ANTIOXIDANT ACTIVITY AND CYTOTOXIC EFFECT OF CASSIA ESSENTIAL OIL

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ANTIOXIDANT ACTIVITY AND CYTOTOXIC EFFECT OF CASSIA ESSENTIAL OIL

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ABSTRACT

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Cassia essential oil is an odorant natural product extracted from the bark of *Cinnamomum cassia* through steam distillation. It comprises of a complex mixture of heterogenous group of secondary metabolites at different concentration with less severe side effects and toxicities. In this study, Cassia essential oil originated from China was purchased from online platform. Thin layer chromatography was conducted to identify metabolites' polarity, followed by the detection of major group of secondary metabolites through qualitative phytochemical analysis. The essential oil was further evaluated for DPPH radical scavenging activity and cytotoxicity using MTT assay on Vero and A549 cells. Cassia essential oil contain both polar and non-polar compounds supported by the well-separated spots in thin layer chromatography in a mixture of hexane and ethyl acetate at ratio 10:3. Based on phytochemical analysis, a positive result was obtained for phenols, terpenoids, alkaloids, tannins, and flavonoids respectively. Nevertheless, saponins, quinones and glycosides were not detected in the essential oil. Cassia essential oil showed higher DPPH radical scavenging activity with EC_{50} values of 0.87 ± 0.110 mg/mL (w/v) and 0.10 ± 0.130% (v/v), respectively. In MTT assay, Cassia essential oil exerted cytotoxicity selectively against A549 cells with IC_{50} values of 24.92 ± 0.079 µg/mL (24 hours), 20.49 ±

0.202 μg/mL (48 hours) and 7.14 ± 0.112 μg/mL (72 hours). No cytotoxicity was observed on Vero cells as the IC_{50} values were above 500 μg/mL in all the incubation periods. Therefore, Cassia essential oil is potential as antioxidant and cytotoxic agents. Future studies in the identification on the specific bioactive components in Cassia essential oil is crucial to correlate with various pharmacological activities.

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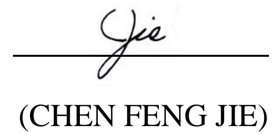
Thank you everyone!

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.



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I hereby give permission to the University to upload the softcopy of my final year project in PDF format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(CHEN FENG JIE)

TABLE OF CONTENTS

	Page
ABSTRACT	**ii**
ACKNOWLEDGEMENTS	**iv**
DECLARATION	**v**
APPROVAL SHEET	**vi**
PERMISSION SHEET	**vii**
TABLE OF CONTENTS	**viii**
LIST OF TABLES	**xi**
LIST OF FIGURES	**xii**
LIST OF ABBREVIATIONS	**xiv**

CHAPTER

1.0	**INTRODUCTION**			**1**
	1.1	Essential Oils		1
	1.2	Significance of the Study		3
	1.3	Objectives		4
2.0	**LITERATURE REVIEW**			**5**
	2.1	*Cinnamomum cassia*		5
		2.1.1	Botanical Description	6
		2.1.2	Geographical Distribution	6
		2.1.3	Taxonomical Classification	7
	2.2	Cassia Essential Oil		8
		2.2.1	Extraction Method	8
		2.2.2	Traditional Uses	9
		2.2.3	Antioxidant Activity	10
		2.2.4	Anticancer Activity	11
	2.3	Cancer		12
	2.4	Cancer Statistics		13
	2.5	Vero Cells		14
	2.6	Thin Layer Chromatography		15
	2.7	Classes of Phytochemicals		16
	2.8	Antioxidant Assay		20
	2.9	Anticancer Assay		22

3.0 MATERIALS AND METHODS **25**
3.1 Materials 25
3.1.1 Cassia Essential Oil 25
3.1.2 Cell Lines 25
3.1.3 Solvents and Chemicals 26
3.1.4 Equipment 27
3.2 Flowchart of the Study 28
3.3 Thin Layer Chromatography 29
3.4 Phytochemical Analysis 31
3.4.1 Test for Phenols 31
3.4.2 Test for Terpenoids 31
3.4.3 Test for Saponins 31
3.4.4 Test for Alkaloids 31
3.4.5 Test for Tannins 32
3.4.6 Test for Quinones 32
3.4.7 Test for Glycosides 32
3.4.8 Test for Flavonoids 32
3.5 DPPH Free Radical Scavenging Assay 33
3.5.1 Preparation of Reagents and Test Samples 33
3.5.2 Protocol and Design 33
3.6 Cell Culture 35
3.6.1 Preparation of Complete Growth Medium 35
3.6.2 Preparation of Phosphate Buffer Saline 35
3.6.3 Preparation of Cryoprotectant 35
3.6.4 Quality Control 35
3.6.5 Thawing of Cell Line 36
3.6.6 Subculturing of Cells 36
3.6.7 Cell Line Cryopreservation 37
3.7 MTT Assay 37
3.7.1 Preparation of Reagents and Test Samples 37
3.7.2 Cell Counting 37
3.7.3 Protocol and Design 38
3.8 Data Analysis 40

4.0 RESULTS **41**
4.1 Thin Layer Chromatography 41
4.2 Phytochemical Analysis 43
4.2.1 Phenols 43
4.2.2 Terpenoids 44
4.2.3 Saponins 45
4.2.4 Alkaloids 46
4.2.5 Tannins 47
4.2.6 Quinones 48
4.2.7 Glycosides 49
4.2.8 Flavonoids 50
4.3 DPPH Assay 51
4.4 MTT Assay 54
4.4.1 Vero Cells 54
4.4.2 A549 Cells 58

5.0 DISCUSSION 63
5.1 Thin Layer Chromatography 63
5.2 Phytochemical Analysis 65
5.3 DPPH Assay 69
5.4 MTT Assay 72
5.4.1 Vero Cells 72
5.4.2 A549 Cells 73
5.5 Limitations of the Study 76
5.6 Future Studies 76

6.0 CONCLUSIONS 78

REFERENCES 79

LIST OF TABLES

Table		Page
2.1	Taxonomical classification of *Cinnamomum cassia*	7
3.1	Solvents and chemicals used in the study	26
3.2	Equipment used in the study	27
3.3	List of combination mixture of organic solvents in developing chamber	30
4.1	Retention factor (R_f) values based on different combination of solvent mixture	42
4.2	DPPH scavenging activity of Cassia essential oil (w/v) and ascorbic acid	52
4.3	The EC_{50} values of the sample tested	53
4.4	The percentage cell viability of Vero cells upon treatment with Cassia essential oil and doxorubicin	57
4.5	The IC_{50} values of Cassia essential oil and doxorubicin on Vero cells	58
4.6	The percentage cell viability of A549 cells upon treatment with Cassia essential oil and doxorubicin	61
4.7	The IC_{50} values of Cassia essential oil and doxorubicin on A549 cells	62

LIST OF FIGURES

Figure		Page
2.1	Different parts of *Cinnamomum cassia* (a) Stem (b) Flowers (c) Leaves (d) Fruits	6
2.2	Isolation flow of Cassia oil from *Cinnamomum cassia* through steam distillation	9
2.3	Age-standardised incidence rate of lung cancer for males and females in the states of Malaysia	14
2.4	Structure of flavonoid	17
2.5	Structure of pyrrolidine and pyridine under alkaloids	19
2.6	The reduction of purple DPPH to yellow diphenylpicryl hydrazine in the presence of antioxidants	22
2.7	The formation of formazan from MTT	24
3.1	The purchased Cinnamon Cassia essential oil	25
3.2	Flowchart of research activities in the study	28
3.3	Layout of thin layer chromatography	30
3.4	The 96-well plate layout for DPPH assay	34
3.5	Open circle represents the cells that must be counted whereas closed circle indicates the cells that are not counted	38
3.6	The 96-well plate layout for MTT assay	40
4.1	TLC results of Cassia essential oil in different combination of organic solvents (A) Hex : EA (0 : 10) (B) Hex : EA (10 : 1) (C) Hex : EA (10 : 3) (D) Hex : EA (1 : 1)	42
4.2	The result of ferric chloride test to detect the presence of phenols in Cassia essential oil	43
4.3	The result of Salkowski test to detect the presence of terpenoids in Cassia essential oil	44

4.4	The result of foam test to detect the presence of saponins in Cassia essential oil	45
4.5	The result of Wagner's test to detect the presence of alkaloids in Cassia essential oil	46
4.6	The result of Braymer's test to detect the presence of tannins in Cassia essential oil	47
4.7	The result of sulphuric acid test to detect the presence of quinones in Cassia essential oil	48
4.8	The result of Keller-Kiliani test to detect the presence of glycosides in Cassia essential oil	49
4.9	The result of Pew's test to detect the presence of flavonoids in Cassia essential oil	50
4.10	Graph of percentage of DPPH scavenging activity against concentration of ascorbic acid, Cassia essential oil (w/v) and Cassia essential oil (v/v)	51
4.11	Morphological observation of Vero cells cultured in DMEM upon 85 to 90% confluency (100x total magnification)	54
4.12	Bar graph representing the percentage cell viability of Vero cells at varying concentrations of Cassia essential oil at 24- and 48-hours	55
4.13	Bar graph representing the percentage cell viability of Vero cells at varying concentrations of doxorubicin at 24- and 48-hours	55
4.14	Morphological observation of A549 cells cultured in DMEM upon 85 to 90% confluency (100x total magnification)	58
4.15	The percentage cell viability of A549 cells at varying concentrations of Cassia essential oil at 24-, 48- and 72-hours	59
4.16	The percentage cell viability of A549 cells at varying concentrations of doxorubicin at 24-, 48- and 72-hours	60

LIST OF ABBREVIATIONS

AKT	Protein kinase B
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CO_2	Carbon dioxide
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Half-maximal effective concentration
EDTA	Ethylenediaminetetracetic acid
EGFR	Epidermal growth factor receptor
FBS	Foetal bovine serum
IC_{50}	Half-maximal inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate buffer saline
PIK3CA (PI3K)	Phosphatidylinositol-3-kinase
PPO	Polyphenol oxidase
PTEN	Phosphatase and tensin homolog
RAF	Rapid accelerated fibrosarcoma
RB	Retinoblastoma
TP53	Tumour protein 53

CHAPTER 1

INTRODUCTION

1.1 Essential Oils

The term natural product is defined as the chemical compounds synthesised by biological, living organism. The presence of primary and secondary forms of metabolites that are present in natural products contribute to various biological and pharmacological activities (Anulika, et al., 2016; Katz and Baltz, 2016). Lipid-soluble essential oils are odorant, transparent and volatile natural product that are synthesised by plants (Aziz, et al., 2018). These synthesised essential oils are found located in glandular hairs, secretory cavities, or cells of different plant parts, for instance, flowers, stems, roots, fruits, seeds, barks, twigs and leaves. Besides, the essential oils can be obtained from various part of the aromatic plants through hydro distillation, steam distillation, cold pressing extraction, supercritical fluid extraction or solvent extraction (Chouhan, Sharma and Guleria, 2017; Aziz, et al., 2018). Mugao, et al. (2020) stated that plant synthesise essential oils in order to protect from predators and pests, to promote seed dispersal and to attracts pollinators. Apart from that, essential oils components reveal a plethora of biological properties like antioxidant, antiviral, anti-inflammatory, antibacterial and anticancer activities (Sharma, et al., 2022).

Blowman, et al. (2018) reported that essential oils enhanced the efficacy of docetaxel and paclitaxel, which are the common chemotherapy agents in cancer treatments. From 1940 to 2006, more than 50% of all internationally approved antitumour agents were plant-derived natural compounds, suggesting the

promising potency of essential oils as candidates for novel anticancer agents due to its tolerability on normal human cells and cytotoxicity against cancer cells (Bhalla, Gupta and Jaitak, 2013; Gautam, Mantha and Mittal, 2014). Plant essential oils contain a complex heterogenous group of plant secondary metabolites with the presence of approximately 20 to 60 molecules at various concentrations. The secondary metabolites from essential oils are originated from the intermediates involved in primary metabolites biosynthesis pathway or from the primary metabolites itself (Pang, et al., 2021). Essential oils contain compounds with low molecular weight of less than 3000 Daltons and is non-essential for cell survival, growth, and development (Kinghorn, Chin and Swanson, 2009).

Generally, secondary metabolites are divided into several classes, which consists of steroids, terpenes, phenolics, flavonoids and alkaloids (Pang, et al., 2021). Terpenes, including monoterpenes and sesquiterpenes are one of the two or three main components that make up roughly 20 to 70% of total essential oils. Therefore, terpenes contribute to the biological effects of oils significantly. Additionally, the presence of phenylpropanoids, diterpenes and nitrogen-containing compounds such as alkaloids are reported to be present in essential oils as well (Sharifi-Rad, et al., 2017). Other than the hydrocarbon terpenes group, oxygenated derivatives such as ethers, ketones, alcohols, aldehydes, and esters are found in relatively trace amount in essential oils (Aziz, et al., 2018). According to Geng, et al. (2011), the extraction techniques, geographical location and the plant's parts involved affect the contents and components in the essential oils significantly (García-Salinas, et al., 2018).

1.2 Significance of the Study

Drug resistance towards conventional chemotherapy has been developed in lung cancer through DNA repair pathways (Li, et al., 2021). For example, the mechanism of action for platinum-based chemotherapy drugs like cisplatin is inhibited due to the upregulation expression of *excision repair cross-complementation group 1* gene in nucleotide excision repair pathway. It acts as a marker of resistance that removes the DNA-platinum adducts produced by cisplatin from the damaged DNA, thereby preventing cisplatin-induced apoptosis (Shanker, et al., 2010). Hence, an urgent demand for a novel effective chemotherapy treatment of lung cancer is needed.

In addition, the side effects and toxicities of cancer therapy are reported, ranging from moderate to significant effects (Wu and Singh, 2011). For example, gastrointestinal disorders, fatigue, hair loss, neuropathies and skin disorders are some common adverse effects induced by chemotherapy (Chan and Ismail, 2014). Based on Zhang, et al. (2019), Cassia essential oil possess little toxicity with less severe side effect, for instance, skin irritation is developed in patient only when the recommended safe dose per day is exceeded and the side effect will subside gradually after treatment. On top of that, it is found that essential oil-mediated therapy can mitigate the side effects of conventional therapies in combination with standard radiotherapy and chemotherapy (Gautam, Mantha and Mittal, 2014).

Despite numerous studies on cancerous cell lines like human colorectal cancer (HCT15) and human breast cancer (HeLa), information with regards to the

cytotoxicity of Cassia essential oil on non-cancerous cells and lung cancer are still limited and inconclusive (Ranjitkar, et al., 2021). Besides, there is still a regrettable dearth of studies that documents the pharmaceutical effects of stem-distilled Cassia oil obtained specifically from the *Cinnamomum cassia* bark as different extraction methods and different part of the plants might result in different biological activities (Geng, et al., 2011; Yang, Li and Chuang, 2012).

Therefore, the findings of this study will be helpful and beneficial particularly as an alternative, traditional plant medicine that can treat cancers effectively with high, sustained efficacy to overcome the problem of increased lung cancer mortality rate, drug resistance and possible side effects from conventional treatments.

1.3 Objectives

The objectives of the study are:

1) To detect the presence of secondary metabolites and their polarity in Cassia essential oil using thin layer chromatography,
2) To determine major groups of secondary metabolites in Cassia essential oil through qualitative phytochemical analysis,
3) To evaluate the antioxidant activity of Cassia essential oil via DPPH free radical scavenging assay,
4) To investigate the cytotoxic effect of Cassia essential oil on A549 cells and Vero cells using MTT assay.

CHAPTER 2

LITERATURE REVIEW

2.1 *Cinnamomum cassia*

2.1.1 Botanical Description

Cinnamomum cassia or Chinese Cinnamon is a medium-large, evergreen aromatic tree belongs to the family of *Lauraceae* (Kazi, Shaikh and Khoharo, 2015). The tree is characterised by the presence of pubescence on the dark brown and cylindrical stem with the shape of the glossy, green leaves ranging from elliptic to sub-lanceolate measured 7 to 18 cm long as illustrated in **Figure 2.1** (Cardoso-Ugarte, López-Malo and Sosa-Morales, 2016). At the apex of the stem, the white conical flowers are about 4.5 mm long with yellowish-brown perianth and the appearance of tomentulose in the inside and outside. The ellipsoid fruit of *C. cassia* is 10 x 7 to 8 (or 9) mm with the colour of black or purple as the fruits mature. The young, cylindrical twig of *C. cassia* is also well known as *Ramulus Cinnamomi* ('Keishi' in Japan, 'Guichi' in China and 'Geiji' in Korea) is estimated 30 to 75 cm long and diameter of approximately 0.3 to 1.0 cm with the presence of reddish-brown to brown-coloured surface, branchlets, punctate lenticels, bud marks, and the knobby leaf marks (Zheng, et al., 2015; Liu, et al., 2019).

