot water extraction which may be due to the loss of thermo-unstable compounds.

CHAPTER SIX

GENERAL DISCUSSION, SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS.

6.1 GERNERAL DISCUSSION

Phytomedicines have shown great potential in providing effective treatment for numerous diseases, including colon cancer and provide a natural blueprint for the development of conventional drugs (Njume et al., 2009). An estimated 352,282 flowering plants have been reported in the world and more than 50,000 are used for therapeutic purposes (Govaerts, 2009; Schippmann et al., 2002). This emphasises the necessity for more research into phytomedicines to assess their potential in managing diseases as supplements and / or alternatives to conventional treatment. In evaluating the importance of plant medicines in ethnomedicine and use as a blueprint for developing conventional drugs, this study aimed to determine the influence of solvent, season and storage on the phytochemicals present in TI and to assess the impact of TI on biomarkers of cyto- and genotoxicity in human cells *in vitro* for potential use in cancer therapy.

The study first developed and validated the procedure for chemical extraction (Soxhlet extraction) of the plant sample (TI) for use in subsequent chapters. The robustness of the extraction procedure was assessed in terms of the efficiency and consistency in yield, phytochemicals identified, and effect on antioxidants and liver cell survival characteristics. These specific biomarkers were used to carry out the method development as they were to be tested subsequently in the research. Six solvents with different polarities were selected for the extraction to capture as many phytochemicals as possible (Table 6.1). For the three subsamples of TI (A, B and C) used for the extraction, the efficiency in yield was very variable (12 – 100 % similarity index) between the six thimbles of the Soxtherm machine (chapter 2; Figure 2.2). This could be as a result of the machine heating faster in the middle beakers thereby producing higher yield than in the beakers at the outside. Dichloromethane extraction showed the lowest similarity (12 – 100 %) in yield while ethyl acetate extraction showed the highest similarity (85 – 100 %) in yield (Table 6.1).

A total of 62 phytochemicals were isolated from TI sample using the six solvents. It was found that the three TI subsamples (A – C) showed a similarity index of 100 % when extracted with acetone, chloroform, ethyl acetate or petroleum ether. Conversely, extraction with dichloromethane or ethanol showed a reduced similarity index of 81.2 and 97.8 % respectively which was associated primarily with the subsamples producing different amounts of 4-hydroacetophenone,

benzoic acid, catechin, ferulic acid, protocatachaldehyde, p-hydroxybenzoic acid, p-hydroxybenzaldehyde, syringic acid, syringin and vanillic acid. It was also found that future extractions could be carried out with four solvents (chloroform, ethanol, ethyl acetate and petroleum ether) to capture similar phytochemicals as with the six solvents used initially. Though the amount of some phytochemicals were different in the subsamples, the Soxhlet extraction procedure was consistent in producing similar phytochemicals.

The study then assessed the consistency in effect of TI subsamples on antioxidant activity, which showed 80 – 100 % similarity in both FRAP and DPPH scavenging activity. A range of TI concentrations (0 - 0.5 mg/ml) was then used to compare the similarity between the effects of the TI subsamples on cell viability and growth which also showed 100 % similarity in effect in both cell survival characteristics (Table 6.1). Therefore, the four solvents (chloroform, ethanol, ethyl acetate and petroleum ether) were used subsequently to carry out the extraction to investigate the impact of season and storage on the phytochemical profile of TI and to test for effects on human liver and colon cells *in vitro*.

Table 6.1: Polarity of solvents and similarity index between different batches of TI samples (A, B and C) prepared using sequential Soxhlet extraction.

TI extract	Polarity index	Similarity in yield (%)	Similarity in phytochemical (%)	Similarity in DPPH activity (%)	Similarity in FRAP (%)	Cell viability similarity (%)	Cell growth similarity (%)
Petroleum ether	0.1	50 - 100	100	99.1	100	100	100
Chloroform	2.7	60 - 100	100	80.1	100	100	100
Dichloromethane	3.1	12 - 100	81.2	95.4	100	100	100
Ethyl acetate	4.4	85 - 100	100	98.8	100	100	100
Acetone	5.1	79 - 100	100	95.8	100	100	100
Ethanol	5.2	68 - 100	97.8	83.1	100	100	100

Phytochemicals were identified by LC/MS MS; antioxidant assays used: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP); MTT assay for cell viability; cell counting for cell proliferation.

Assessment of the impact of season on the phytochemical profile of TI showed that more phytochemicals were identified from the TI sample obtained in the dry season compared to the sample obtained in the rainy season. This shows that the dry season is more ideal for the collection of TI sample.

