In vitro **investigation on synergistic anticancer effects between vitamin E isomers, pure compounds and crude alkaloid plant extracts on human cancerous cells**

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ABSTRACT

Anticancer chemotherapeutic treatment using single dose has been limited due to drug resistance and potential metabolic degradation. For instance, high-dose tocotrienols undergo metabolic degradation that limits the availability of therapeutic dose thereby limiting the potency *in vivo*. Combined treatment of tocotrienols at low dosage has been suggested as alternative to circumventing this limitation. This study was designed to investigate the cytotoxic effects and subsequently the apoptotic mechanisms of individual doses and combined treatments at lower dosages of tocotrienols (delta and gamma), jerantinines (A and B) and extracts (ethanol and alkaloid crude) from leaves and bark of *Ficus hispida*, *Ficus fistulosa* and *Ficus schwarzii* on lung (A549), brain (U87MG) and colon (HT-29) cancer cell lines. Neutral red uptake assay was conducted to evaluate the antiproliferative effect of individual and combined treatments. Staining techniques (histochemical and fluorescence), COMET assay flow cytometric analysis and immunofluorescence were conducted to evaluate cell morphology, DNA damage, cell cycle arrest pattern and antimicrotubule effects. Finally cell and molecular based assays were conducted to investigate the pathways for induction of apoptosis.

Cell viability study revealed that alkaloid crude extracts of leaves and bark of *F. fistulosa* demonstrated the highest potency with IC_{50} range of $0.96 - 46.81$ µg/ml compared to *F. schwarzii* ($8.79 - 107.9$ µg/ml) and *F. hispida* ($15.14 - 49.58$ µg/ml) on A549, U87MG and HT-29 cells. Both delta- and gamma-tocotrienols induced antiproliferative effects on A549, U87MG and HT-29 cells with IC_{50} values of $3.12 - 12.40$ µg/ml and $3.17 - 16.36$ µg/ml, respectively. Potent antiproliferative effects were also evident for jerantinine A (IC_{50} $0.62 - 1.74$ µg/ml), jerantinine B (IC_{50} $0.58 -$

1.48 µg/ml) and vinblastine (IC_{50} 0.03 – 0.71 µg/ml). However, similar toxic effects on these three compounds were also evident on non-cancerous lung fibroblast (MRC5) cells. The leaf and bark alkaloid crude extracts of *F. fistulosa* and *F. schwarzii* were selected for combined treatments. The combined treatment of IC_{20} doses of *F. fistulosa* with both delta- and gamma-tocotrienols induced synergistic antiproliferative effects (combination index (CI) < 1) on U87MG and HT-29 with up to 34.7-fold and 4-fold dose reductions for tocotrienols and *F. fistulosa* extracts, respectively. In contrast, additive (CI = 0.98) or antagonistic effects (CI > 1) were observed for IC_{20} doses of *F. schwarzii* extracts combined with delta- and gamma-tocotrienols on HT-29 cells. On the other hand, combined treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) induced synergistic effects (CI < 1) on A549, U87MG and HT-29 cells causing up to 4.48-fold dose reduction of tocotrienols thus reducing toxicity towards MRC5 cells compared to cancer cells.

Further morphological and DNA damage assessment focusing on tocotrienols (delta and gamma), jerantinines (A and B) and combined low-dose treatments revealed anticancer features including cell shrinkage, nuclear chromatin condensation and fragmentation, membrane blebbing, apoptotic bodies and induction of predominantly double stranded DNA breaks. Cell cycle analysis demonstrated the induction of G0/G1 and G2/M cell cycle arrests by tocotrienols (delta and gamma) and jerantinines (A and B), respectively on U87MG, A549 and HT-29 cells. Meanwhile, G2/M (A549) and G0/G1 (U87MG and HT-29) cell cycle arrests were evident for combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B). Jerantinines A and B and combined low-dose treatments with tocotrienols