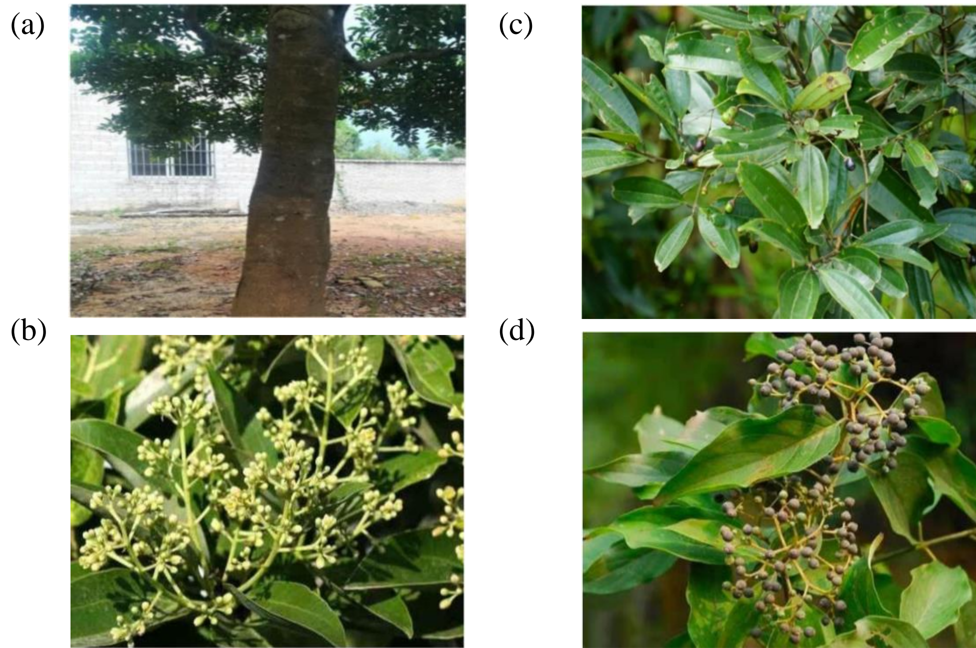


Figure 2.1: Different parts of *Cinnamomum cassia* (a) Stem (b) Flowers (c) Leaves (d) Fruits (Adapted from Liu, et al., 2019; Zhang, et al., 2019).

2.1.2 Geographical Distribution

Cinnamomum cassia is found on the valleys or slopes at 400 to 1100 m altitude in the evergreen forests that are broadleaved. The plant grows well in warm, frost-free climate in the subtropical regions and require about 26 to 30°C optimum temperature with over 70% humidity and a plethora of rainfall to grow (Liu, et al., 2019). *C. cassia* is concentrated predominantly in the subtropical areas of Guangdong, Hainan, Guangxi, Yunnan, Fujian, Guizhou, Sichuan and Taiwan provinces in China while at the same time, this species is also found distributed in many other countries in the southern and eastern Asia, for instance, Malaysia, Thailand, Laos, Vietnam, India where *C. cassia* is cultivated extensively (Kuete, 2017; Zhang, et al., 2019).

2.1.3 Taxonomical Classification

Cinnamomum cassia are grouped under the genus *Cinnamomum* with estimated more than 250 species (Kuang, 2011). The taxonomical hierarchy of *C. cassia* is presented in **Table 2.1**.

Table 2.1: Taxonomical classification of *Cinnamomum cassia* (Adapted from Menggala and Damme, 2018).

Scientific Classification	
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Magnoliidae
Order	Laurales
Family	Lauraceae
Genus	*Cinnamomum*
Species	*Cinnamomum cassia*

2.2 Cassia Essential Oil

2.2.1 Extraction Method

In this study, Cassia essential oil used was isolated from *C. cassia* through steam distillation as illustrated in **Figure 2.2**. Steam distillation is a prevalent, conventional extraction method that uses superheated steam produced by steam generator to isolate and vaporise the plant essential oils which comprise of temperature sensitive compounds.

Based on Božović, et al. (2017), steam distillation enables the distillation of constituents from essential oils at a temperature that is below the boiling point of each individual compound to prevent decomposition. In the first place, the steam generator, for instance, boiling water supplies steam to the distillation apparatus from the base to top to heat the barks of *C. cassia* in the absence of water in the alembic. The cell structures of raw *C. cassia* barks are broken down in the presence of steam as the separation agent so that the volatile constituents of Cassia essential oil from *C. cassia* are released, volatilised, and evaporated (Tongnuanchan and Benjakul, 2014). The vaporised oils are further condensed and liquefied in a condenser to yield a mixture of essential oil and some aromatic water (Rassem, Nour and Yunus, 2016). The Cassia essential oil are found rising on top of aromatic water in the separator owing to the lower density of oil and henceforth, it enables the extraction of oil from the mixture to yield pure Cassia essential oil for further analysis.

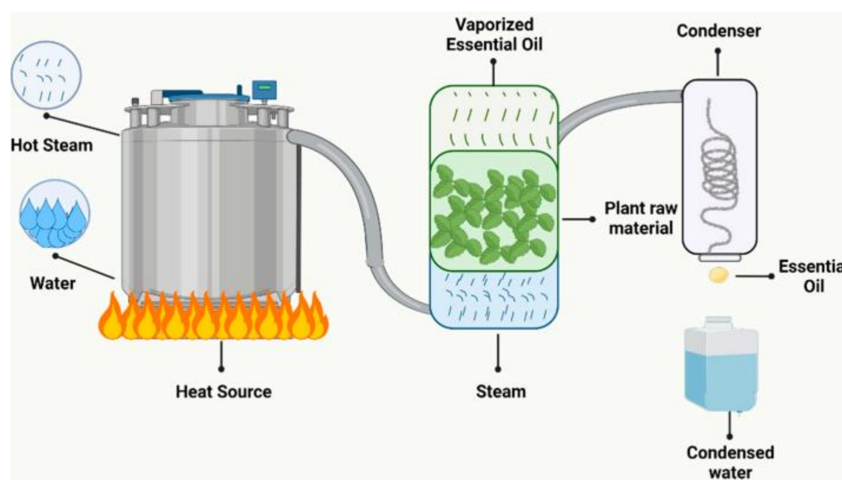


Figure 2.2: Isolation flow of Cassia essential oil from *Cinnamomum cassia* through steam distillation (Adapted from Machado, et al., 2022).

2.2.2 Traditional Uses

Cassia essential oil are frequently utilised as antiseptic, astringent, and carminative agents traditionally (Al-Samydai, et al., 2018). In addition, the volatile oil has been used in the area of aromatherapy, which involves the absorption of plant volatile essential oils into the body through olfactory system or skin for therapeutic purpose. Hur, et al. (2012) stated that the usage of cinnamon oil isolated from natural plant in aromatherapy massage is believed to alleviate the level of menstruation pain as compared to the consumption of acetaminophen. Besides, according to Liao, et al. (2009), conditions like dysmenorrhea, gastrointestinal necrosis, diarrhoea, amenorrhea, oedema, impotency, tussis and arthralgia can be treated with the involvement of barks from *C. cassia*.

Apart from that, Cassia essential oil are commonly being used as traditional Chinese medicine in the treatment of hyperuricemia which can lead to the development of gout, which is then associated with renal failure, diabetes

mellitus, alcoholism, obesity, cardiovascular diseases, and others (Chang, et al., 2013).

2.2.3 Antioxidant Activity

Antioxidant activity of Cassia essential oil had been assessed and documented in numerous studies suggesting that it is a potential antioxidant agent. Brodowska, et al. (2016) had conducted a study to examine the antioxidant profile of hydro-distilled bark *C. cassia* essential oil using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay by calculating the concentration of Cassia essential oil needed to scavenge 50% of DPPH free radicals (EC_{50}). Based on the study, Cassia essential oil was concluded as a good antioxidant agent with EC_{50} of 147.23 ± 0.04 μg/L for DPPH assay with increasing radical scavenging activity alongside with increasing concentration. This is further supported by the findings reported by De-Montijo-Prieto, et al. (2021) where the hydro-distilled Cassia essential oil isolated from *C. cassia* bark contain higher antioxidant property with EC_{50} of 0.05 mg/mL for DPPH assay in contrast to essential oil extracted from other aromatic plants, for instance, *Thymus vulgaris*, *Salvia lavandulifolia* and *Rosmarinus officinalis*.

On top of that, based on another study from Feng, et al. (2019), the antioxidant activity of Cassia essential oil was confirmed to be significant with the highest percentage of DPPH scavenging activity, which is approximately 92.4% compared to 89.1% of peppermint oil, 80.1% nutmeg oil and 25.5% lemon oil.

2.2.4 Anticancer Activity

Cassia essential oil has an effective antitumour property against cancer cells as reported by Błaszczyk, Rosiak and Kaluzna-Czaplinska (2021) in which the study described cinnamon oil as the natural product that contain cinnamaldehyde and eugenol compounds which demonstrated promising result in treating tumours like melanoma, stomach cancer, lymphoma and leukaemia.

Sharma, et al. (2017) utilised 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the effect of cinnamaldehyde and eugenol on the cell viability of PC-3 prostate cancer cell line. As the concentration of cinnamaldehyde and eugenol concentration increased, a significant drop of the cell viability of PC-3 from 87% to 25% and from 91% to 36% was detected respectively indicating an excellent cytotoxic activity of Cassia essential oil and their compounds against PC-3 cancerous cell (Larasati and Meiyanto, 2018). The IC_{50} value of eugenol and cinnamaldehyde on PC-3 cell line were 85.43 µM and 73.38 µM, correspondingly (Sharma, et al., 2017).

Similar studies had been conducted by Chang, et al. (2016) to analyse the cytotoxic effect of Cassia essential oil on HSC-3 human oral squamous cell carcinoma in a dose-dependent manner using MTT assay. As expected, the viability of the cancer cell line treated with Cassia essential oil had reduced after 24 and 48 hours.

Yan, et al. (2017) documented that eugenol exerted cytotoxicity against the MCF10A-ras cells with decreasing cell viability of 64.2%, 52.7%, 22.7%, 14.8%, 9.3% at concentration of 200 µM, 160 µM, 120 µM, 80 µM and 40 µM, respectively. On the contrary, the cell viability of non-cancerous MCF-10A cells were not influenced by eugenol. Hence, it was concluded that Cassia essential oil only cause cytotoxicity specifically and selectively on cancerous cells, but not against the non-cancerous cell lines (Hussain, et al., 2011; Larasati and Meiyanto, 2018).

2.3 Cancer

Normal cells transformed into cancer cells via a multi-step carcinogenesis process that involved the accumulation of multiple genetic mutations which subsequently lead to the suppression of tumour suppressor pathways and the activation of pathways that promote cell growth (Cooper, et al., 2013). For example, Tsay, et al. (2017) revealed that constitutive activation of signal transduction pathway can be caused by KRAS gene mutations, which comprises of 20 to 30% of lung cancer patients. GTPase is encoded by KRAS gene that acts as central mediator to the downstream signalling pathways. Activating mutations involving the substitution of amino acids Gly 12 results in a decrease in the activity of GTPase, causing an increase in the protein bounded by GTP which consequently increases the growth signalling via RAF (Greulich, 2010).

Besides, lung cancer also develops when the function of tumour suppressor genes like TP53, PTEN, CDKN2A and RB that normally act as negative regulators to control the growth of cells is altered through loss of heterozygosity

(LOH) due to the allelic loss (Cooper, et al., 2013). For instance, roughly 27% lung cancer patients contain mutations in p53 gene, and the affected region is chromosome 17p13, in which 80% involves missense mutations, 5% involves silent mutations, 7% consists of nonsense mutations and 9% contains deletions or insertions which subsequently disrupt the normal cell cycle progression, resulting in lung cancer formation (Mogi and Kuwano, 2011; Tsay, et al., 2017).

Lung adenocarcinoma cell line (A549) which is human alveolar epithelial cells, was isolated and cultured by Giard, et al. in 1972 from explant of a Caucasian male who is 58 years old (American Type Culture Collection, 2022a). The doubling time of the cells is approximately 21.8 hours on average. Hence, the cells must be monitored frequently for confluency and subculture accordingly (Wiesmann, et al., 2021).

2.4 Cancer Statistics

Based on Malaysia National Cancer Registry Report in the year 2012 to 2016 written by Azizah, et al. (2019), there were 11 256 cases of lung, trachea, and bronchus cancers in total among Malaysia population compared to 10 608 cases in the year from 2007 to 2011, which showed a 6.11% increase. Out of the 11 256 reported lung cancer cases, males were more prevalent in the development of lung cancer which consisted of approximately 68.3% and hence, it was said to be the second most common tumour among males. On the other hand, lung cancer was ranked as the fifth most common cancer among Malaysian females with roughly 31.7% cases. According to the statistics from World Health Organization (WHO) in 2014, 4088 patients had died from lung cancer per year

with 19.1 deaths for every 100 000 Malaysia population (Siang and John, 2016). **Figure 2.3** displays incidence rate of lung cancer according to gender and the state in Malaysia in an age-specific manner.

Figure 2.3: Age-standardised incidence rate of lung cancer for males and females in the states of Malaysia (Adapted from Azizah, et al., 2019).

2.5 Vero Cells

The non-cancerous, immortal fibroblastic Vero cell line is isolated by Y. Kawakita and Y. Yasumura in 1962 from the kidney of non-diseased grown-up *Ceropithecus aethiops*, which is known as an African green monkey (Osada, et al., 2014; American Type Culture Collection, 2022b). For every 24 hours, the number of Vero cells double and therefore, subculture is needed upon confluency to avoid cell degeneration and detachment of cells (Ammerman, Beier-Sexton and Azad, 2008; Adams, et al., 2015). It is pivotal because unlike cancerous cells, the non-transformed Vero cells possess contact inhibition, in which the growth of cells are terminated, and the cells will begin to die upon confluency.

Vero cells have been widely utilised to study cytotoxicity, differentiation, and growth (Gonçalves, et al., 2006). Meanwhile, it is also involved in bacteriology, toxicology, virology, and parasitology research fields to screen for *Escherichia coli* toxin or as eukaryotic parasites and viruses host cells (Adams, et al., 2015; Kiesslich and Kamen, 2020). Vero cells are used widely in these areas due to their characteristics as a continuous cell line, which generates large cell banks for cell characterisation extensively since they contain indefinite passage ability unlike primary cell lines such as chicken embryo fibroblast which cannot be passaged unlimitedly (Kiesslich and Kamen, 2020).

2.6 Thin Layer Chromatography

Thin layer chromatography (TLC) or planar chromatography, is defined as the separation of more than two nonpolar, mid polar or polar components in a mixture. It involves differential partitioning between two phases, which are the stationary and mobile phase based on "Like Dissolves Like" rule of solubility (Kumar, Jyotirmayee and Sarangi, 2013; Lade, et al., 2014). TLC is considered as a solid-liquid chromatographic technique, in which it depends on the competition between compound adsorption on solid stationary phase and solute desorption by liquid mobile phase to separate the components from one another (Akash and Rehman, 2020).

TLC utilises a silica gel or aluminium oxide-coated thin glass or aluminium foil solid plate as the solid stationary phase whereas the liquid mobile phase involves one or a combination of eluting solvents that draw up via capillary action through the stationary phase, for instance, hexane, ethyl acetate and methane (Lade, et

al., 2014). According to Bele and Khale (2011), in normal-phase chromatography, the silica gel stationary phase is more polar as compared to the mobile phase. Reversely, stationary phase is considered less polar than mobile phase for reversed-phase TLC (Shantha and Napolitano, 2009). Thus, depending on the molecular structure, functional groups as well as the physical properties of the solutes, the components separate from the mixture and travel up the stationary phase at different extent (Lade, et al., 2014). In normal phase TLC, a polar solute in the mixture contains higher affinity and stronger interaction with the polar stationary phase owing to the similarity in polarity. Therefore, the solute retains in the stationary phase and will move at a slower rate with a low retention factor (R_f) value. On the other hand, the nonpolar compound will travel at a greater distance over the stationary phase surface as it has stronger interaction and affinity towards the nonpolar mobile phase, resulting in a higher R_f value. Through this, the separated compounds are detected on the TLC plate at different positions in the form of spots (Sjursnes, Kvittingen and Schmid, 2015; Akash and Rehman, 2020).

According to Santiago and Strobel (2013), TLC is simple, low cost, high sensitivity, accuracy, and reproducibility. Furthermore, less amount of solvent per sample is required when performing TLC with fast analysis time, enabling simultaneous analysis of numerous samples (Sherma, 2006).

2.7 Classes of Phytochemicals

Phenolic compounds are one of the secondary metabolites that are commonly found in plants. Based on Vuolo, Lima and Junior (2019), phenolics are

biosynthesised through shikimate/phenylpropanoid pathway and further categorised into simple phenols, flavonoids, lignans, phenolic acids, tannins, quinones, stilbenes and xanthones.

Phenols are the simplest phenolic compounds with chemical formula of C_6H_5OH whilst the ubiquitous and most studied flavonoids, which are also known as polyphenolic compounds are considered as the largest phenolic compounds of plants, consisting of more than one phenol as illustrated on **Figure 2.4** (Saxena, et al., 2013). Depending on the attachment of C ring on B ring carbon and the extent of C ring oxidation and unsaturation, flavonoids are further classified into several subgroups, recapitulating isoflavones, chalcone, anthocyanin, flavone, flavanone and flavonol. For example, flavonol is resulted from the linkage of C ring to position 2 (C_2) of B ring (Panche, Diwan and Chandra, 2016; Mamari, 2021).

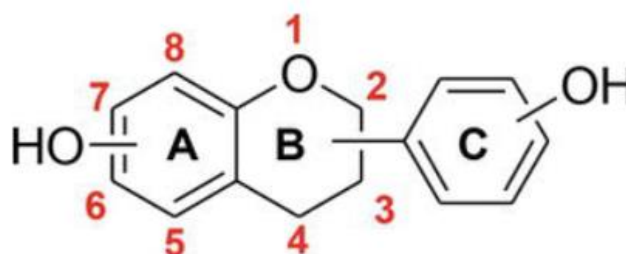


Figure 2.4: Structure of flavonoid (Adapted from Saxena, et al., 2013).

Moreover, tannins are also categorised under the group of polyphenolic compounds, and further can be subdivided into four groups, including condensed tannins, complex tannins, and hydrolysable tannins like ellagitannins and gallotannins based on the structure (Koche, Shirsat and Kawale, 2016).