Moreover, evaluation of the impact of storage on TI phytochemicals showed that 4 years of storage resulted in the loss of only 12 % of the total metabolites present in the fresh TI. The absence of 2-hydroxybenzyl alcohol, biochanin A, chlorogenic acid, formononetin, hesperidin, imperatorin, neohesperidin and p-anisic acid accounted for fewer metabolites identified in the stored TI sample compared with the fresh sample. Comparison of water extraction with chemical extraction also showed similar number of phytochemicals using both methods of extractions. With a combined total of 82 phytochemicals identified using water extraction and chemical extraction, approximately 75 % of the phytochemicals were common to both water and chemical solvents but in different amounts. This shows that the traditional extraction method would give a similar medicinal efficacy as the chemical extraction. Among the four chemical solvents, ethyl acetate was the most efficient solvent for isolation of phytochemicals and recorded 80.5 % of the total phytochemicals identified. Conversely, 43.9 % of the phytochemicals were isolated using petroleum ether. This showed that TI stem bark contained more polar metabolites than non-polar metabolites, since petroleum ether was the least polar solvent (polarity index=0.1) among the four solvents. Conclusively, the phytochemicals produced from TI depend on the solvent, season and storage, hence, the medicinal efficacy may vary dependent on these conditions.

The extracts from the four chemical solvents were used to investigate the impact of TI on human liver. The liver plays a central role in the metabolism of both endogenous and exogenous substances in the body, and hence, is prone to the toxic effects of these substances and /or their metabolites (Almazroo et al., 2017). Previous studies have reported the use of TI in ethnomedicine for the treatment of various human conditions including diuresis, general body pains, haemorrhoids, malaria and ulcers (Akinyemi et al., 2006; Etukudo, 2003; Sitapha et al., 2013). Therefore, this study investigated the impact of the four TI extracts on human liver cell viability, proliferation, genomic stability and DME.

A dose-response of TI (0 – 5 mg/ml) was used to evaluate the impact of TI on human liver cell survival characteristics. It was found that all four TI extracts showed a concentration dependent negative effect on liver cell viability, growth and genomic stability (DNA SSB) and were toxic at concentrations ≥1 mg/ml. TI ethanol extract showed the highest cyto- (IC_{50}=1.6 mg/ml) and genotoxic effect (LC_{50} = 1.1 mg/ml) on the human liver cells. Some studies reported that TI ethanol extract showed low toxicity in mice (LD_{50} = 2236.06 mg/kg) and rats (LD_{50} ≥5000 mg/kg) (Ben-Azu et al., 2016; Moomin et al., 2020; Zaza et al., 2016). This study was intended to investigate whether TI extracts were toxic in human liver. However, higher concentrations (≥1 mg/ml) of TI extract were found to induce DNA SSB and to decrease liver cell viability and growth.

Moreover, all six TI extracts used previously (chapter 2) showed similar effects on cell viability (84 – 98 %) and growth (78 – 94 %) (chapter 2; Figures 2.9 and 2.10) when compared to the four solvents used subsequently in chapter 3 (chapter 3; Figures 3.1 and 3.2). This confirmed the robustness of the extraction protocol in yielding similar phytochemicals with similar effects on human liver cells and justified the decision to proceed with the research using only four solvents. Furthermore, a non-toxic concentration of TI (0.5 mg/ml) was selected from the dose-response studies to investigate the impact of TI on DME and GSH in the liver up to 7 days of treatment.

Treatment of liver cells with TI ethanol extract showed a slight induction (15 %) in Phase I DME (cytochrome P450) while showing higher induction in Phase II DME: catalase, GPx and GSR by 43, 47 and 86 % respectively, and moderate (25 %) induction in GSH. On the other hand, TI chloroform extract showed a very high inhibition of both Phase I and Phase II DME with the exception of catalase. Treatment with ethyl acetate or petroleum ether extract was shown to have a slight effect on some DME but showed high induction in GSR activity by 63 and 30 % respectively. Treatment with petroleum ether extract also inhibited cytochrome P450 by 65 % (Table 6.2). These findings imply that treatment with ethanol extract might enhance the liver metabolism of other drugs whereas exposure to chloroform extract might inhibit DME and delay the excretion of other drugs.

Table 6.2: Impact of TI extracts at a concentration of 0.5 mg/ml on human liver cells survival characteristics and DME *in vitro.*

	TI extract (0.5 mg/ml)			
Biomarker	Petroleum ether	Chloroform	Ethyl acetate	Ethanol
Cell viability (%)	95	95	96	95
Cell growth (%)	90	82	85	78
DNA damage (AU)	94	83	79	93
Cytochrome P450 activity (%)	-65	-75	+1.3	+15
Catalase activity (%)	-11	-8	+6	+43
GSH levels (%)	-5	-34	-16	+20
GSR activity (%)	+30	-66	+63	+86
GPx activity (%)	-13	-46	-23	+47
Phytochemicals associated with the activities of DME by mathematical modelling.	Inhibition of activity by: 4-hydroxyphenylpyruvic acid, bergapten, morin, p-asinic acid and pinoresinol.	Inhibition of activity by: 4-hydroxyphenylpyruvic acid, bergapten, morin, p-asinic acid and pinoresinol.	Induction of activity by: 1,2,3-trianthranilic acid; caffeic acid; epicatechin; epigallocatechin; gallocatechin, luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol.	Induction of activity by: 1,2,3-trianthranilic acid; caffeic acid; epicatechin; epigallocatechin; gallocatechin, luteolin; myricetin; neohesperidin; phloretin; psolaren and resveratrol.