Gallotannins, for instance, tannic acid, are produced when galloyl unit is attached to the polyol unit such as D-glucose whilst ellagitannins are defined as the tannins which contain two or more C-C coupled galloyl units without catechin unit that are linked glycosidically (Khanbabaee and Ree, 2001). Other phytochemicals such as quinones are produced from the oxidation of phenolic compounds that involves enzymes such as peroxidase and polyphenol oxidases (PPOs) which recapitulate laccase as well as catechol oxidase. PPOs yield quinones from *o*-diphenols via oxidation through catecholase activity or in another way, PPOs induce the catalysation of monophenols hydroxylation to form *o*-diphenols via cresolase activity (Schieber, 2018). The general structure of quinones comprise of two carbonyl groups containing benzene core in which two oxygen atoms substitute two of the hydrogen atoms (Niaz, Khan and Muhammad Ajmal, 2020).

Besides, alkaloids synthesised from shikimic acid pathway with aromatic amino acids are also important secondary metabolites as well (Shu, 2007; Mendoza and Silva, 2018). Depending on the location of nitrogen atom, alkaloids consist of non-heterocyclic nitrogen atoms, for example, tropolone-derived alkaloids as well as heterocyclic basic nitrogen atoms such as derivative of L-lysine, L-tryptophan, and L-tyrosine (Bhambhani, Kondhare and Giri, 2021). There are several classes of alkaloids, including pyridine, pyrrolidine, quinolines, indoles, isoquinolines and pyrrolizidines based on their heterocyclic ring in their chemical structure (Kurek, 2019). For example, pyridine alkaloids made up of hexahydropyridine or piperidine ring whilst a tetrahydropyrrole or pyrrolidine

ring is present in pyrrolidine group as illustrated in **Figure 2.5** (Saxena, et al., 2013).

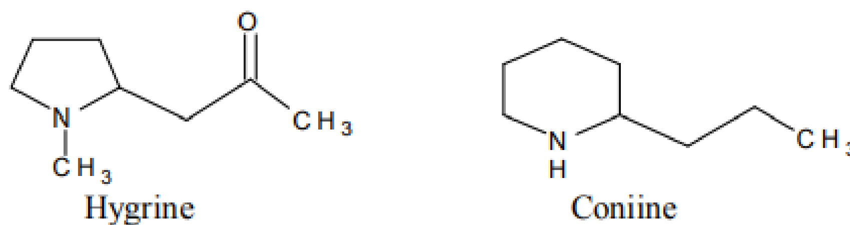


Figure 2.5: Structure of pyrrolidine and pyridine under alkaloids (Adapted from Saxena, et al., 2013).

Saponins, which are biosynthesised through mevalonate pathway contain at least one hydrophilic carbohydrate chain connected to a sapogenin or aglycone unit which comprises of a hydrophobic triterpene or sterol unit (Moses, Papadopoulou and Osbourn, 2014). Monodesmoside saponins are synthesised if only a sugar chain is linked at position C3 of aglycone whereas when two or more sugar residues are attached to the C-3 and C-22 of aglycone, bidesmoside as well as polydesmodise saponins are produced (Upadhyay, et al., 2018).

Based on Habtemariam (2019), terpenoids are formed via mevalonate pathway and they consist of five-carbon isoprene units which are the building block with the formula of $CH_2=C(CH_3)-CH=CH_2$ or $(C_5H_8)n$. Terpenoids are classified into several classes depending on the number of isoprene (n) that are present. Hemiterpenes with molecular formula of C_5H_8 contain only an isoprene unit; Monoterpenes ($C_{10}H_{16}$) built up from two isoprene in acyclic, bicyclic, or monocyclic types; Sesquiterpenes ($C_{15}H_{24}$) with three isoprene units and diterpenes ($C_{20}H_{32}$) comprise of four isoprene units, followed by sesterpenes

($C_{30}H_{48}$), triterpenes ($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$), polyterpenes ($C_{40}H_{64}$)n with five, six, eight or more isoprene units, respectively (Mabou and Yossa, 2021).

On top of that, other secondary metabolites found in plant is the glycosides. Glycosides are produced when aglycone is linked to anomeric carbon of glycone via a heteroatom (Brito-Arias, 2022). According to Soto-Blanco (2021), glycosides can be further subdivided into several groups based on the glycosidic bond types, aglycone group as well as the saccharides number. For example, C-(carbon), N-(nitrogen from NH), S-(sulfur) and O-(oxygen) glycosides are classified referring to the formed glycosidic linkage.

2.8 Antioxidant Assay

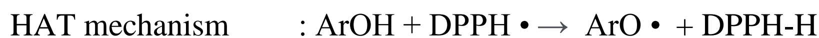
Free radicals are known as the molecular species with at least one unpaired electron that behave as either oxidants or reductants owing to their reactive and unstable characteristics that tend to donate or receive an electron from another molecules (Lobo, et al., 2010). Examples of radicals which contain oxygen recapitulates oxygen singlet, hydrogen peroxide, hypochlorite, hydroxyl radical and others (Lobo, et al., 2010; Zeb, 2020). Although the production of these oxidants is considered as a normal cellular process, nonetheless, an elevated level of free radicals contributes to oxidative stress due to the downregulation of antioxidant system in the cell and imbalance in biochemical pathways (Zeb, 2020). As a result, these reactive oxygen species (ROS) attacks and damages various macromolecules like lipids, carbohydrates, proteins and particularly,

DNA, leading to malignant transformation (Lobo, et al., 2010; Aggarwal, et al., 2019).

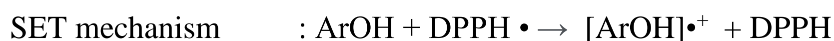
Antioxidant is defined as the chemical compound that prevents, terminates or regulates the substrate from oxidation by scavenging the free radicals through the donation of one of their electrons to inhibit the unstable radicals from removing electron from other particles (Singh, et al., 2018). Antioxidant is divided into two categories, including the primary as well as secondary form according to their mechanism of action. Primary antioxidants neutralise free radicals actively to produce stable products through hydrogen atom transfer, single electron transfer or sequential proton loss electron transfer mechanism whereas secondary antioxidants neutralise the catalysts of prooxidant via chelation of prooxidant transition metal ions, for example, iron. Citric acid is one of the examples of secondary antioxidant (Zeb, 2020; Flieger, et al., 2021).

DPPH assay has been utilised extensively as the antioxidant assay as it provides accurate, reproducible results to be compared with the findings from other antioxidant assays. It is an inexpensive, fast, and easy method to determine the antioxidant capacity of either liquid or solid samples (Kedare and Singh, 2011). Free radicals interact with antioxidants based on two proposed mechanisms, which are the single electron transfer (SET) and hydrogen atom transfer (HAT) as shown in the following equation. Originally, DPPH free radicals (DPPH•) yield solution with intense purple colour in methanol, which absorbs strongly at the wavelength of 517 nm. In the presence of antioxidants (ArOH), a product with yellow colour (DPPH-H) is yielded through decolourisation due to the

ability of antioxidants to donate its hydrogen atom to scavenge the DPPH radicals as displayed in **Figure 2.6**. The yellow colour is directly proportional to the antioxidant concentration (Sadeer, et al., 2020).



HAT mechanism : ArOH + DPPH • → ArO • + DPPH-H



SET mechanism : ArOH + DPPH • → $[ArOH]^{\bullet+}$ + DPPH

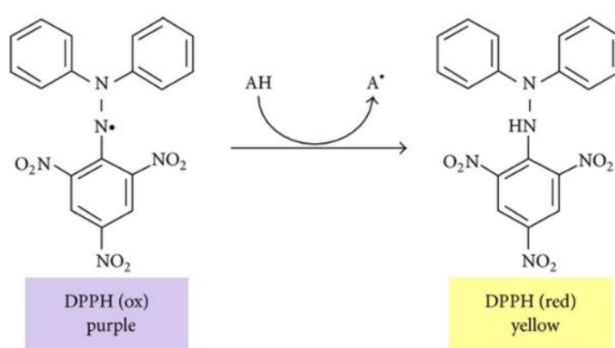


Figure 2.6: The reduction of purple DPPH to yellow diphenylpicryl hydrazine in the presence of antioxidants (Adapted from Teixeira, et al., 2013).

2.9 Anticancer Assay

Anticancer or cytotoxic agents reduce the viability of cancer cells through various mechanism of action, for instance, apoptosis induction, arrest of cell cycle (G_1, S, G_2 and metaphase), enhancement of detoxification enzymes, antiangiogenesis and others (Gautam, Mantha and Mittal, 2014). In response to the essential oil treatment, an elevated levels of reactive oxygen species (ROS) and a reduced amount of glutathione in the cancer cells initiate the process of programmed cell death which results in cell death. Apoptosis is also activated when the expression of *Bax* and *Bcl-2* genes change which causes the release of cytochrome C and subsequently leads to caspase-9 and caspase-3 activation.

Furthermore, according to Blowman, et al. (2018) and Gautam, Mantha and Mittal (2014), phase I enzymes recapitulate cytochrome P450 and phase II enzymes, including transferases such as glutathione-S-transferase are responsible for the detoxification of harmful components. Some constituents in essential oil can serve as the inducer for the detoxification enzymes to inhibit the formation of cancer or induced-toxicity (Gautam, Mantha and Mittal, 2014; El-Kenawi and El-Remessy, 2013). Through these essential oil-mediated antitumour mechanisms, the clonal expansion and metastasis of cancer cells are regulated and prevented, making Cassia essential oil a potential anticancer therapeutic candidate (Gautam, Mantha and Mittal, 2014).

MTT assay is widely utilised as cell viability assay due to its high sensitivity, reproducibility, rapid and it enables high-throughput screening (Tonder, Joubert and Cromarty, 2015). Generally, the mitochondrial activity in most alive cells is constant. Therefore, the number of alive cells is associated with an increase or decrease in mitochondrial activity, which is reflected by the amount of formazan crystals produced (Meerloo, Kaspers and Cloos, 2011). When treated with cytotoxic agents, the metabolic active viable cells convert yellow-coloured tetrazolium MTT that are soluble in water to purple-coloured formazan, also known as (E, Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan that are insoluble in water through dehydrogenase and oxidoreductase that are NAP(P)H-dependent as shown in **Figure 2.7** (Bahuguna, et al., 2017). The solubilisation of water-insoluble formazan in dimethyl sulfoxide (DMSO) produces a solution with intense purple colour that can be detected spectrophotometrically at 570 nm. Hence, the intensity of purple colour which

is measured in term of absorbance is directly proportional to the alive cells number (Riss, et al., 2016). Meanwhile, death cells lack the ability to reduce MTT to formazan which makes colour production a convenient and significant marker to identify viable cells.

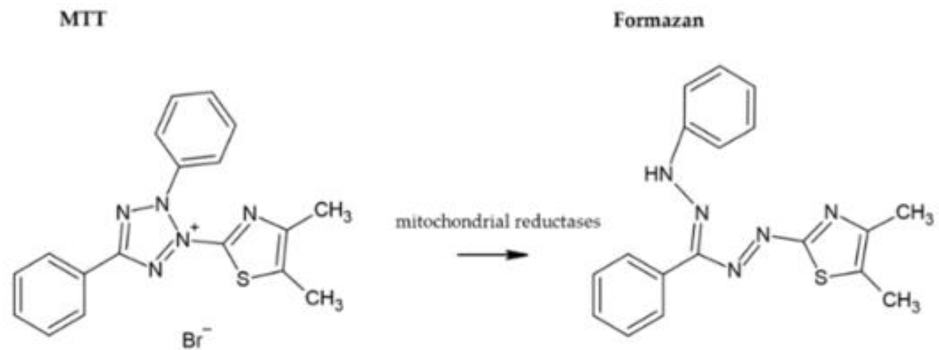


Figure 2.7: The formation of formazan from MTT (Adapted from Ligasová and Koberna, 2021).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cassia Essential Oil

The United States Department of Agriculture (USDA) certified organic Cassia essential oil was purchased from authorised Plant Therapy store as shown in **Figure 3.1**.

Figure 3.1: The purchased Cinnamon Cassia essential oil.

3.1.2 Cell Lines

Vero cells (CCL-81) and lung adenocarcinoma cells (A549) (CCL-185) were used in this study.

3.1.3 Solvents and Chemicals

A list of solvents and chemicals utilised in this study is listed in **Table 3.1**.

Table 3.1: Solvents and chemicals used in the study.

Solvents and Chemicals	Brand/Manufacturer
Chloroform	Synerlab
Dimethyl sulfoxide (DMSO)	Nacalai Tesque Inc.
Dulbecco's modification of Eagle's medium (DMEM)	Capricorn Scientific
Doxorubicin hydrochloride	MedChemExpress
1,1-diphenyl-2-picrylhydrazyl (DPPH) powder	Sigma-Aldrich
Ethyl acetate (Industrial grade)	ChemSoln
Ethyl alcohol (95%)	ChemSoln
Foetal bovine serum (FBS)	Tico Europe
Hexane (Industrial grade)	Rank Synergy Sdn Bhd
Hydrochloric acid (37%)	QReC
L-ascorbic acid powder	Fisher Scientific Pte Ltd
Methanol	GENE Chemicals
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder	ChemSoln
Phosphate buffered saline (PBS) tablet	Oxoid
Silica-coated aluminium sheet	Merck
Sulphuric acid (98%)	RCI Labscan Limited
Trypan blue solution (0.4%)	Sigma-Aldrich
Trypsin-EDTA (0.25%)	GE Healthcare Life Sciences HyClone Laboratories

3.1.4 Equipment

A list of equipment utilised in this study is shown in **Table 3.2**.

Table 3.2: Equipment used in the study.

Equipment	Brand/Manufacturer
Biosafety cabinet	TELSTAR Bio-II-A
Carbon dioxide (CO_2) incubator	BINDER
Drying incubator	Memmert
Electronic balance	A&D Company
Freezer (-20°C)	Pensonic
Inverted phase contrast microscope	Nikon ECLIPSE TS100
Laminar hood	Edamix
Microplate reader	BMG LABTECH
Refrigerated centrifuge	SIGMA 3-18KS
Refrigerator (4°C)	Samemax
Ultraviolet (UV) lamp	Spectroline CM-10A
Vortex	Velp Scientifica
Water bath	PMI-Labortechnik GmBH

3.2 Flowchart of the Study

A summary of the methodology in the study is displayed as in **Figure 3.2**.

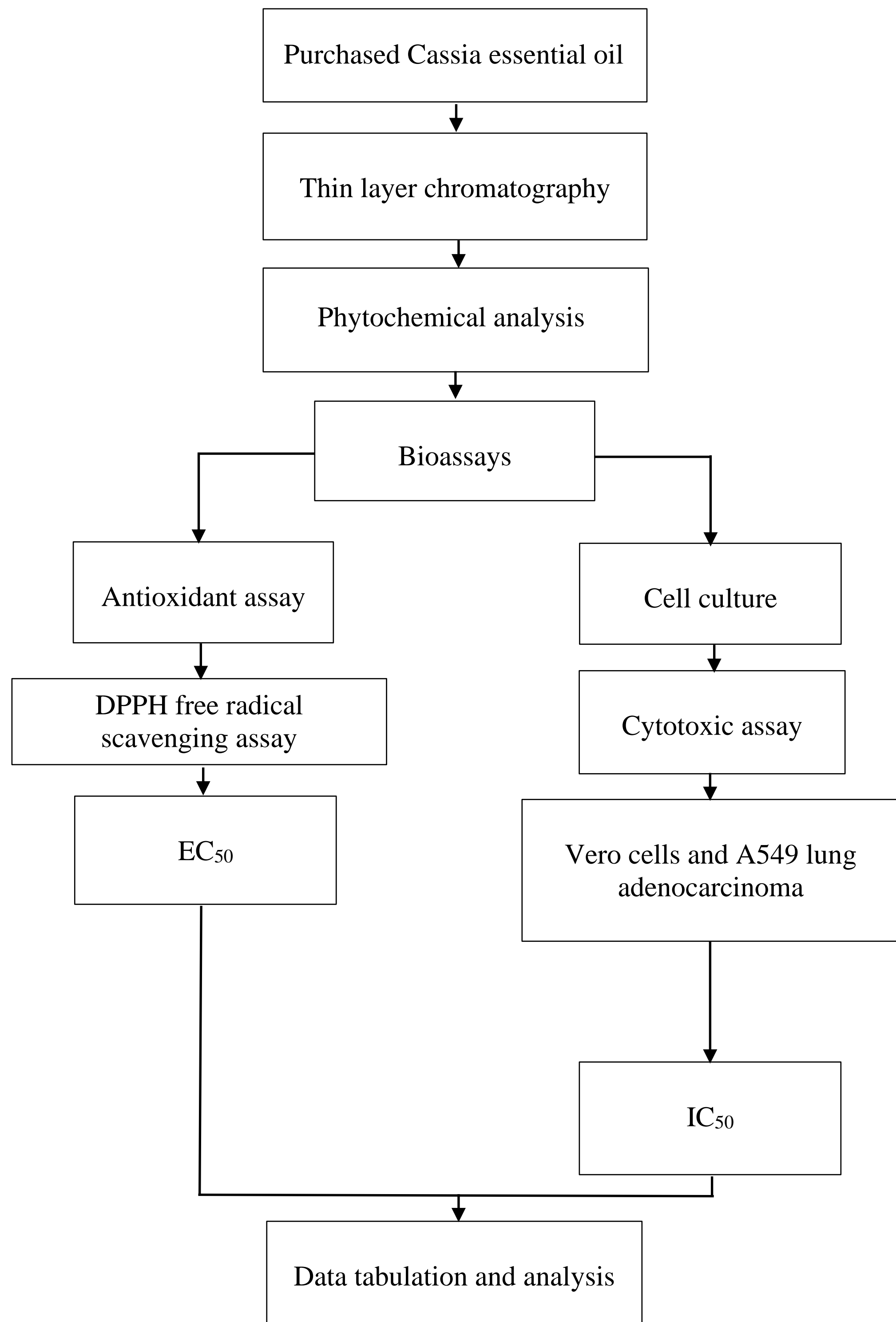


Figure 3.2: Flowchart of research activities in the study.