- mean inhibition and + mean induction of enzyme activity or GSH at 7 days.

Similar phytochemicals were observed to be associated with the effects on DME activities with both short- and long-term treatment (appendix 2; Tables 8.1 – 8.5). This suggests that the phytochemicals identified by PLS-DA may be responsible as having the greatest influence on the actual measured effects of the TI extracts. Some of the phytochemicals that were most strongly associated with DME activities were present in all four TI extracts but were present in different amounts, while other phytochemicals were found exclusively in some extracts. For instance, syringin was associated with an inhibitory effect on both Phase I and Phase II DME and GSH (appendix 2; Tables 8.1 – 8.5). However, the amount measured in chloroform extract (77 pg/mg) was at least 3-fold higher than the amount found in petroleum ether (21 pg/mg), ethanol (10 pg/mg) and ethyl acetate (1 pg/mg) extracts (chapter 5; Tables 5.1 – 5.4). Therefore, further research is required on individual phytochemicals to confirm this finding, as the present crude extracts contained numerous phytochemicals. The four TI extracts were further used to determine the potential therapeutic efficacy in colon cancer.

Similar to the work carried out in human liver cells, a dose-response (0 – 5 mg/ml) was used to evaluate the impact of TI on both cancerous and non-malignant colon cell survival characteristics. It was found that all four TI extracts showed a concentration dependent inversely related effect on colon cell viability, growth and genomic stability. However, the TI extracts were more toxic to both colon cells (at concentrations ≥0.5 mg/ml) than to the liver cells (at concentrations ≥1 mg/ml). TI extracts were also more toxic to the cancerous colon (CaCo-2) cells than to the non-malignant colon (NCM460) cells. This was evident from comparison of the two colon cell lines which showed that while TI affected the growth of both cells equally (chapter 4; Table 4.4), it was approximately 2-fold more cytotoxic (chapter 4; Table 4.2), DNA damage-inducing (chapter 4; Table 4.6) and DNA repair-inhibiting (chapter 4; Table 4.8) in the colon cancer cells compared with the non-malignant colon cells. TI also showed anticancer properties by inhibiting colon cell migration. Cancer cell migration was inhibited by 73 and 80 % by ethanol and ethyl acetate respectively. This selective toxicity measured in cancerous colon cells could serve as the basis for further anticancer research using TI.

6.2 SUMMARY OF FINDINGS

This study aimed to investigate the influence of solvent, season and storage on the phytochemicals present in TI and to determine the impact of TI on biomarkers of cyto- and genotoxicity in human cells *in vitro*. The study used sequential Soxhlet extraction to isolate phytochemicals present in TI and measured the effect on human liver and colon cells' survival and characteristics. Analysis of the results indicated that:

1) The chemical extraction procedure was consistent and robust for carrying out the study as it yielded comparable and consistent phytochemicals from different batches of extractions.

2) The phytochemicals isolated and identified from TI was significantly dependent on the solvent, season and storage.

3) The traditional extraction which uses water yielded similar phytochemicals as the chemical extraction, hence, gives similar medicinal efficacy as the chemical.

4) Different extracts of TI showed differential activity on hepatic DME activities in human liver cells in vitro. Chloroform extract significantly ($p<0.05$) inhibiting the activities of cytochrome P450, GSH, GPx and GSR. Contrary, ethanol extract significantly ($p<0.05$) induced the activities of cytochrome P450, catalase, GSH, GPx and GSR.

5) TI was more toxic in human colon cells than in liver cells and showed potential anticancer effects by affecting some hallmarks of cancer such as cell viability and proliferation, DNA damage, DNA repair and cell migration. Ethyl acetate and ethanol extracts were selectively more toxic in colon cancer cells than in non-malignant colon cells.

6.3 CONCLUSIONS

The phytochemicals isolated from TI are dependent on the solvent, season and storage. Different extracts of TI showed differential effects on hepatic DME activities notably chloroform and ethanol extracts causing inhibition and

induction respectively, hence, TI should be used with caution in cells. TI also showed potential anticancer effects by affecting some hallmarks of cancer. Ethyl acetate and ethanol extracts were selectively more toxic in colon cancer cells than in non-malignant colon cells. The potential anticancer properties could possibly be associated with the different compounds present in the extracts. Hence, the further work is required to isolate the bioactive compounds and test them individually or combination of several compounds for potential anticancer effects.

6.4 RECOMMENDATIONS

This study has contributed to the scientific knowledge on NP by showing the impact of change in season on secondary metabolites of TI and recommended that the dry season is the better time than rainy season for collection of the plant sample for medicinal purposes.

This PhD study also added to knowledge on literature of phytomedicines by showing that traditional extraction, which uses mostly water, yielded similar phytochemicals as the chemical extraction, hence, might have similar medicinal efficacy as the chemical. Hence, it is recommended that traditional medicine might possibly be used as an alternative treatment option for conventional medicine.

This study also investigated the impact of TI on human liver cell survival and DME, it is recommended that future work should test how the liver metabolises TI.

This study also provided basics for further research into the use of TI as a potential anticancer agent by showing the selective toxicity of TI extract on human colon cancer cells than in non-malignant colon cells. It also used a mathematical model to predict the possible phytochemicals that might be associated with the effects. Future work should isolate key phytochemicals from the crude TI extracts and test them individually for effects on cancer. Future work should use Organisation for Economic Co-operation and Development (OECD) guidelines to test acute and chronic toxicity of TI on cancer and study the teratogenicity in animal models.

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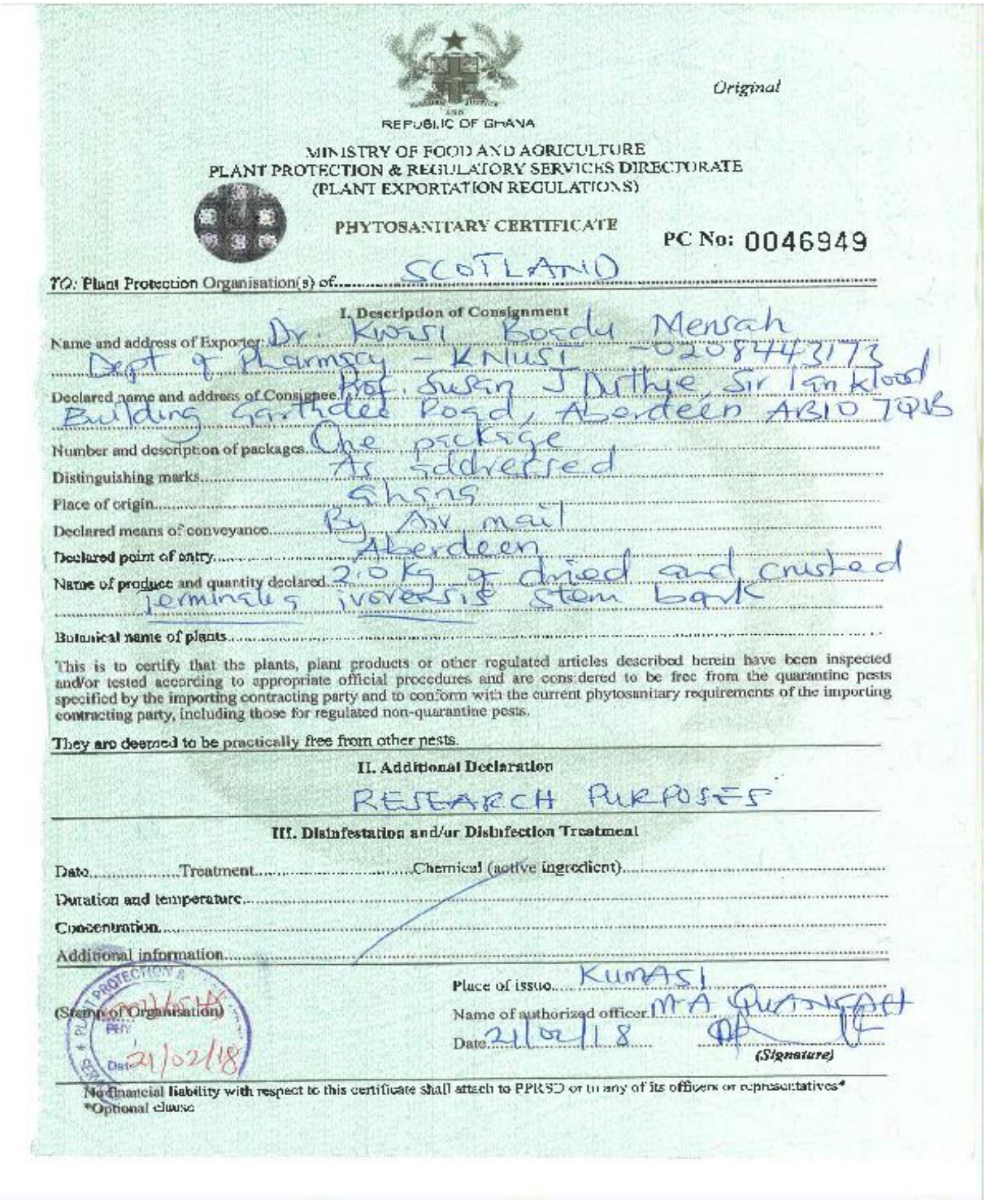
8.0 APPENDICES

8.1 APPENDIX 1: CERTIFICATES FOR THE COLLECTION AND AUTHENTICATION OF TI SAMPLE.

Figure 8.1: Phytosanitary certificate for the collection of TI sample.