3.3 Thin Layer Chromatography

The aluminium sheet coated with silica was used in thin layer chromatography (TLC). The sheets were cut to the length and width of 10 cm and 2 cm, respectively. As illustrated in **Figure 3.3**, the solvent front and baseline were drawn on the plate about 1 cm from the top and the bottom using pencil. Chloroform was used to dissolve the Cassia essential oil for spotting on TLC plate. The developing chamber was pre-saturated with a combination mixture of organic solvents as tabulated in **Table 3.3**.

Using thin capillary tubes, two small drops of Cassia essential oil were spotted on the baseline of the plate. The plate was placed into the developing chamber covered with aluminium lid to allow separation by capillary action. When the solvent had reached to the solvent front, the TLC plate was taken out and observed under the UV with short (254 nm) and long (365 nm) wavelength. The spots observed under the UV lamp were circled with pencils and the retention factor, R_f value was calculated using the following formula (Bele and Khale, 2011).

$$\text{Retention factor } (R_f) = \frac{Distance\ travelled\ by\ sample\ spot\ from\ baseline}{Distance\ travelled\ by\ solvent\ from\ baseline}$$

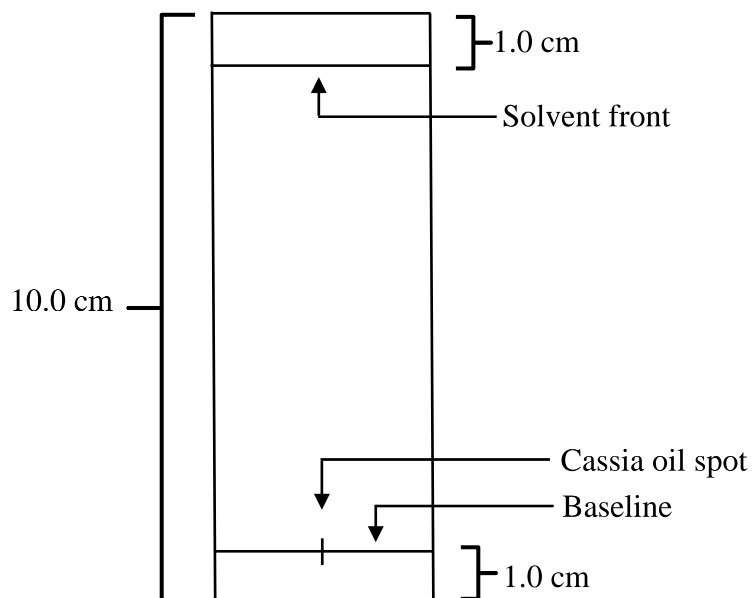


Figure 3.3: Layout of thin layer chromatography.

Table 3.3: List of combination mixture of organic solvents in developing chamber.

Mixture of organic solvents	Ratios
Hexane : Ethyl acetate	0:10
Hexane : Ethyl acetate	10:1
Hexane : Ethyl acetate	10:3
Hexane : Ethyl acetate	1:1

3.4 Phytochemical Analysis

3.4.1 Test for Phenols

Ferric chloride test was performed by adding one drop of 5% ferric chloride ($FeCl_3$) solution to Cassia essential oil and the observation was compared with that of gallic acid positive control. Formation of red, green, purple, or blue colour is the expected observation for a positive result (Rao, Abdurrazak and Mohd, 2016).

3.4.2 Test for Terpenoids

Salkowski test was conducted by mixing Cassia essential oil with 200 µL of chloroform and followed by the addition of one drop of 1M sulphuric acid (H_2SO_4) on top to form a layer. The expected colour for positive terpenoids is the formation of reddish-brown at the interface (Soni and Sosa, 2013; Mumtaz, et al., 2014).

3.4.3 Test for Saponins

Foam test was carried out by adding 1 mL distilled water to Cassia essential oil and the mixture was shaken vigorously. Presence of persistent foam that measured around 1 cm for 10 minutes indicates a positive result (Mumtaz, et al., 2014; Efiong, et al., 2020).

3.4.4 Test for Alkaloids

Through Wagner's test, one drop of Wagner's reagent added to the Cassia essential oil at the tube side to confirm the presence of alkaloids. The production

of reddish-brown precipitate confirmed the presence of alkaloids (Soni and Sosa, 2013; Banu and Cathrine, 2015; Shaikh and Patil, 2020).

3.4.5 Test for Tannins

Braymer's test was performed by the addition of one drop of 10% of $FeCl_3$ solution and 600 µL of distilled water to the Cassia essential oil. The result was compared to the tannic acid as a positive control. The formation of blue green colour suggests the presence of tannins (Shaikh and Patil, 2020).

3.4.6 Test for Quinones

Sulphuric acid test was carried out by adding 200 µL of 1M H_2SO_4 to Cassia essential oil. A positive result is represented by the production of red colour (Rajesh, et al., 2014; Shaikh and Patil, 2020).

3.4.7 Test for Glycosides

Through Keller-Kiliani test, Cassia essential oil was tested with 120 µL 1M glacial acetic acid, followed by one drop of 5% $FeCl_3$ solution and five to ten drops of 1M H_2SO_4 at the side of tube. The presence of blue-coloured acetic acid layer is the expected to be observed (Shaikh and Patil, 2020).

3.4.8 Test for Flavonoids

Pew's test was carried out, in which 0.01 g of metallic zinc powder was added to Cassia essential oil, followed by 1M hydrochloric acid (HCl) in a dropwise manner. Purple red colour is the expected observation for a positive result (Joseph, Kumbhare and Kale, 2013).

3.5 DPPH Free Radical Scavenging Assay

3.5.1 Preparation of Reagents and Test Samples

About 0.2 mM DPPH reagent was prepared by weighing 0.004 g DPPH powder and dissolved it to 50 mL of methanol. The solution was vortexed to dissolve the powder completely and was covered with aluminium foil to be incubated for around 30 minutes in the dark.

Ascorbic acid at 100 mg/mL (w/v) were prepared by dissolving 5 g ascorbic acid powder in 50 mL of methanol. The solutions were mixed using vortex and further incubated in dark for 30 minutes. On the other hand, methanol was utilised as a negative control in the study. About 0.1 g of Cassia essential oil was mixed in 1 mL of methanol to yield 100 mg/mL (w/v) sample and 100% (v/v) Cassia essential oil sample was used as well.

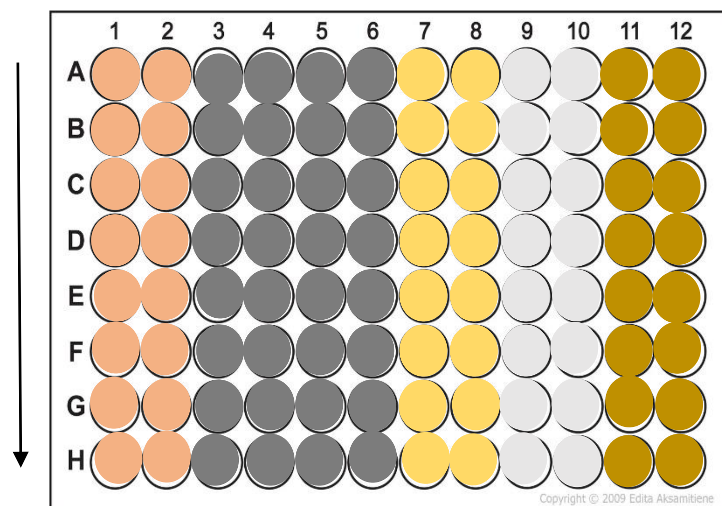
3.5.2 Protocol and Design

Figure 3.4 reveals the 96 well-plate layout for DPPH antioxidant assay. First, 100 µL methanol was added into the wells from columns 3 to 12, followed by addition of 100 µL of ascorbic acid, Cassia essential oil both in (w/v) and (v/v) into the first well of the columns, correspondingly. Two-fold serial dilution was conducted from rows A to H, yielding concentrations from 0.39 to 50.00 mg/mL. About 100 µL of methanol was added lastly into columns 1 and 2 to reduce evaporation. DPPH reagent (20 µL) was then added into all the wells except for columns 11 and 12. The plate was covered with aluminium foil immediately and was incubated in dark at room temperature for 20 minutes. The absorbances were measured using microplate reader at 515 nm spectrophotometrically and were

used to calculate the DPPH free radical scavenging activity using the following equation (Lu, et al., 2014). Graphs of percentage of DPPH free radical scavenging activity versus the concentration were plotted and the EC_{50} values were identified (Nadarajan and Pujari, 2014).

$$\text{Percentage of DPPH scavenging activity} = [(A_0 - A_1)/ A_0] \times 100\%$$

Where A_0 was the mean absorbance of negative control consists of DPPH reagent and methanol whilst A_1 was the absorbance of Cassia essential oil in average.



: Methanol (Negative control)

: Methanol + Ascorbic acid (Positive control)

: Methanol + Cassia essential oil (w/v)

: Methanol + Cassia essential oil (v/v)

: Cassia essential oil background

: Two-fold serial dilution

Figure 3.4: The 96-well plate layout for DPPH assay.

3.6 Cell Culture

3.6.1 Preparation of Complete Growth Medium

Complete growth medium (CGM) was prepared by adding 90 mL of basic DMEM and 10 mL of FBS to make a final volume of 100 mL. The prepared CGM was sealed with parafilm and was kept in 4°C refrigerator.

3.6.2 Preparation of Phosphate Buffer Saline

PBS was prepared by dissolving four PBS tablets into 400 mL distilled water in the Schott bottle and was autoclaved. The autoclaved PBS was left at room temperature to cool down and was filtered through bottle top vacuum filter connected to the pump filter into a sterile Schott bottle aseptically. The prepared PBS was sealed with parafilm and was kept in 4°C refrigerator.

3.6.3 Preparation of Cryoprotectant

A total of 10 mL of cryopreservation solution was prepared by mixing 9.5 mL (95%) of complete growth medium and 500 µL (5%) of 100% DMSO in a 15 mL centrifuge tube. The cryoprotectant was sealed with parafilm and stored in -20°C freezer.

3.6.4 Quality Control

Quality control of the CGM, FBS and PBS were performed by transferring 3 mL of the solution into a sterile petri dish. The petri dish was sealed with parafilm and was placed in 37°C, 5% CO_2 incubator overnight. The condition of the medium before and after the overnight incubation was observed under the inverted phase contrast microscope.

3.6.5 Thawing of Cell Line

First, the cryovials containing Vero and A549 cells were thawed for about 1 to 2 minutes in a 37℃ water bath. The cryovials were aseptically brought into a laminar hood by spraying the tube with 70% alcohol. The cells in the cryovial were transferred to a 9 mL CGM-containing centrifuge tube using micropipette. The cells were centrifuged for 10 minutes at 1000 rpm at 25℃. After centrifugation, the supernatant was removed, and the pellet was resuspended to be transferred to a T25 flask pre-filled with 5 mL of CGM. The culture was kept in 5% CO_2 incubator at 37℃ for growth (Huang, et al., 2012).

3.6.6 Subculturing of Cells

Subculture was performed once the cells reached around 80% to 90% confluency. The old CGM from the flask was first discarded and the cells were rinsed with 5 mL of PBS twice. Subsequently, 2.5 mL of 0.25% trypsin-EDTA solution was added to the flask and was incubated for 10 to 15 minutes at 37℃ in 5% CO_2 incubator to facilitate detachment of cells. The cells were then observed under inverted phase contrast microscope to make sure complete detachment from the culture flask, followed by addition of 5 mL of complete medium to stop trypsinisation. The cells were transferred to a centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The supernatant was removed, and the pellet was resuspended. The pellet was then transferred to two new T25 flasks each that were pre-filled with 5 mL of CGM. The original T25 flask was added with 5 mL of CGM as well. The flasks were placed at 37℃, 5% CO_2 incubator to allow the growth of cells.

3.6.7 Cell Line Cryopreservation

The suspension cells after centrifugation were added with 3 mL of cryoprotectant. The cells were transferred to few cryovials and was kept at -80°C freezer for storage.

3.7 MTT Assay

3.7.1 Preparation of Reagents and Test Samples

Stock concentration of Cassia essential oil (100 mg/mL) was prepared by mixing 0.1 g of essential oil in 1 mL of 100% DMSO. A total of 5 mL of 1 mg/mL working solution of Cassia essential oil was obtained by dissolving 0.05 mL of Cassia essential oil stock solution in 4.95 mL of basic DMEM.

In addition, 0.01 mL of stock concentration (50 mg/mL) of doxorubicin was added into 6.99 mL of basic DMEM to obtain 7 mL of 100 µg/mL doxorubicin as positive control for MTT assay. Negative control was prepared by adding 0.5 mL of 100% DMSO into 49.5 mL of basic DMEM to obtain 50 mL of 1% DMSO.

Approximately 5 mg/mL of MTT reagent was prepared by dissolving 0.15 g of MTT powder in tube pre-filled with 30 mL PBS. The tube was wrapped with aluminium foil to avoid degradation by direct lights and kept at -20°C for further usage.

3.7.2 Cell Counting

Based on 1:1 dilution of cells, 50 µL of 0.4% trypan blue solution was mixed with 50 µL of suspension cells in microcentrifuge tube and incubated for 2 to 5

minutes. The cells were then loaded onto haemocytometer through capillary action and was examined under inverted phase contrast microscope at a magnification of 100x (Strober, 2015). The amount of viable alive cells was counted in the four squares located at the corner site. According to Green and Sambrook (2019), cells touching the left and top boundary lines of square were included in the count whilst the cells located on the right and bottom boundary lines of square were excluded from counting as demonstrated in **Figure 3.5**. The following equation of cell count expressed in cells per mL (Green and Sambrook, 2019).

$$\text{Cell count} = \text{Average viable cells per square} \times \text{Dilution factor} \times 10^4$$

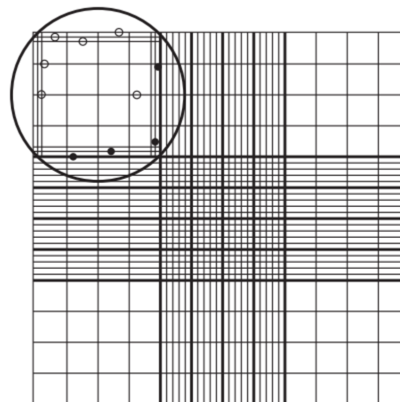


Figure 3.5: Open circle represents the cells that must be counted whereas closed circle indicates the cells that are not counted (Adapted from Green and Sambrook, 2019).

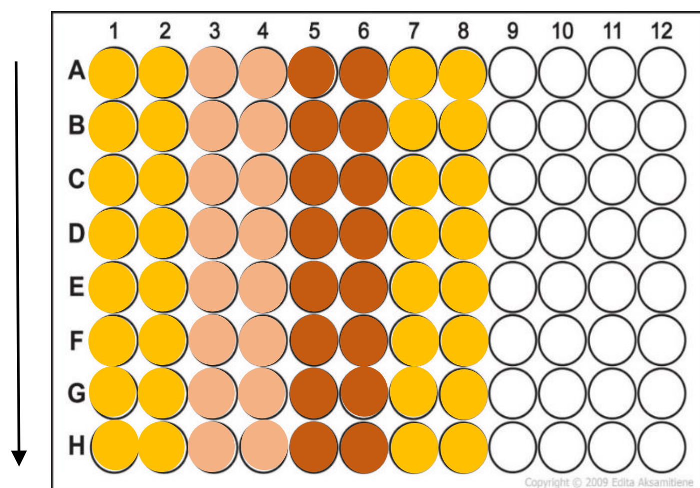
3.7.3 Protocol and Design

Roughly 1×10^4 to 1×10^5 cells/mL were used for MTT assay. The counted cells were added into each well in the flat bottom 96 well plate. The plate was sealed with parafilm and was kept overnight at 37°C in 5% CO_2 for attachment (Ranjitkar, et al., 2021).

On the next day, the medium was discarded using multichannel pipette. Two-fold serial dilution of samples was performed from rows A to H on another 96 well plate before transferring to the wells seeded with cells as shown in **Figure 3.6**. This concentrations of samples and doxorubicin were ranged from 7.81 to 1000.00 µg/mL and from 0.78 to 100.00 µg/mL, respectively. The 96-well plate was placed in 5% CO_2 incubator for 24, 48 and 72 hours, correspondingly.

For every respective incubation, 5 mg/mL MTT reagent (20 µL) was added into all the wells, followed by 4 hours of incubation. Approximately 100 µL of 100% DMSO was added into each well and resuspended gently using micropipette. The plate was further incubated at room temperature for 20 minutes to allow complete dissolution of the formazan crystals that were purple in colour before measuring the absorbance spectrophotometrically at 570 nm using microplate reader (Sharma, et al., 2017). Duplicate results were obtained, and the viability of cells was calculated in percentage using the following equation (Kim, et al., 2022). Graphs of percentage of cell viability versus concentration were drawn and used to determine the IC_{50} values.

$$\text{Percentage of cell viability} = \frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} \times 100\%$$



: 1% DMSO + Cells (Negative control)

: Doxorubicin + Cells (Positive control)

: Cassia essential oil + Cells (Sample)

↓ : Two-fold serial dilution

Figure 3.6: The 96-well plate layout for MTT assay.

3.8 Data Analysis

DPPH and MTT assays were repeated thrice and twice, respectively. The results were displayed as mean value and standard deviation, which were analysed either using student t-test or one-way ANOVA using GraphPad Prism. The data with $P < 0.05$ is considered significantly different.

CHAPTER 4

RESULTS

4.1 Thin Layer Chromatography

Different combination of organic solvents had been utilised as the mobile phase in TLC. **Table 4.1** shows the respective R_f values and number of spots that were observed. There were two spots observed on the TLC plate when only ethyl acetate was used as the mobile phase. This organic solvent displays the smallest number of spots among the four other organic solvent system involved with the R_f values ranged from 0.44 to 0.68.