Original

REPUBLIC OF GHANA

MINISTRY OF FOOD AND AGRICULTURE
PLANT PROTECTION & REGULATORY SERVICES DIRECTORATE
(PLANT EXPORTATION REGULATIONS)

PHYTOSANITARY CERTIFICATE

PC No: 0046949

TO: Plant Protection Organisation(s) of: SCOTLAND

I. Description of Consignment

Name and address of Exporter: Dr. Kwasi Boadu Mensah
Dept of Pharmacy - KNUST - 0208443173

Declared name and address of Consignee: Prof. Susan J Duthie, Sir Ian Wood Building, Garthdee Road, Aberdeen AB10 7QB

Number and description of packages: One package

Distinguishing marks: As addressed

Place of origin: Ghana

Declared means of conveyance: By Air mail

Declared point of entry: Aberdeen

Name of produce and quantity declared: 2.0 kg of dried and crushed terminalia ivorensis stem bark

Botanical name of plants:

This is to certify that the plants, plant products or other regulated articles described herein have been inspected and/or tested according to appropriate official procedures and are considered to be free from the quarantine pests specified by the importing contracting party and to conform with the current phytosanitary requirements of the importing contracting party, including those for regulated non-quarantine pests.

They are deemed to be practically free from other pests.

II. Additional Declaration

RESEARCH PURPOSES

III. Disinfestation and/or Disinfection Treatment

Date: Treatment: Chemical (active ingredient):

Duration and temperature:

Concentration:

Additional information:

(Stamp of Organisation) PLANT PROTECTION & REGULATORY SERVICES DIRECTORATE PPRSD Date 21/02/18

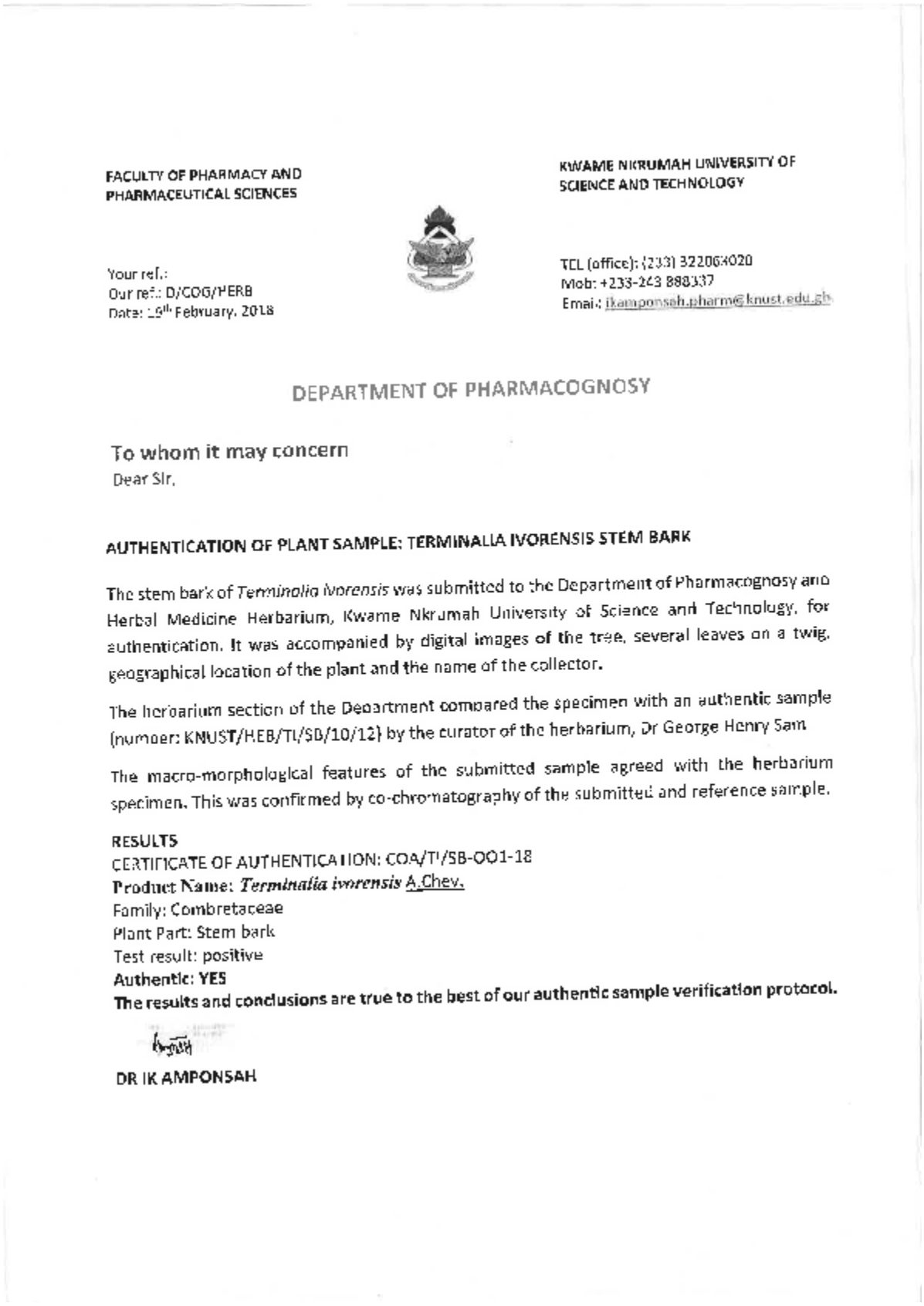
Place of issue: KUMASI

Name of authorized officer: MA QUAINOAH

Date: 21/02/18 (Signature)

No financial liability with respect to this certificate shall attach to PPRSD or to any of its officers or representatives*
*Optional clause

Figure 8.2: Authentication of TI sample from Kwame Nkrumah University of Science and Technology, Ghana.



FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

Your ref.:
Our ref.: D/COG/HERB
Date: 19th February, 2018

TEL (office): (233) 322064020
Mob: +233-243 888337
Email: ikamponsah.pharm@knust.edu.gh

DEPARTMENT OF PHARMACOGNOSY

To whom it may concern
Dear Sir,

AUTHENTICATION OF PLANT SAMPLE: TERMINALIA IVORENSIS STEM BARK

The stem bark of *Terminalia ivorensis* was submitted to the Department of Pharmacognosy and Herbal Medicine Herbarium, Kwame Nkrumah University of Science and Technology, for authentication. It was accompanied by digital images of the tree, several leaves on a twig, geographical location of the plant and the name of the collector.

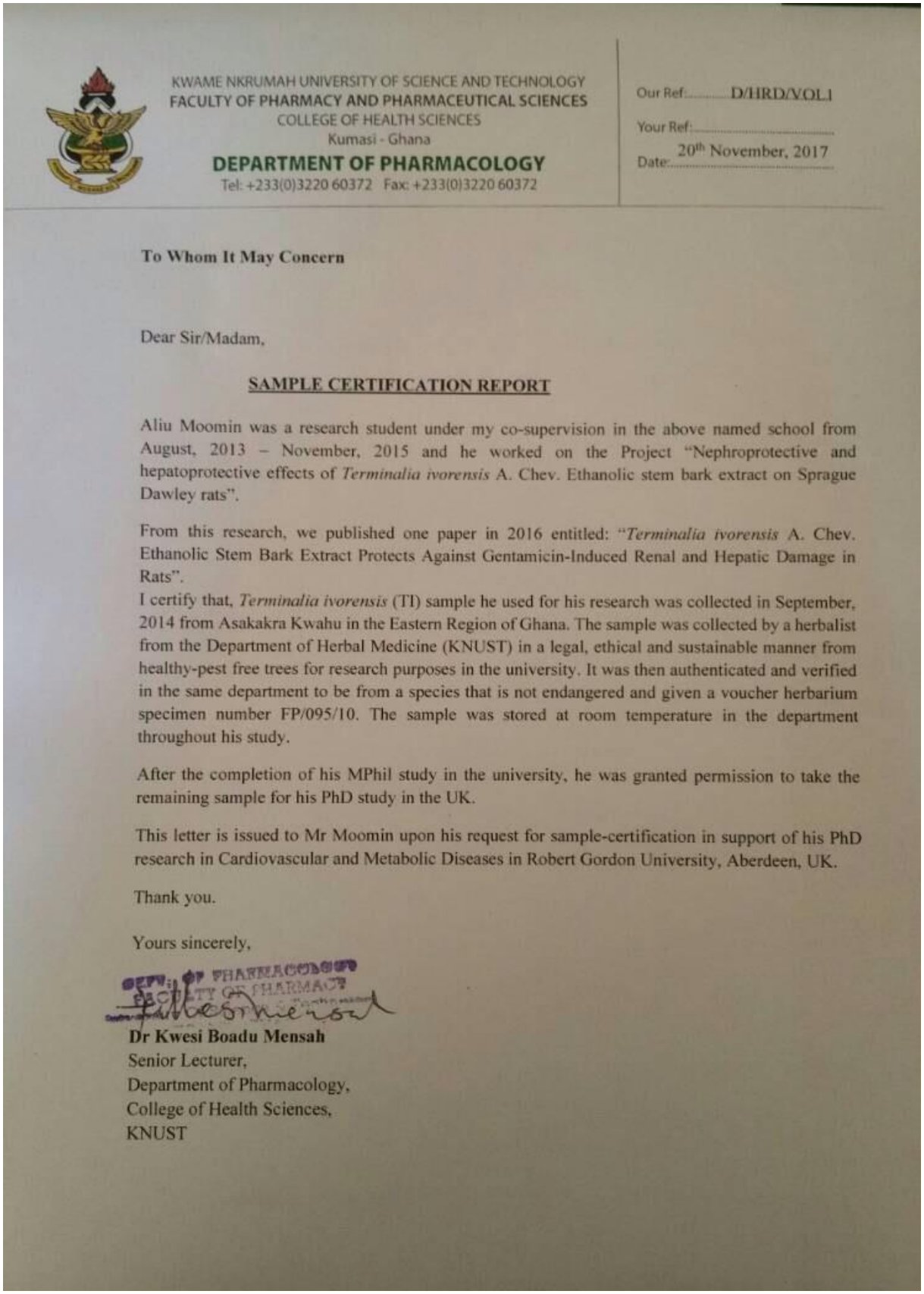
The herbarium section of the Department compared the specimen with an authentic sample (number: KNUST/HEB/TI/SB/10/12) by the curator of the herbarium, Dr George Henry Sam.