Figure 4.1 shows the that the combination of hexane and ethyl acetate with a ratio of 10:1 and the ratio of 10:3 resulted in the formation of six spots on the TLC plate with different R_f values. In Hex : EA (10 : 1), the R_f values were ranged from 0.06 to 0.34 whereas a wider R_f range was calculated in Hex : EA (10 : 3) from 0.06 to 0.51. Among all, the widest R_f value that ranged from 0.21 to 0.89 with a total of five spots were obtained in the combination of hexane and ethyl acetate with 1:1 ratio.

Table 4.1: Retention factor (R_f) values based on different combination of solvent mixture.

Organic solvents combination	Ratios	Number of spot(s)	R_f values range
Hex : EA	0:10	2	0.44-0.68
Hex : EA	10:1	6	0.06-0.34
Hex : EA	10:3	6	0.06-0.51
Hex : EA	1:1	5	0.21-0.89

Hex- Hexane; EA- Ethyl acetate

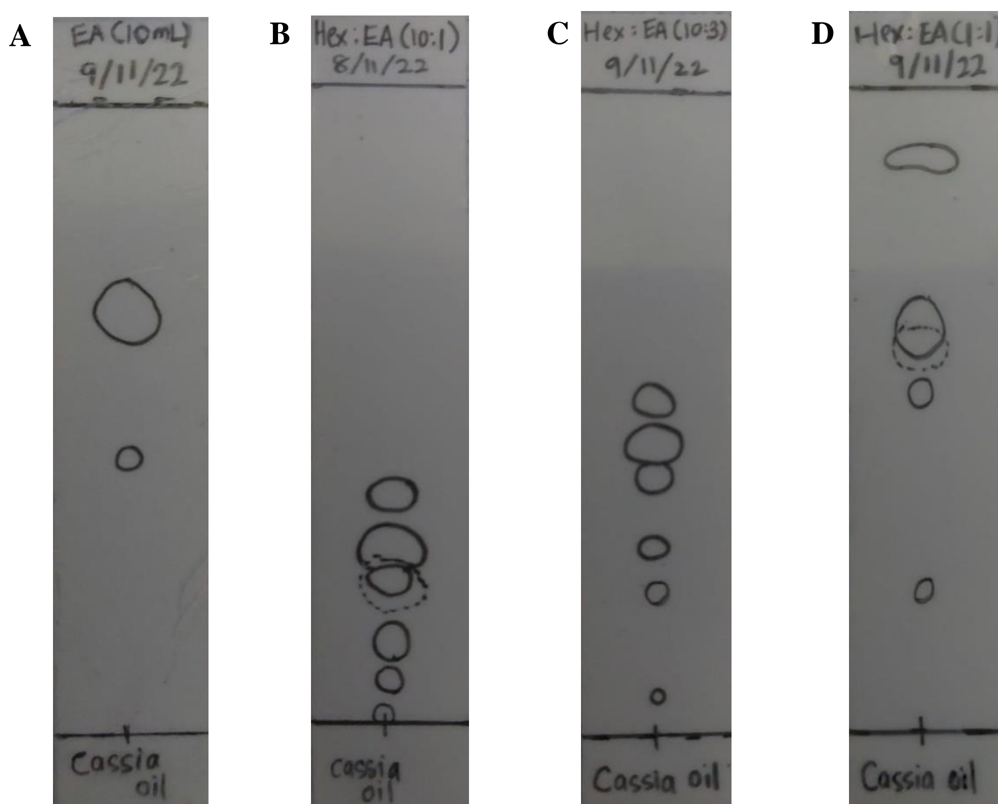


Figure 4.1: TLC results of Cassia essential oil in different combination of organic solvents (A) Hex : EA (0 : 10) (B) Hex : EA (10 : 1) (C) Hex : EA (10 : 3) (D) Hex : EA (1 : 1).

4.2 Phytochemical Analysis

4.2.1 Phenols

In ferric chloride test, gallic acid (P) has changed from transparent yellow to dark green colour as shown in **Figure 4.2**. Besides, the colour of the Cassia essential oil (S) turned to dark red colour from its initial transparent yellow colour as compared to the negative control (N) indicating a positive result for phenols in Cassia essential oil.

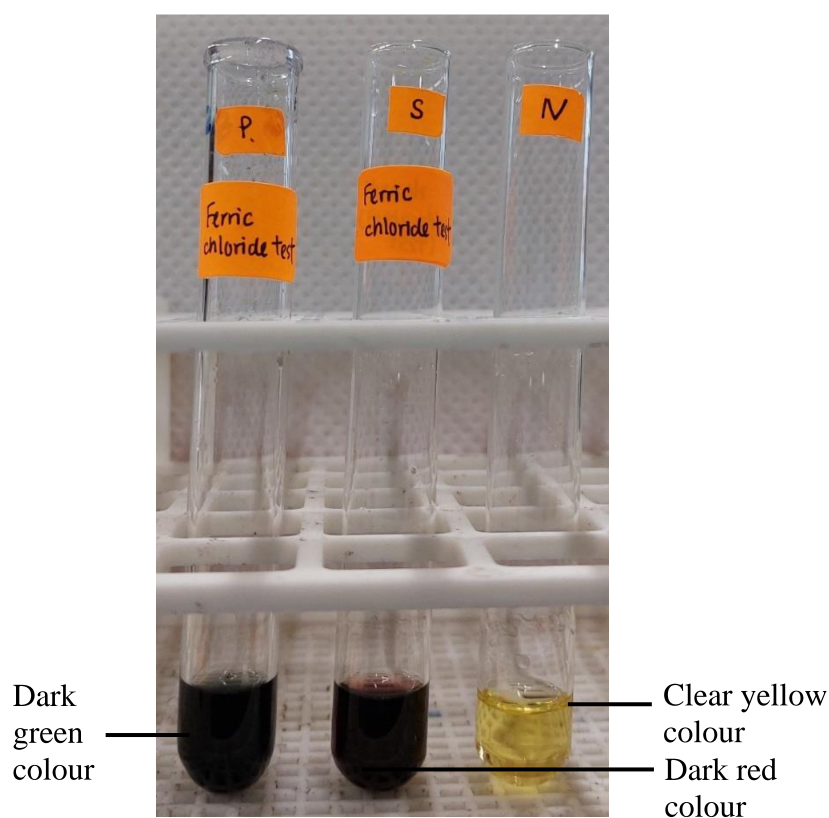


Figure 4.2: The result of ferric chloride test to detect the presence of phenols in Cassia essential oil. P, positive control; S, Cassia essential oil sample; N, negative control.

4.2.2 Terpenoids

In **Figure 4.3**, a positive result for the presence of terpenoids was observed in Cassia essential oil (S) when the solution has changed from the original transparent yellow to cloudy yellow colour. In contrast, the colour of the negative control (N) solution was clear without any cloudy appearance.

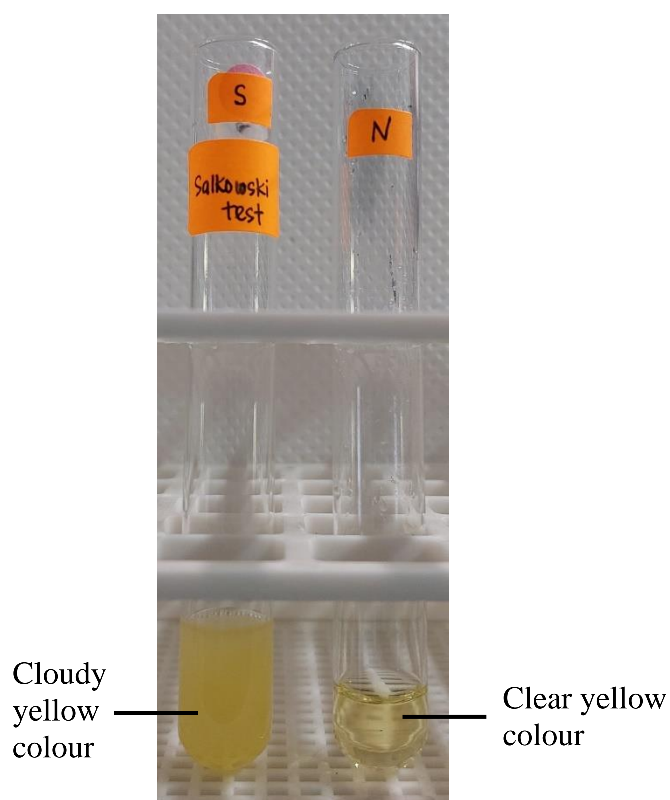


Figure 4.3: The result of Salkowski test to detect the presence of terpenoids in Cassia essential oil. S, Cassia essential oil sample; N, negative control.

4.2.3 Saponins

As compared to the transparent yellow colour of negative control (N), Cassia essential oil (S) had turned to cloudy yellow in colour as shown in **Figure 4.4**. However, no persistent foam formation was noted which was similar like the negative control, confirming a negative foam test for saponins in Cassia essential oil.

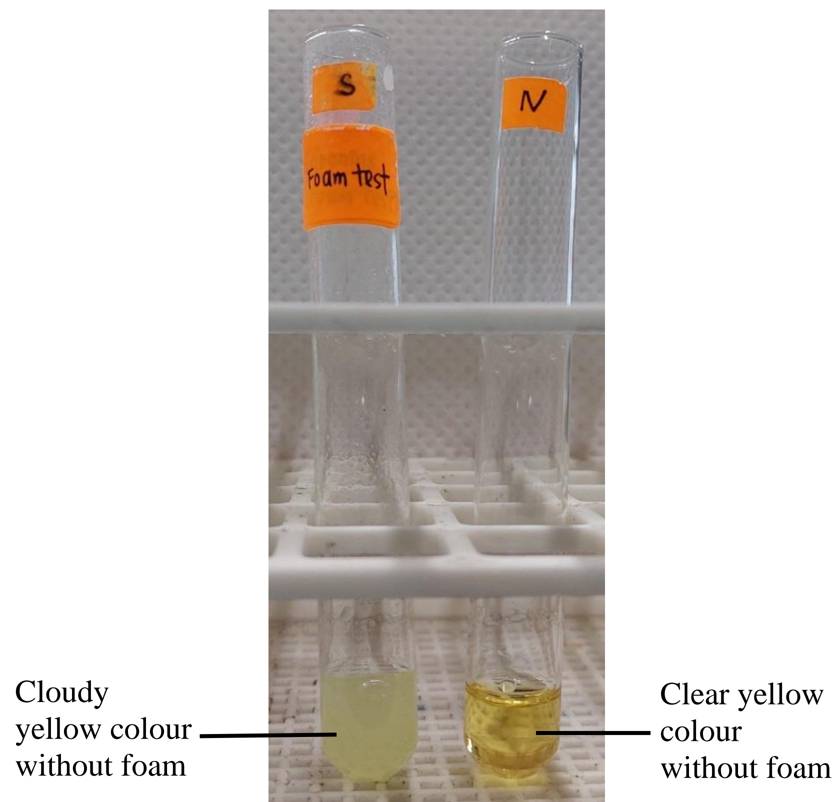


Figure 4.4: The result of foam test to detect the presence of saponins in Cassia essential oil. S, Cassia essential oil sample; N, negative control.

4.2.4 Alkaloids

As demonstrated in **Figure 4.5**, a reddish-brown precipitate was produced in Cassia essential oil (S) during the Wagner's test as compared to the negative control (N) which was clear yellow in colour without the formation of precipitation. Therefore, the results confirmed positive for the presence of alkaloids.

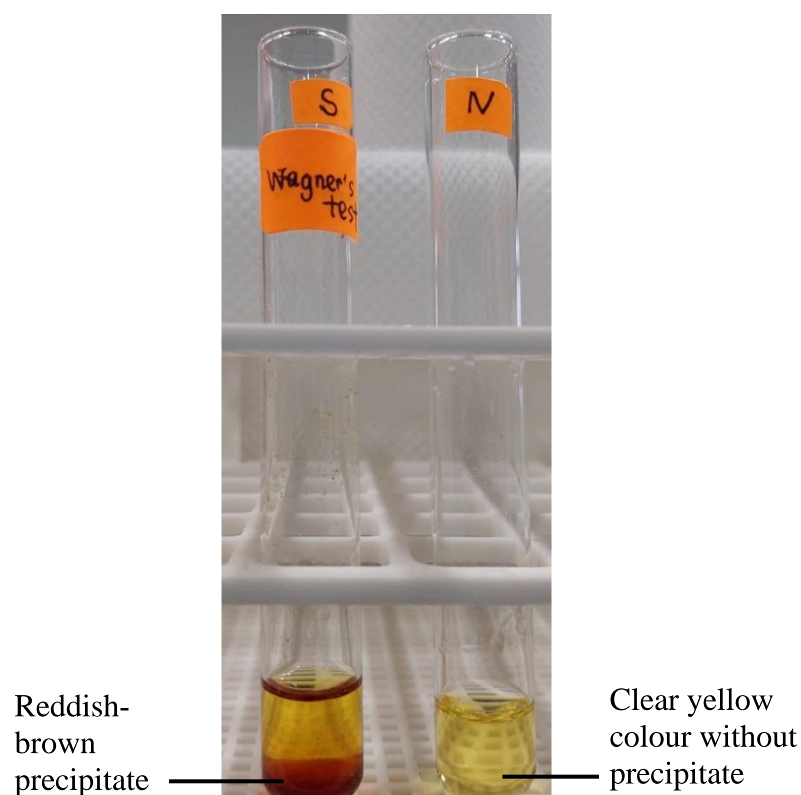


Figure 4.5: The result of Wagner's test to detect the presence of alkaloids in Cassia essential oil. S, Cassia essential oil sample; N, negative control.

4.2.5 Tannins

In Braymer’s test, positive tannins were determined in Cassia essential oil (S) by the production of dark red colour from the initial yellow colour as in negative control (N). The positive control of tannins (P), tannic acid has changed to dark blue colour instead as illustrated in **Figure 4.6**.

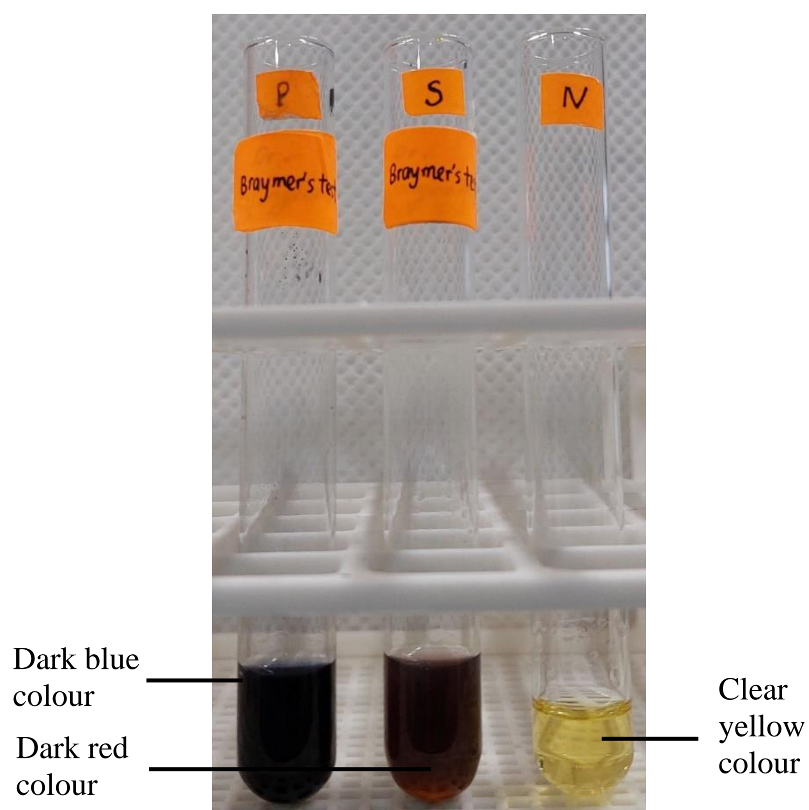


Figure 4.6: The result of Braymer’s test to detect the presence of tannins in Cassia essential oil. P, positive control; S, Cassia essential oil sample; N, negative control.

4.2.6 Quinones

As shown in **Figure 4.7**, the solution colour in test tube before and after the sulphuric acid test remained unchanged as transparent yellow colour in both Cassia essential oil (S) and negative control (N). This suggested the absence of quinones in Cassia essential oil leading to a negative detection.

Figure 4.7: The result of sulphuric acid test to detect the presence of quinones in Cassia essential oil. S, Cassia essential oil sample; N, negative control.

4.2.7 Glycosides

In **Figure 4.8**, through Keller-Kiliani test, a negative result of glycosides was obtained. The colour of Cassia essential oil (S) has remained clear yellow colour and was similar as in negative control (N).

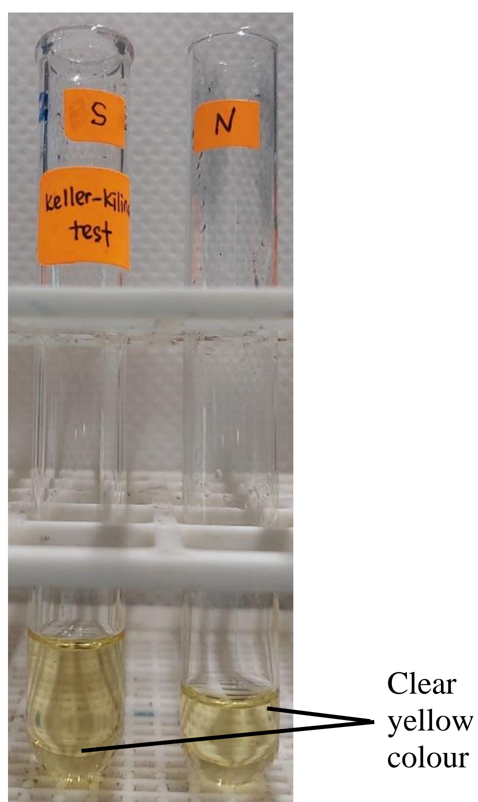


Figure 4.8: The result of Keller-Kiliani test to detect the presence of glycosides in Cassia essential oil. S, Cassia essential oil sample; N, negative control.

4.2.8 Flavonoids

A positive result for the presence of flavonoids was confirmed through Pew's test as shown in **Figure 4.9**. A dark green colour was yielded in Cassia essential oil (S) in contrast to the negative control (N) which contained the original clear yellow colour.

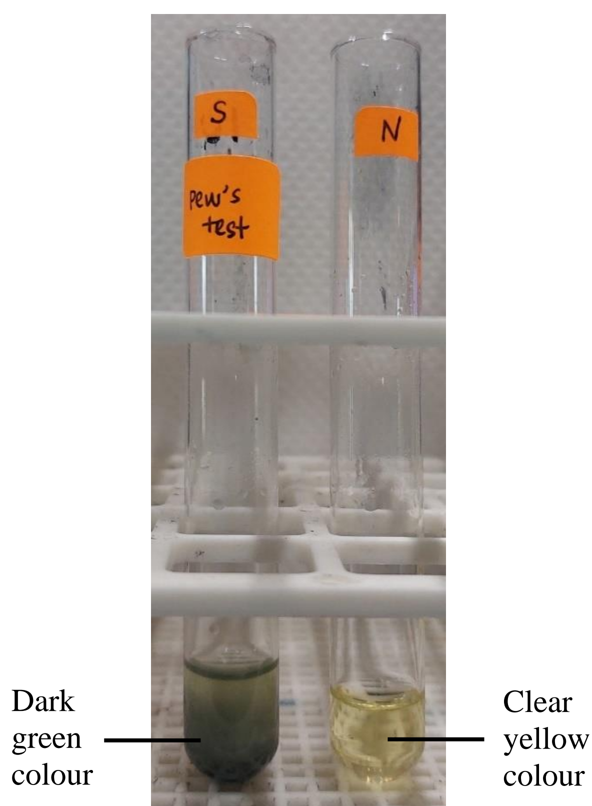


Figure 4.9: The result of Pew's test to detect the presence of flavonoids in Cassia essential oil. S, Cassia essential oil sample; N, negative control.

4.3 DPPH Assay

The overall trend of DPPH scavenging activity of ascorbic acid, Cassia essential oil (w/v) and (v/v) were displayed in **Figure 4.10**. As the concentration of ascorbic acid, Cassia essential oil (w/v) and (v/v) increases from 0.00 to 50.00 mg/mL, the percentage of DPPH scavenging activity increases.

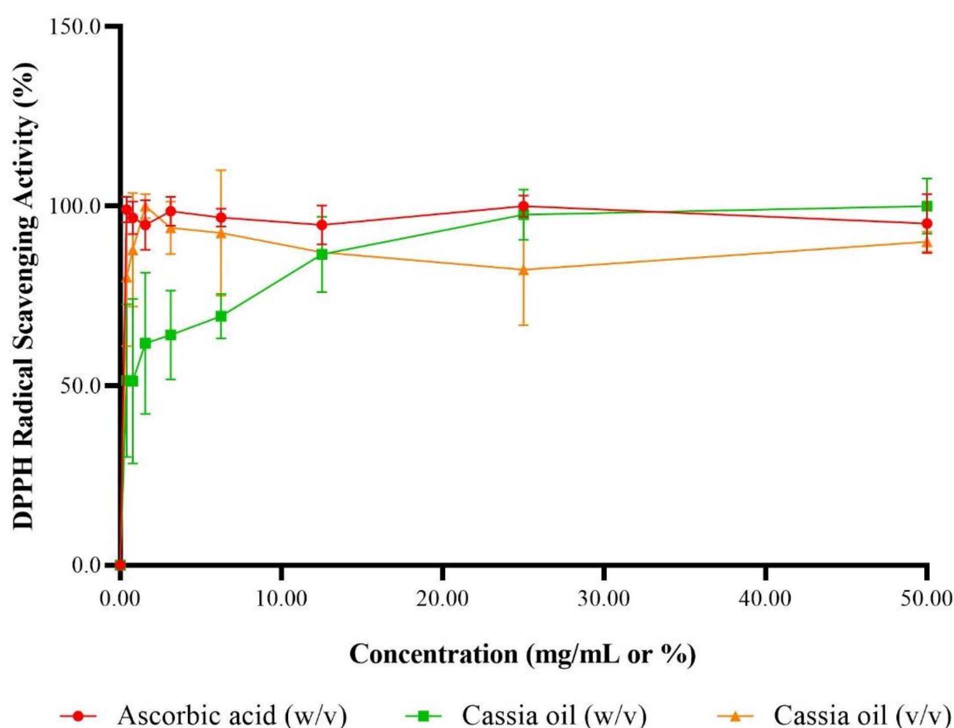


Figure 4.10: Graph of percentage of DPPH scavenging activity against concentration of ascorbic acid (w/v), Cassia essential oil (w/v) and Cassia essential oil (v/v).

Table 4.2 shows the percentage of DPPH scavenging activity of ascorbic acid, Cassia essential oil (w/v) and (v/v), respectively. There was a fluctuation in the percentage scavenging activity of Cassia essential oil (v/v) and ascorbic acid along with increasing concentration. The highest percentage DPPH scavenging activity of 88.1 ± 0.077% at a concentration of 50.00 mg/mL was obtained in Cassia essential oil (w/v) and the lowest percentage was at 0.39 mg/mL which

was around 45.1 ± 0.213%. Oppositely, Cassia essential oil (v/v) yielded 87.8 ± 0.034% as the highest percentage DPPH scavenging activity at 1.56% concentration whilst the lowest percentage of 70.4 ± 0.193% was identified at the concentration of 0.39%. Additionally, ascorbic acid with the highest percentage of DPPH scavenging activity of 86.0 ± 0.030% and the lowest of 81.4 ± 0.054% were obtained at 25.00 mg/mL and 12.50 mg/mL, correspondingly.

Table 4.2: DPPH scavenging activity of Cassia essential oil (w/v) and ascorbic acid.

Concentration (mg/mL; %)	**DPPH Radical Scavenging Activity (%)**		
	Ascorbic Acid	**Cassia Essential Oil (w/v)**	**Cassia Essential Oil (v/v)**
0.39	85.2 ± 0.036	45.1 ± 0.213	70.4 ± 0.193
0.78	83.2 ± 0.045	45.4 ± 0.230	77.2 ± 0.158
1.56	81.5 ± 0.069	54.4 ± 0.197	87.8 ± 0.034
3.13	84.7 ± 0.041	56.5 ± 0.124	82.5 ± 0.073
6.25	83.3 ± 0.025	61.1 ± 0.062	81.3 ± 0.175
12.50	81.4 ± 0.054	76.3 ± 0.105	76.5 ± 0.000
25.00	86.0 ± 0.030	86.1 ± 0.007	72.2 ± 0.154
50.00	81.8 ± 0.082	88.1 ± 0.077	79.1 ± 0.028

Data is expressed as mean ± standard deviation (SD), n = 3 ($P < 0.05$).

Based on one-way ANOVA, ascorbic acid, Cassia essential oil (w/v) and (v/v) at different incubation periods were not significantly different ($P > 0.05$). Referring to **Table 4.3**, ascorbic acid contained EC_{50} values of 0.01 ± 0.010 mg/mL. It was much lower than the Cassia essential oil (w/v) which contained EC_{50} values of 0.87 ± 0.110 mg/mL. Moreover, Cassia essential oil (v/v) were identified to have the EC_{50} values of 0.10 ± 0.130% in DPPH assay.

Table 4.3: The EC_{50} values of the sample tested.

Sample	EC_{50} value (mg/mL or %)
Ascorbic acid	0.01 ± 0.010
Cassia essential oil (w/v)	0.87 ± 0.110
Cassia essential oil (v/v)	0.10 ± 0.130

Data is expressed as mean ± standard deviation (SD), n = 3 ($P < 0.05$).

4.4 MTT Assay

4.4.1 Vero Cells

Figure 4.11 illustrates the morphology of cultured Vero cells. Vero cells is an anchorage dependent cells and grow in monolayers. It features fibroblast-like elongated shape under the inverted phase contrast microscope.

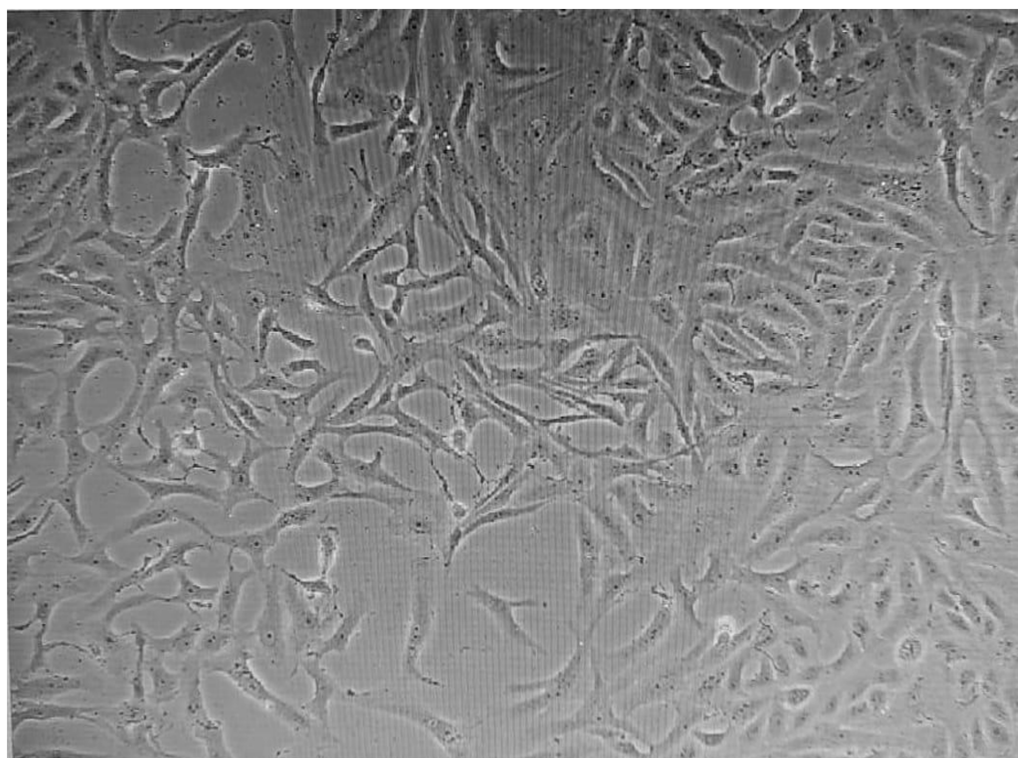


Figure 4.11: Morphological observation of Vero cells cultured in DMEM upon 85 to 90% confluency (100x total magnification).

Figures 4.12 and **4.13** shows the bar graph of percentage cell viability with increasing concentration of Cassia essential oil (w/v). The percentage of cell viability of Vero cells was almost constant as the concentration of Cassia essential oil and doxorubicin increases from 7.81 to 1000.00 µg/mL and from 0.78 to 100.00 µg/mL. for both 24-and 48-hours, respectively.

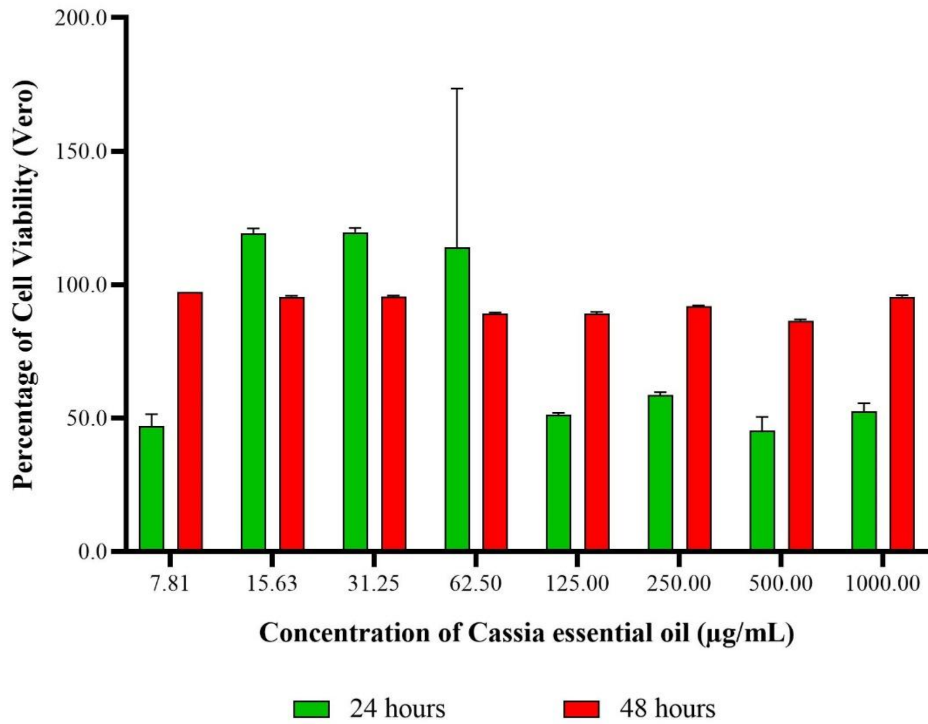


Figure 4.12: Bar graph representing the percentage cell viability of Vero cells at varying concentrations of Cassia essential oil at 24-and 48-hours.

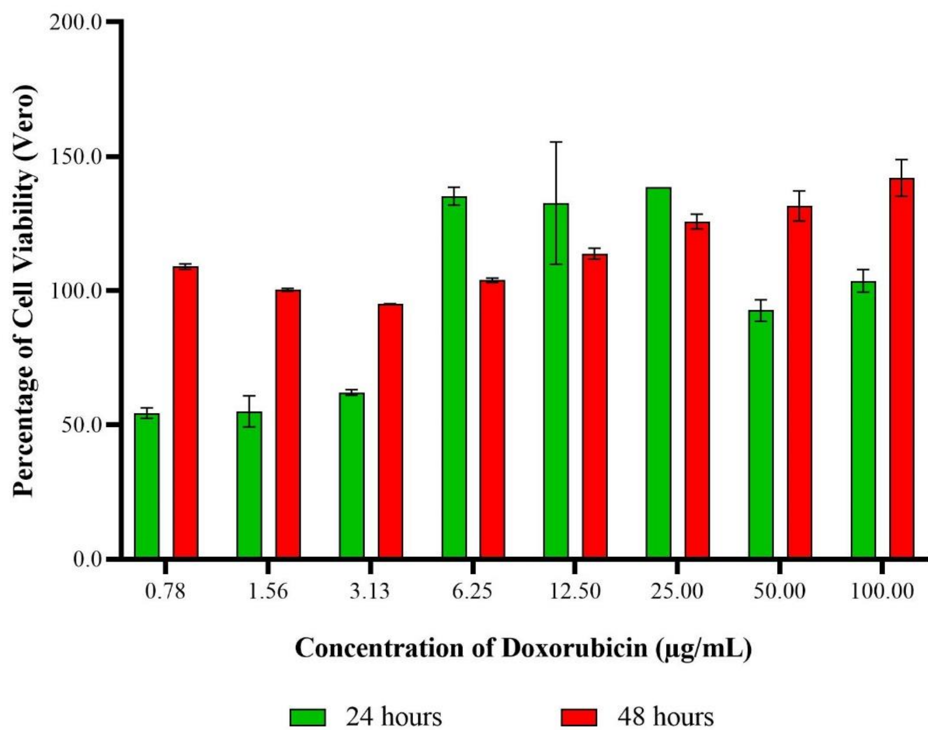


Figure 4.13: Bar graph representing the percentage cell viability of Vero cells at varying concentrations of doxorubicin at 24- and 48-hours.

Table 4.4 displays the percentage cell viability of Vero cells when treated with various concentration of doxorubicin and Cassia essential oil at 24- and 48-hours. As the incubation time increased, the percentage of cell viability for Cassia essential oil raised. As an example, the percentage cell viability of Cassia essential oil at 125.00 µg/mL for 24 hours was 51.2 ± 0.001% and remained as high as 89.3 ± 0.008% for 48 hours. Thus, Cassia essential oil have no killing effect on Vero cells.

Based on student t-test, Cassia essential oil and doxorubicin at different incubation periods were not significantly different ($P > 0.05$). **Table 4.5** revealed the IC_{50} values from the time-dependent and dose-response graph. The IC_{50} values of Cassia essential oil increases from 512.60 ± 0.029 µg/mL at 24 hours to 7147.00 ± 0.019 µg/mL at 48 hours. Likewise, the IC_{50} values of doxorubicin increased with time. At 24- and 48-hours, the obtained IC_{50} values of doxorubicin were 4.66 ± 0.388 µg/mL and 44.69 ± 0.048 µg/mL, respectively.

Table 4.4: The percentage cell viability of Vero cells upon treatment with Cassia essential oil and doxorubicin.

Sample	Concentration (μg/mL)	Cell Viability (%)	
		24 hours	48 hours
Cassia essential oil	7.81	47.1 ± 0.062	97.2 ± 0.023
	15.63	119.2 ± 0.026	95.5 ± 0.006
	31.25	119.6 ± 0.024	95.6 ± 0.006
	62.50	114.0 ± 0.842	89.2 ± 0.004
	125.00	51.2 ± 0.001	89.3 ± 0.008
	250.00	58.6 ± 0.173	92.0 ± 0.004
	500.00	45.2 ± 0.745	86.6 ± 0.007
	1000.00	52.6 ± 0.433	95.5 ± 0.008
Doxorubicin	0.78	54.4 ± 0.028	109.0 ± 0.015
	1.56	55.0 ± 0.081	100.3 ± 0.007
	3.13	62.1 ± 0.016	95.0 ± 0.001
	6.25	135.2 ± 0.047	103.9 ± 0.115
	12.50	132.6 ± 0.322	113.8 ± 0.029
	25.00	138.5 ± 0.000	125.8 ± 0.039
	50.00	92.6 ± 0.555	131.5 ± 0.079
	100.00	103.7 ± 0.589	142.0 ± 0.096

Data is expressed as mean ± standard deviation (SD), n = 2 ($P < 0.05$).