The macro-morphological features of the submitted sample agreed with the herbarium specimen. This was confirmed by co-chromatography of the submitted and reference sample.

RESULTS
CERTIFICATE OF AUTHENTICATION: COA/TI/SB-001-18
Product Name: ***Terminalia ivorensis*** A.Chev.
Family: Combretaceae
Plant Part: Stem bark
Test result: positive
Authentic: YES
The results and conclusions are true to the best of our authentic sample verification protocol.

DR IK AMPONSAH

Figure 8.3: TI sample certification report obtained from the Department of Pharmacology, KNUST, Ghana.



KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES
COLLEGE OF HEALTH SCIENCES
Kumasi - Ghana
DEPARTMENT OF PHARMACOLOGY
Tel: +233(0)3220 60372 Fax: +233(0)3220 60372

Our Ref: D/HRD/VOL1
Your Ref:
Date: 20th November, 2017

To Whom It May Concern

Dear Sir/Madam,

SAMPLE CERTIFICATION REPORT

Aliu Moomin was a research student under my co-supervision in the above named school from August, 2013 – November, 2015 and he worked on the Project "Nephroprotective and hepatoprotective effects of *Terminalia ivorensis* A. Chev. Ethanolic stem bark extract on Sprague Dawley rats".

From this research, we published one paper in 2016 entitled: "*Terminalia ivorensis* A. Chev. Ethanolic Stem Bark Extract Protects Against Gentamicin-Induced Renal and Hepatic Damage in Rats".
I certify that, *Terminalia ivorensis* (TI) sample he used for his research was collected in September, 2014 from Asakakra Kwahu in the Eastern Region of Ghana. The sample was collected by a herbalist from the Department of Herbal Medicine (KNUST) in a legal, ethical and sustainable manner from healthy-pest free trees for research purposes in the university. It was then authenticated and verified in the same department to be from a species that is not endangered and given a voucher herbarium specimen number FP/095/10. The sample was stored at room temperature in the department throughout his study.

After the completion of his MPhil study in the university, he was granted permission to take the remaining sample for his PhD study in the UK.

This letter is issued to Mr Moomin upon his request for sample-certification in support of his PhD research in Cardiovascular and Metabolic Diseases in Robert Gordon University, Aberdeen, UK.

Thank you.

Yours sincerely,

Dr Kwesi Boadu Mensah
Senior Lecturer,
Department of Pharmacology,
College of Health Sciences,
KNUST

8.2 APPENDIX 2: LIST OF TABLES SHOWING PHYTOCHEMICALS ASSOCIATED WITH DME, DNA SBR AND CELL MIGRATION.

Table 8.1: Relationship between phytochemicals isolated from TI extracts and the impact on total cytochrome P450 activity in Hep G2 cells after treatment for 24 h or 7 days.

TI	Metabolites showing the greatest impact on cytochrome P450 levels	
	24 h	7 day
Petroleum ether extract	3,4-dimethoxybenzaldehyde; 4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; p-asinic acid; syringin and vanillin.	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; p-asinic acid; syringin and vanillin.
Chloroform extract	3,4-dimethoxybenzaldehyde; 4-hydroxyacetophenone; 4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid; benzoic acid; bergapten; p-asinic acid; syringin and vanillin.	4-hydroxyacetophenone; 4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid; benzoic acid; bergapten; p-asinic acid; syringin and vanillin.
Ethyl acetate extract	4-hydroxyacetophenone; benzoic acid; syringin and vanillin.	4-hydroxyacetophenone; benzoic acid; syringin and vanillin.
Ethanol extract	4-hydroxyacetophenone; benzoic acid; syringin and vanillin.	4-hydroxyacetophenone; benzoic acid; syringin and vanillin.

Table 8.2: Relationship between phytochemical compounds isolated from TI extracts and the impact on catalase activity in Hep G2 cells after treatment for 24 h or 7 day.

TI	Metabolites showing the greatest impact on catalase activity	
	24 h	7 day
Petroleum ether extract	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Chloroform extract	4-hydroxyacetophenone; 4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; bergapten; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Ethyl acetate extract	4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin; syringin; taxifolin and vanillin.	4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin; syringin; taxifolin and vanillin.
Ethanol extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin;	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin;

	epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; quercetin-3-glucoside; resveratrol; scopoletin; syringin; taxifolin and vanillin	epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; quercetin-3-glucoside; resveratrol; scopoletin; syringin; taxifolin and vanillin.

Table 8.3: Relationship between phytochemical compounds isolated from TI extracts and the impact on GSR activity in Hep G2 cells after treatment for 24 h or 7 day.