Table 4.5: The IC_{50} values of Cassia essential oil and doxorubicin on Vero cells.

Sample	IC_{50} values (µg/mL)	
	24 hours	**48 hours**
Cassia essential oil	512.60 ± 0.029	7147.00 ± 0.019
Doxorubicin	4.66 ± 0.388	44.69 ± 0.048

Data is expressed as mean ± standard deviation (SD), n = 2 ($P < 0.05$).

4.4.2 A549 Cells

The adherent lung cancer (A549 cells) grows in monolayers with the shape of a fibroblast. In addition, the A549 cell sizes were small, and the boundaries of the cells were clear as shown in **Figure 4.14**.

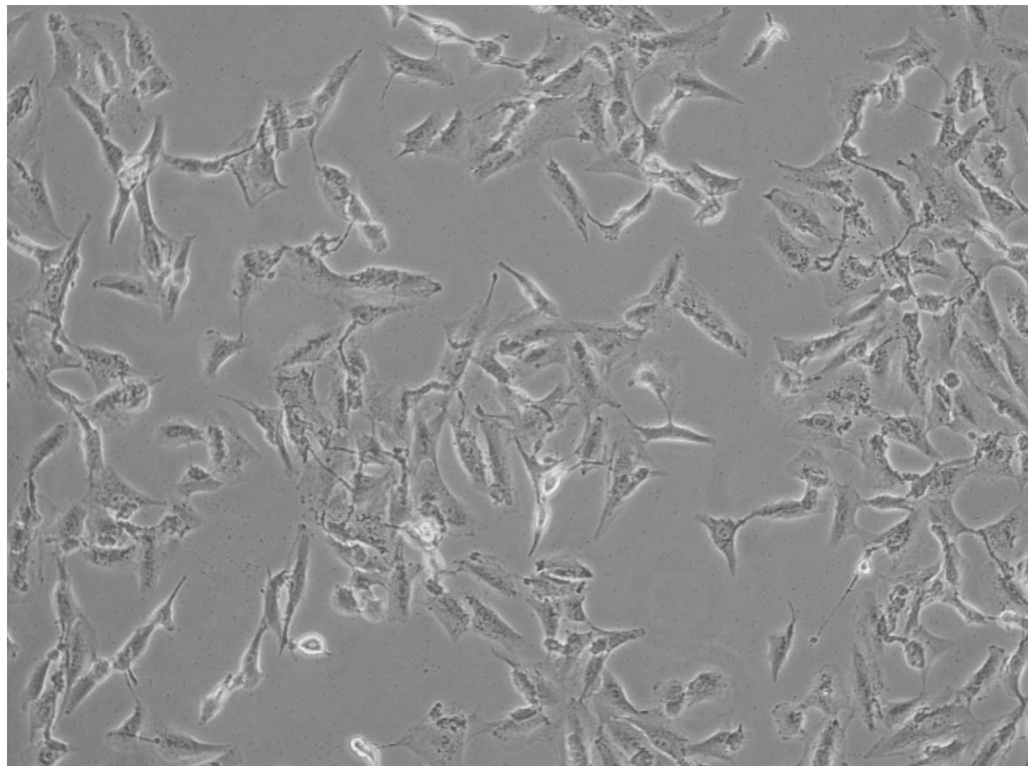


Figure 4.14: Morphological observation of A549 cells cultured in DMEM upon 85 to 90% confluency (100x total magnification).

Figures 4.15 reveals an overall decreasing trend, in which the percentage of cell viability reduced with increasing concentration of Cassia essential oil from 7.81 to 1000.00 µg/mL for 24-, 48- and 72-hours, respectively. Meanwhile, an increased trend in the percentage of cell viability was observed in doxorubicin at higher concentration based on **Figure 4.16**.

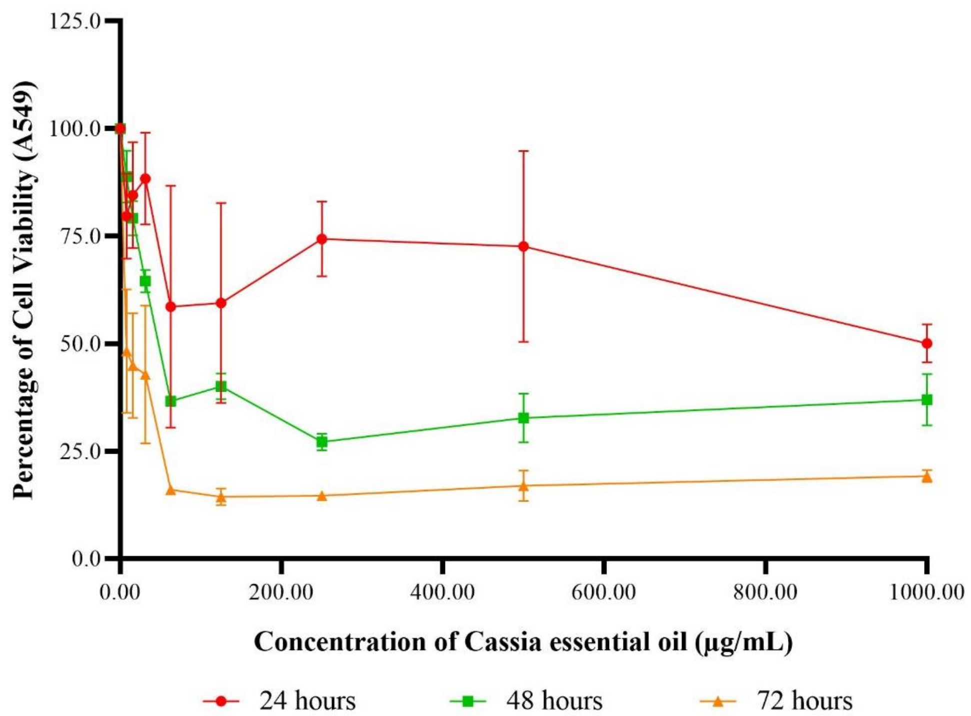


Figure 4.15: The percentage cell viability of A549 cells at varying concentrations of Cassia essential oil at 24-, 48- and 72-hours.

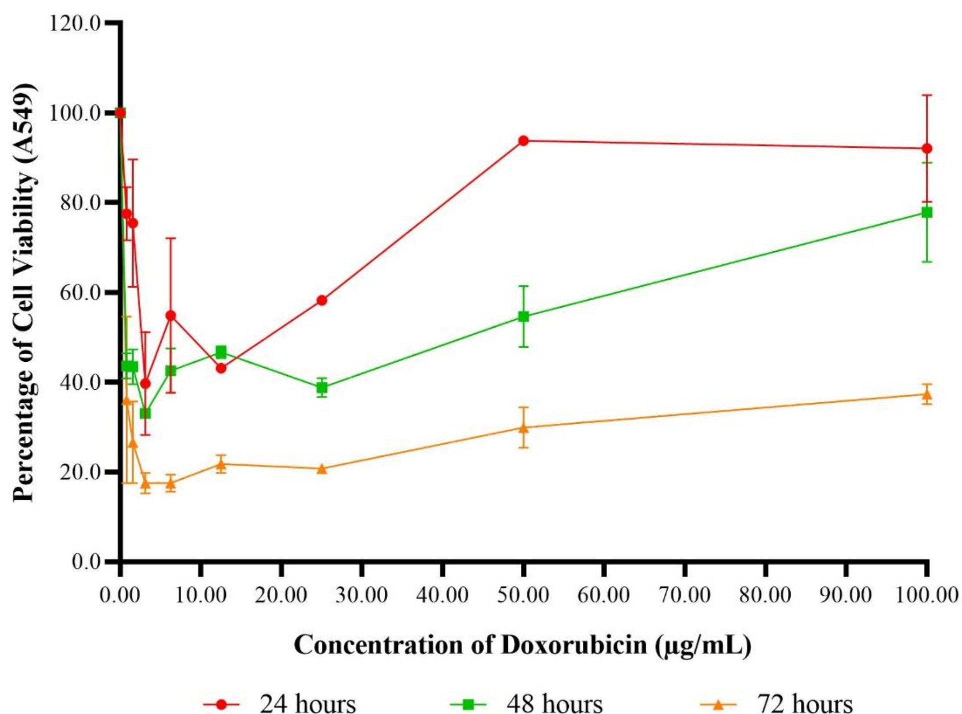


Figure 4.16: The percentage cell viability of A549 cells at varying concentrations of doxorubicin at 24-, 48- and 72-hours.

Table 4.6 shows the percentage of viability of A549 cells when treated with doxorubicin and Cassia essential oil for 24-, 48- and 72-hours. The percentage of cell viability dropped with increasing incubation time, in which the highest percentage viability of A549 cells treated with Cassia essential oil declined from the original 88.3 ± 0.151% for 24 hours to only 42.9 ± 0.228% for 72 hours at a concentration of 31.25 μg/mL. Similarly, percentage of cell viability as high as 93.8 ± 0.000% was noted for 24 hours and decreased to only 29.9 ± 0.063% for 72 hours at the doxorubicin concentration of 50.00 μg/mL.

Table 4.6: The percentage cell viability of A549 cells upon treatment with Cassia essential oil and doxorubicin.

Sample	Concentration (µg/mL)	Cell Viability (%)		
		24 hours	48 hours	72 hours
Cassia essential oil	7.81	79.6 ± 0.139	88.8 ± 0.085	48.3 ± 0.203
	15.63	84.6 ± 0.179	79.2 ± 0.056	44.9 ± 0.173
	31.25	88.3 ± 0.151	64.6 ± 0.036	42.9 ± 0.228
	62.50	58.6 ± 0.397	36.6 ± 0.005	16.1 ± 0.005
	125.00	59.5 ± 0.329	40.1 ± 0.043	14.4 ± 0.027
	250.00	74.3 ± 0.122	27.2 ± 0.028	14.7 ± 0.006
	500.00	72.6 ± 0.314	32.7 ± 0.008	16.9 ± 0.005
	1000.00	50.1 ± 0.063	37.0 ± 0.084	19.2 ± 0.002
Doxorubicin	0.78	77.5 ± 0.084	43.6 ± 0.004	36.1 ± 0.262
	1.56	75.4 ± 0.200	43.4 ± 0.056	26.6 ± 0.128
	3.13	39.7 ± 0.162	33.0 ± 0.012	17.5 ± 0.032
	6.25	54.9 ± 0.244	42.5 ± 0.071	17.5 ± 0.027
	12.50	43.1 ± 0.000	46.7 ± 0.019	21.7 ± 0.027
	25.00	58.2 ± 0.000	38.8 ± 0.030	20.7 ± 0.009
	50.00	93.8 ± 0.000	54.6 ± 0.096	29.9 ± 0.063
	100.00	92.1 ± 0.168	77.8 ± 0.157	37.3 ± 0.032

Data is expressed as mean ± standard deviation (SD), $n = 2$ ($P < 0.05$).

Based on one-way ANOVA, Cassia essential oil and doxorubicin at different incubation periods were significantly different ($P < 0.05$). As tabulated in **Table 4.7**, the IC_{50} values of Cassia essential oil were 24.92 ± 0.079 µg/mL, 20.49 ± 0.202 µg/mL, and 7.14 ± 0.112 µg/mL for 24-, 48- and 72-hours, respectively showing a decreasing trend. Similarly, the IC_{50} values of doxorubicin reduced with time as well, in which the obtained IC_{50} values for 24- and 72-hours were 0.12 ± 0.003 µg/mL and 0.09 ± 0.042 µg/mL, correspondingly whereas at 48 hours, the IC_{50} values cannot be determined.

Table 4.7: The IC_{50} values of Cassia essential oil and doxorubicin on A549 cells.

Sample	**IC_{50} values (µg/mL)**		
	24 hours	**48 hours**	**72 hours**
Cassia essential oil	24.92 ± 0.079	20.49 ± 0.202	7.14 ± 0.112
Doxorubicin	0.12 ± 0.003	Could not be determined	0.09 ± 0.042

Data is expressed as mean ± standard deviation (SD), n = 2 ($P < 0.05$)

CHAPTER 5

DISCUSSION

5.1 Thin Layer Chromatography

Normal phase thin layer chromatography involved in this study is the standard chromatography techniques that utilises polar silica-coated aluminum sheet as stationary phase and non-polar organic solvents as mobile phase (Lade, et al., 2014). Polarity index is the polarity of solvent when comparing to water and it determines the degree of interaction between organic solvent and any polar compounds. The polarity index of 0 for hexane characterises it as nonpolar solvent. On the contrary, ethyl acetate is identified as polar organic solvent with the polarity index of 4.4 (Harb, et al., 2020).

From this study, when ethyl acetate alone was used, the mobile phase has become intermediate polar owing to the presence of hydrophilic ethyl acetate. Hence, polar constituents that are discovered in Cassia essential oil were displaced from hydrophilic silica binding sites (stationary phase) by the intermediate polar ethyl acetate (mobile phase) through the competition for absorption sites on stationary phase (Akash and Rehman, 2020). This contributes to high R_f values as tabulated in **Table 4.1**. Nonpolar compounds, on the other hand, are not likely to dissolve in polar organic solvents. As a result, the components of Cassia essential oil traveled further up the TLC plate at a rapid rate and therefore, there was little separation of metabolites in the Cassia essential oil mixture, yielding only two spots on TLC plate (Bele and Khale, 2011).

Furthermore, a poor separation of the components was observed in the mixture of hexane and ethyl acetate at ratio 1:1, in which five spots was detected and with overlapping between the spots. In this case, probably the non-polar compounds from Cassia essential oil dissolves in mobile phase and moves up the plate at a faster rate. Meanwhile, a speedy movement of polar metabolites in Cassia essential oil through TLC plate was noticed as well with little resolution due to the rapid displacement of polar constituents from the stationary phase before they can equilibrate on the plate. Hence, a wide range of R_f values was obtained as displayed in **Table 4.1** (Aljamali, Thamer and Redha, 2015).

Additionally, in hexane and ethyl acetate mixture at 10:1, the mobile phase is predominantly nonpolar due to the higher volume of hexane as compared to ethyl acetate. Thus, a low resolving power was obtained in which there was little separation of the components in the mixture of Cassia essential oil due to increased retention of polar solutes that causes low mobility of hydrophilic components on the TLC plate. Therefore, spots that formed were nearer to the baseline with small R_f values for any polar compounds as shown in **Table 4.1** and **Figure 4.1**. Oppositely, nonpolar compounds dissolved in nonpolar solvents to migrate further up and hence yielding greater R_f values (Robards and Ryan, 2022).

Referring to **Table 4.1** and **Figure 4.1**, Hex : EA (10 : 3) is the best organic solvent combination with the best separation of spots among all the tested solvent combination. This solvent system provided time for Cassia essential oil sample to equilibrate on TLC plate and enabled all the constituents to be fully

resolved. This causes the formation of well-separated spots on TLC plate with wider R_f values in contrast to Hex : EA (10 : 1) (Aljamali, Thamer and Redha, 2015). In this case, the polar metabolites in Cassia essential oil were trapped or retained at polar silica stationary phase by adhering to the binding sites via hydrogen bonding, Van der Waals forces and dipole-type interaction (Spangenberg, Poole and Weins, 2011). Thus, it migrates up the TLC plate at a slower rate. Oppositely, nonpolar compounds travel up the plate more rapidly together with the organic solvent due to its less affinity towards the polar stationary phase (Santiago and Marina, 2013).

One of the examples of possible polar compounds that may be present in Cassia essential oil includes quercetin, which is categorised under the polar flavonoids group (Rao and Gan, 2014; Etika and Iryani, 2019). On the other hand, cinnamaldehyde which contains a nonpolar aromatic aldehde functional group, linalool which carries acyclic non-polar hydrocarbon chain and eugenol with nonpolar cyclic structure may possible present in Cassia essential oil as reported previously by many studies (Chen, et al., 2016; Jiang, Kempinski and Chappell, 2016; Sucipto, Kuswandi and Wibawa, 2022).

5.2 Phytochemical Analysis

A positive result for the presence of phenols was obtained in ferric chloride test when Cassia essential oil has changed to dark red colour during the reaction as shown in **Figure 4.2**. Example of major bioactive phenolic compounds also known as phenylpropanoids that may present in Cassia essential oil includes cis-2-methoxycinnamic acid and cinnamaldehyde with 43.06% and 42.37% contents,

correspondingly. On top of that, cinnamyl acetate, *o*-methoxycinnamaldehyde, eugenol, benzaldehyde and *cis*-2-methoxycinnamic acid may be identified as well (Chang, et al., 2013; Zachariah and Leela, 2014). The presence of phenolic compounds is closely associated with antioxidant, antimicrobial, anticancer and anti-inflammatory activities (Sauceda, et al., 2017).

According to **Figure 4.3**, the observed result for terpenoid test revealed the production of cloudy yellow colour that was different from the expected observation with reddish-brown colour at the interface (Singh, et al., 2016). However, it was still considered as positive finding as reported by Goswami, Thakker and Dhandhukia (2015) because of the reaction between other compounds or derivatives in the Cassia essential oil in causing non-specific reaction which interferes with the colour changes. Study by Zhang, et al. (2019) showed presence of terpenes components such as monoterpenes, sesquiterpenes and diterpenes primarily in the *Lauraceae* family generally. Linalool, β-pinene and camphor are some examples of monoterpenoids that may be discovered in Cassia essential oil. Likewise, active chemical constituent like germacrene D which has been categorised under sesquiterpenoids may also be detected in Cassia essential oil (Chang, et al., 2013). Based on Yang, et al. (2020), anticancer, antioxidant, antimalarial and antiviral activities are the pivotal pharmacological effect possessed by the terpenoids group.

Based on **Figure 4.4**, negative foam test was detected for saponins in Cassia essential oil due to the absence of persistent foam (Mumtaz, et al., 2014; Efiong, et al., 2020). This negative finding is consistent with the studies carried out by

Ahmad, Capoor and Khatoon (2013) and Julianti, Rajah and Fidrianny (2017) in which the negative frothing test revealed the absence of saponins in *C. cassia* bark. Hence, saponins cannot be detected in Cassia essential oil extracted from the bark as well.

Referring to **Figure 4.5**, the formation of reddish-brown precipitate confirmed the presence of alkaloids in Cassia essential oil (Soni and Sosa, 2013; Banu and Cathrine, 2015). This is supported by the studies performed by Wu, et al. (2022), in which they listed down some examples of alkaloids that may be isolated from *C. cassia*, for instance, pyrrolidines like 3-glyceroylindole and amines like N-trans-feruloyl-5-methoxytyramine. Hence, Cassia essential oil isolated through steam distillation is most likely to still possess the alkaloid group with anticancer, antibacterial, and anti-inflammatory activities (Astuti, Fauzi and Mustikasari, 2022).

In **Figure 4.6**, the finding from Braymer's test for tannins was still considered to be positive although the colour of the Cassia essential oil has turned to dark red instead of the expected blue green colour due to the non-specific reaction (Dahlin, Baell and Walters, 2015). There is very limited information available for the presence of tannins isolated from steam-distilled *C. cassia* bark essential oil. However, the presence of tannins was identified by Al-Numair, et al. (2007), which concluded that approximately 0.65% catechin equivalent of total tannin content can be detected in *C. cassia*. Some examples of tannins that may be identified from the bark of Chinese cinnamon includes cinnamtannin D1, procyanidin B2 and cinnamtannin B1 (Chen, et al., 2014). Thus, the obtained

Cassia essential oil may possess these metabolites (Astuti, Fauzi and Mustikasari, 2022). Some common pharmacological activities of tannins include antioxidant, anticancer, antimicrobial, and anti-nutritional (Smeriglio, et al., 2016).

Referring to **Figure 4.7**, quinones was not found in Cassia essential oil due to a negative observation, in which the expected red colour was not generated (Rajesh, et al., 2014). The result obtained contradicted with the finding discovered in a study carried out by Julianti, Rajah and Fidrianny (2017), in which a positive result for the detection of quinones in *C. cassia* was identified. This may be due to the changes in the chemical composition in Cassia essential oil isolated from *C. cassia* bark during steam distillation using high temperature (140 to 212°F) that may lead to the degradation of quinones and leads to negative result in sulphuric acid test (Gahukar, 2014; Bakar, et al., 2020).

A negative result was also identified for glycosides due to the absence of blue-coloured layer as displayed in **Figure 4.8** (Shaikh and Patil, 2020). Likewise, according to Ahmad, Capoor and Khatoon (2013) and Julianti, Rajah and Fidrianny (2017), the negative Keller-Kiliani test confirmed the absence of glycosides in *C. cassia* bark. This explains the negative result obtained for glycosides detection in Cassia essential oil which was extracted from the *C. cassia* bark.

In positive Pew's test for flavonoids, a dark green colour was yielded instead of the expected purple red colour as shown in **Figure 4.9** because of the non-specific reaction between mixture of bioactive components (Joseph, Kumbhare

and Kale, 2013; Dahlin, Baell and Walters, 2015). Adisakwattana, et al. (2011) stated that the presence of flavonoids in *C. cassia* bark was confirmed with total flavonoids content of 63.49 ± 1.42 mg quercetin equivalent/g. Moreover, Rao and Gan (2014) showed approximately 0.172% quercetin and 0.016% kaempferol which are the compounds of flavonoids, were extracted from cinnamon bark. The detection of flavonoids in *C. cassia* bark suggested the possibility of the presence of flavonoids in Cassia essential oil through distillation process, thereby yielding a positive result (Astuti, Fauzi and Mustikasari, 2022). Additionally, flavonoids are associated with antiviral, antioxidant, anti-inflammatory, and anticancer activities (Ullah, et al., 2020).

5.3 DPPH Assay

Based on **Figure 4.10**, the percentage of DPPH scavenging activity increases as the concentration of Cassia essential oil increases. The antioxidant activity is therefore confirmed in both concentrations of Cassia essential oil (w/v) and (v/v). Afshari and Sayyed-Alangi (2016) stated that a smaller EC_{50} value indicates a higher antioxidant activity. Thus, ascorbic acid that showed EC_{50} value of 0.01 ± 0.010 mg/mL will have the highest antioxidant activity as compared to the Cassia essential oil (EC_{50} of 0.87 ± 0.110 mg/mL) (w/v). This high antioxidant activity of ascorbic acid can be explained by its capacity to donate electrons to scavenge the DPPH radicals, while oxidising to form dehydroascorbic acid. More electrons can be released when dehydroascorbic acid is transformed back to ascorbic acid (Pehlivan, 2017). In addition, ascorbic acid is a pure compound, unlikely the Cassia essential oil which consist of mixture of bioactive components as shown by many spots in thin layer chromatography.

The concentration of Cassia essential oil (v/v) showed higher antioxidant activity compared to (w/v). This is shown when Cassia essential oil (v/v) revealed a higher percentage of DPPH scavenging activity that ranged from 70.4 to 87.8% in contrast to (w/v) with a lower percentage radical scavenging activity from 45.1 to 88.1%. The possible explanation to this can be due to the diluted concentration of (w/v) Cassia essential oil as it was prepared by dissolving in methanol. In term of (w/v), Cassia essential oil was measured in a weighing scale to obtain the desired concentration. Thus, the content as compared to (v/v) may lesser and hence it exerts less antioxidant effect as mentioned by Torre, et al. (2019).

The antioxidant activity of Cassia essential oil may be contributed by the presence of certain phytochemicals. As an example, phenolic compounds comprise of phenolic hydroxyl group that plays a role in free radical scavenging activity by donating hydrogen atom to DPPH free radicals to terminate the propagation chain during oxidation (Morales and Lucas, 2010). The position and the number of phenolic hydroxyl group is directly proportional to the antioxidant activity of Cassia essential oil (Chen, et al., 2020). Based on the previous study, phenylpropanoids such as cinnamaldehyde is found to exhibit around 23 to 57% DPPH scavenging activity with antioxidant activity ranging from low to moderate. Moreover, the presence of eugenol, as one of the compounds found in Cassia essential oil contributes about 58 to 81% DPPH scavenging activity with a strong antioxidant activity detected (Sharma, Sharma and Pandey, 2016).

Terpenoids are another compound that may contribute to antioxidant activity owing to the presence of phenolic hydroxyl group as well as double bonds. In oxidative stress circumstances, these two functional groups will react with the *O*- and *N*-centered free radical by donating electrons to scavenge the unstable radicals (Ziyatdinova and Kalmykova, 2023). Linalool under the monoterpenoids group and β-caryophyllene, a member of sesquiterpenoids which made up about less than 10% of total Cassia essential oil contain antioxidant property (Stevens and Allred, 2022). This is confirmed in previous study conducted by Jabir, et al. (2018), in which roughly 50.6% DPPH scavenging activity was exhibited by linalool. Moreover, as an antioxidant agent, the presence of NH and OH groups in alkaloids from Cassia essential oil enable the donation of hydrogen atom to DPPH (Sabah, Al-Atbi and Mukhaiti, 2021). On the other hand, oxidation can also be inhibited by the ability of tannins to chelate metal ions and disrupt one of the steps involved in Fenton reaction (Okuda and Ito, 2011).

Lastly, flavonoids as one of the Cassia essential oil constituents may confer antioxidant activity because of the formation of C-ring in double bands. Depending on the arrangement of hydroxyl group and the structure, the DPPH scavenging activity may vary. For example, the highest DPPH free radical scavenging activity is determined in Cassia essential oil which consists of quercetin and kaempferol with the structure of B ring *ortho* 3, 4-dihydroxy and ring A 5, 7-dihydroxy, respectively (Loganayaki, Siddhuraju and Manian, 2013).

5.4 MTT Assay

5.4.1 Vero Cells

In **Figures 4.12 and 4.13**, an overall constant percentage of cell viability in Vero cells was observed with increasing concentrations of Cassia essential oil and doxorubicin for both 24- and 48-hours. From **Table 4.5**, the IC_{50} values increased from 24- to 48-hours for Cassia essential oil and reduced with incubation time for doxorubicin. Based on National Cancer Institute (NCI), IC_{50} values of less than 20 µg/mL indicates strong cytotoxicity; IC_{50} values that fall between 21 to 200 µg/mL suggests moderate cytotoxicity; IC_{50} values ranging from 201 to 500 µg/mL shows weak cytotoxicity and there is no cytotoxicity when IC_{50} value is more than 501 µg/mL (Nguyen, et al., 2020). This confirms that Cassia essential oil with the IC_{50} value of 512.60 ± 0.029 µg/mL (24 hours) and 7147.00 ± 0.019 µg/mL (48 hours) exerted no cytotoxicity on Vero cells from 24- to 48-hours, which shares the similar conclusion as the study conducted by Ye, et al. (2013). According to the study, the IC_{50} value of cinnamaldehyde, a constituent of Cassia essential oil on Vero cells is 6300 µg/mL (more than 501 µg/mL), confirming that Cassia essential oil and their constituent are not cytotoxic to the normal Vero cells. Meanwhile, doxorubicin exerted cytotoxicity on Vero cells with strong cytotoxicity observed in the first 24 hours and reduced to moderate cytotoxicity at 48 hours according to the IC_{50} value. This suggests that, in this study, doxorubicin cytotoxic effect was more effective at 24 hours as compared to 48 hours.

The possible reason that the normal cells were not killed by Cassia essential oil may be due to their specific and selective cytotoxic effect. In normal cells, the

presence of nuclear factor erythroid 2-related factor 2 (Nrf2) shields the cells from ROS-induced cell death by controlling the antioxidant response inside the cells so that oxidative stress can be avoided. Nonetheless, Nrf2 is overexpressed in cancer cells which includes the non-small cell lung cancer, shielding tumour cells from the cytotoxic effect induced by chemotherapeutic drugs. The component of Cassia essential oil such as quercetin acts as Nrf2 inhibitor to inhibit the Nrf2. Thus, Cassia essential oil only causes death specifically in the Nrf2-addicted cancer cells by inducing oxidative stress (Hsu, et al., 2022).

5.4.2 A549 Cells

According to **Figure 4.15**, the percentage of cell viability of A549 cells decreases when the concentration of the Cassia essential oil increases. This can be explained by concentration-dependent manner, in which more bioactive constituents are present at high concentration to exert high cytotoxic effect. Likewise, IC_{50} values of Cassia essential oil also reduced as the incubation period prolonged from 24- to 72-hours as shown in **Table 4.7**. The possible reason for this can be elucidated in term of time-dependent manner in which more time is required for bioactive components with complex structure to penetrate the membrane of cells to exert cytotoxicity (Menichetti, Kanekal and Bereau, 2019). Referring to guidelines provided by NCI, it is suggested that Cassia essential oil is a potential anticancer agent by exerting moderate cytotoxicity against A549 cells for the first 48 hours and progress to strong cytotoxicity at 72 hours. The cytotoxicity of Cassia essential oil towards A549 cells in this study is further exemplified by the study carried out by Farrag, et al. (2021), in which quercetin significantly killed the A549 cells with IC_{50} of 8.65,

7.96 and 5.14 µg/mL for 24-, 48- and 72-hours, correspondingly. Meanwhile, doxorubicin exerted cytotoxicity on A549 cells with strong cytotoxicity from 24- to 72-hours, which was shown by a reduced percentage of A549 cell viability and IC_{50} values from 24- to 72-hours as in **Tables 4.6** and **4.7** (Nguyen, et al., 2020). An increased in the percentage of cell viability at high doxorubicin concentration can be due to its ineffectiveness in inducing the death of A549 cells when doxorubicin is present at higher concentration as reported in Tacar, Sriamornsak and Dass (2012).

Induction of apoptosis is one of the possible anticancer mechanisms of Cassia essential oil which includes the intrinsic (mitochondria-driven) and extrinsic (receptor-driven) pathways (Kopustinskiene, et al., 2020). Extrinsic pathway is activated during the attachment of ligand to the intracellular death domain-containing death receptor. Subsequently, trimerization of death receptor recruit caspase-8 as well as Fas-associated death domain (FADD), producing death-inducing signalling complexes (DISC). The activation of DISC driven by the oligomerisation of caspase-8 via auto-proteolytic cleavage then generates caspase-3 and -7 (Kopustinskiene, et al., 2020). On the other hand, intrinsic pathway involves Bax, proapoptotic protein that integrates into the outer membrane of mitochondria, promoting permeabilization of mitochondria outer membrane. Therefore, cytochrome c is released into the cytosol from mitochondria to further oligomerise apoptotic peptidase activating factor-1 (Apaf-1). Caspase-9 is activated in apoptosome to act as initiator caspase that activates effector caspases-3 and -7, which consequently breaks the key regulatory protein and result in the death of A549 cells (Yang and Dou, 2012).

Such anticancer property can be triggered by the presence of terpenoids like linalool by elevating the apoptotic protein and decreasing the antiapoptotic protein (Pereira, et al., 2018). Flavonoids such as quercetin in the Cassia essential oil upregulates the transcription of ligands and death receptors that act as imperative beginning points for extrinsic pathway while at the same time, causes Bcl2/Bax ratio imbalance (Mukherjee and Khuda-Bukhsh, 2015).

Other anticancer mechanism that can be induced by Cassia essential oil against A549 cells includes cell cycle arrest. CDK-cyclin complexes are the central regulators of cell cycle progression to control cell division. Anticancer agents disturb the growth and proliferation of cancer cells by inhibiting cell cycle events. As an example, eugenol, the compound from steam-distilled bark Cassia essential oil down-regulate the expression of cyclin A. Hence, cyclin A cannot associate with CDK 2, and S phase cannot progress leading to cancer cell death. It can also be triggered by flavonoids like quercetin which halt G2-M phase; terpenoids like linalool that involves in G0-G1 phase arrest (Rodenak-Kladniew, et al., 2020; Zari, Zari and Hakeem, 2021).

Doxorubicin was used as the positive control in this experiment. According to Johnson-Arbor and Dubey (2022), there are several anticancer mechanisms of action exerted by doxorubicin. For instance, doxorubicin causes RNA and DNA synthesis inhibition by intercalating into the base pairs which disturbs the DNA strands. Additionally, it also stimulates apoptosis and DNA damage through the inhibition of topoisomerase II enzyme while meanwhile, generating free radical to induce oxidative stress in cancer cells. Thus, doxorubicin is widely used as

anticancer agent in the treatment of cancer in small cell lung cancer, bladder, breast, and others (Johnson-Arbor and Dubey, 2022).

5.5 Limitations of the Study

Due to time constraints and the availability of chemicals and reagents, only duplicate data were obtained for MTT assay which is not a reliable result. Incomplete solubilisation can be observed in the preparation of phytochemical reagents for phytochemical analysis, which can lead to false negative and inaccuracy results in this study. Besides, there was a lack of positive control in the certain phytochemical analysis and thus, comparison of colour changes between Cassia essential oil and positive control cannot be done.

5.6 Future Studies

Gas chromatography-mass spectrophotometry (GC-MS) is recommended to be conducted as the method to study metabolomics by identifying and quantifying a variety of compounds present in Cassia essential oil (Wang, et al., 2015). The spectrum of the metabolites in Cassia essential oil will be compared with the reported database to determine the identity of the constituents so that it can be linked to various pharmacological activities of Cassia essential oil.

Moreover, different types of antioxidant assays can be performed apart from DPPH assay to produce reliable results. For example, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, ferric reducing/antioxidant power (FRAP) assay and ferric thiocyanate (FTC) assay (Moon and Shibamoto, 2009). Similarly, several other assays can be used to study cytotoxicity to

increase the accuracy of the findings, such as adenosine triphosphate (ATP) assay and protease viability marker assay.

CHAPTER 6

CONCLUSIONS

Cassia essential oil consists of a mixture of polar and non-polar compounds. The presence of secondary metabolites in Cassia essential oil was detected using TLC with the best resolving power of constituents in the organic system consisted of Hex : EA (10 : 3) that revealed many well-separated spots. Apart from that, the classes of compounds were determined through qualitative phytochemical analysis, in which positive finding for phenols, terpenoids, alkaloids, tannins and flavonoids were observed whilst a negative result for saponins, quinones and glycosides was identified.

In DPPH assay, Cassia essential oil possesses antioxidant activity with EC_{50} values of 0.10 ± 0.130% and 0.87 ± 0.110 mg/mL for (v/v) and (w/v), respectively. In MTT assay, Cassia essential oil exerted cytotoxicity specifically and selectively against the A549 cells with decreasing IC_{50} values of 24.92 ± 0.079 μg/mL, 20.49 ± 0.202 μg/mL and 7.14 ± 0.112 μg/mL at 24-, 48- and 72-hours, respectively. Hence, Cassia essential oil exerted moderate cytotoxicity in A549 cells at 24-, 48-hours and progress to strong cytotoxic activity at 72 hours. However, Cassia essential oil was not cytotoxic to Vero cells as the IC_{50} values were 512.60 ± 0.029 μg/mL and 7147.00 ± 0.019 μg/mL at 24- and 48-hours, correspondingly. In a nutshell, Cassia essential oil can be a potential antioxidant and cytotoxic agents. Further purification using various chromatographic techniques and evaluation of other biological assays are crucial to develop the cassia essential oil into possible drug along with chemotherapeutic agents.

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