TI	Metabolites showing the greatest impact on GSR activity	
	24 h	7 day
Petroleum ether extract	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Chloroform extract	4-hydroxyacetophenone; 4-hydroxyphenylpropionic acid; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; morin; p-asinic acid; pinoresinol; protocatechuic acid; quercetin; scopoletin;	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; morin; p-asinic acid; pinoresinol; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.

	syringin; taxifolin and vanillin.	
Ethyl acetate extract	4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin; syringin; taxifolin and vanillin.	4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin; syringin; taxifolin and vanillin.
Ethanol extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; quercetin-3-glucoside; resveratrol; scopoletin; syringin; taxifolin and vanillin	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; quercetin-3-glucoside; resveratrol; scopoletin; syringin; taxifolin and vanillin.

Table 8.4: Relationship between phytochemical compounds isolated from TI extracts and the impact on GPx activity in Hep G2 cells after treatment at 7 days.

TI	7 day
Petroleum ether extract	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Chloroform extract	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; bergapten; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Ethyl acetate extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin; syringin; taxifolin and vanillin.
Ethanol extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; quercetin-3-glucoside; resveratrol; scopoletin; syringin; taxifolin and vanillin.

Table 8.5: Relationship between phytochemical compounds isolated from TI extracts and the impact on GSH levels in Hep G2 cells after treatment for 7 days.

TI	7 day
Petroleum ether extract	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; p-asinic acid; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Chloroform extract	4-hydroxyacetophenone; 4-hydroxyphenylpyruvic acid; benzoic acid; bergapten; catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; morin; p-asinic acid; pinoresinol; protocatechuic acid; quercetin; scopoletin; syringin; taxifolin and vanillin.
Ethyl acetate extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin; syringin; taxifolin and vanillin.
Ethanol extract	1,2,3-trihydroxybenzene; 4-hydroxyacetophenone; anthranilic acid; benzoic acid; caffeic acid; catechin; epicatechin; epigallocatechin; epigallocatechin gallate; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; neohesperidin; phloretin; protocatechuic acid; psolaren; quercetin; quercetin-3-glucoside; resveratrol; scopoletin; syringin; taxifolin and vanillin.

Table 8.6: Relationship between phytochemical compounds isolated from TI extract and the impact on DNA SBR in colon cells.

TI	Phytochemical metabolites showing the greatest impact on colon cells	
	NCM460	CaCo-2
Petroleum ether extract	2,3-dihydroxybenzoic acid; ferulic acid and phenylacetic acid.	2,3-dihydroxybenzoic acid; 3,4,5-trimethoxyacetophenone and phenylacetic acid.
Chloroform extract	Catechin; ferulic acid; scopoletin and taxifolin.	Catechin; scopoletin and taxifolin.
Ethyl acetate extract	2,3-dihydroxybenzoic acid; anthranilic acid; caffeic acid; catechin; cinnamic acid; epicatechin; epigallocatechin; ferulic acid; gallocatechin; indole-3-pyruvic acid; luteolin; myricetin; niacin; phenylacetic acid; phloretin; resveratrol; scopoletin; sinapic acid and taxifolin.	2,3-dihydroxybenzoic acid; 3,4,5-trimethoxyacetophenone; 4-hydroxy-3-methoxyphenylpropionic acid; anthranilic acid; caffeic acid; catechin; cinnamic acid; epicatechin; epigallocatechin; gallocatechin; hydroxytyrosol; indole-3-acetic acid; indole-3-pyruvic acid; luteolin; myricetin; niacin; phenylacetic acid; phenyllacetic acid; phloretin; resveratrol; scopoletin; sinapic acid and taxifolin.
Ethanol extract	Anthranilic acid; caffeic acid; catechin; epicatechin; epigallocatechin; ferulic acid; gallocatechin; luteolin; myricetin; niacin; phenylacetic acid; phloretin; resveratrol; scopoletin and taxifolin.	3,4,5-trimethoxyacetophenone; anthranilic acid; caffeic acid; catechin; epicatechin; epigallocatechin; gallocatechin; luteolin; myricetin; niacin; phenylacetic acid; phenyllacetic acid; phloretin; resveratrol; scopoletin and taxifolin.

Table 8.7: Relationship between phytochemical compounds isolated from TI extracts and the impact on migration in colon cells.

TI	Phytochemical metabolites showing the greatest impact on CaCo-2 cell migration
Petroleum ether extract	Ferulic acid; indole-3-carboxaldehyde; phenylacetic acid and phloridzin.
Chloroform extract	Catechin; ferulic acid; gallic acid; indole-3-carboxaldehyde; kaempferol; protocatechuic acid; quercetin; scopoletin and taxifolin.
Ethyl acetate extract	1,2-dihydroxybenzene; anthranilic acid; caffeic acid; catechin; cinnamic acid; epicatechin; epigallocatechin; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; niacin; neohesperidin; phenylacetic acid; phloretin; phloridzin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin and taxifolin.
Ethanol extract	anthranilic acid; caffeic acid; catechin; epicatechin; epigallocatechin; ferulic acid; gallic acid; gallocatechin; indole-3-carboxaldehyde; kaempferol; luteolin; myricetin; niacin; neohesperidin; phenylacetic acid; phloretin; phloridzin; protocatechuic acid; psolaren; quercetin; resveratrol; scopoletin and taxifolin.