(delta and gamma) caused disruption of microtubule networks and induction of caspase 8-, 9- and 3-mediated apoptosis with caspase-independent growth inhibition evidenced in the presence of caspase inhibitors on U87MG, A549 and HT-29 cells. In contrast, although treatments of tocotrienols (delta and gamma) alone caused similar apoptotic features as those of combined, disruption of microtubule networks were not characterized on these three cancer cell lines. Further mechanistic study on U87MG cells revealed that the apoptosis triggered by individual doses of tocotrienols (delta and gamma) involved the activation of TRAIL and Bid as well as the release of cytochrome C, thus confirming the recruitment of the death receptor and mitochondria-mediated pathways. On the other hand, individual doses of jerantinines (A and B) and combined low-dose treatments with tocotrienols resulted in the activation of TRAIL, FAS, p53 and Bid, as well as the release of cytochrome C. The activation of both death receptors, p53 and microtubule disruption by combined low-dose treatments demonstrates an improved mechanism of action comparing to individual doses of tocotrienols and jerantinines. In addition, the combined low-dose treatments also caused a reduction of required potent doses thereby minimizing the toxicity of jerantinines (A and B) towards the non-cancerous MRC5 cells. In conclusion, this research has presented valuable combined treatment candidates which are warranted for further investigations as future chemotherapeutic agents against cancers.

LIST OF PUBLICATIONS

Abubakar, I.B., Lim, K.-H., Kam, T.-S., Loh, H.-S., 2016. Synergistic cytotoxic effects of combined δ-tocotrienol and jerantinine B on human brain and colon cancers. J. Ethnopharmacol. 184, 107–118.

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Abubakar, I.B., Lim, K.-H., Loh, H.-S., 2014. Alkaloid extracts of Ficus species and palm oil-derived tocotrienols synergistically inhibit proliferation of human cancer cells. Nat. Prod. Res. 29, 2137–2140.

CONFERENCE ABSTRACTS

Abubakar, I.B., Lim, K.-H., Loh, H.-S., 2016. Synergistic apoptotic effect of combined low-dose gamma Tocotrienol and Jerantinine A treatments on cancer cells, in: International Conference of Translational Molecular Imaging and Aerospace Medicine and Physiology Showcase.

Abubakar, I.B., Lim, K.-H., Loh, H.-S., 2014. Combination of low dose δ-tocotrienol and Jerantinine A induces synergistic growth inhibition on human lung adenocarcinoma, in: Joint Malaysia-UK Symposium on Natural Product Chemistry and Drug Discovery.

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combined low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B).

LIST OF ABBREVIATIONS

Name	Abbreviation
Delta tocotrienol	δ-T3, δ-Tocotrienol
Gamma tocotrienol	γ-T3, γ-Tocotrienol
Jerantinine A	JA
Jerantinine B	JB
Alkaloid extract	ALK
Ethanol extract	ET
Ficus fistulosa	FF
Ficus hispida	FH
Ficus schwarzii	FS
Combination index	CI
Dose reduction index	DRI
Half maximal inhibitory concentration	IC_{50}
Acridine orange/propidium iodide	AO/PI
Hematoxylin & eosin	H&E
Single stranded breaks	SSBs
Double stranded breaks	DSBs
Ataxia-telangiectasia mutated	ATM
ATM and Rad3-related	ATR
Dithiothreitol	DTT
p-nitroanilide	pNA
Fluorescein isothiocyanate	FITC
4',6-diamidino-2-phenylindole	DAPI
BH3 interacting domain	BID
Cytochrome C	Cyto c
X-linked inhibitor of apoptosis protein	XIAP
BcL2 associated death promoter	Bad
BcL2 associated X protein	Bax
TNF-related apoptosis inducing ligand	TRAIL
Phosphate buffered saline	PBS
Dimethyl sulfoxide	DMSO
Roswell park memorial institute-1640	RPMI-1640
Minimum essential medium	MEM
Fetal bovine serum	FBS
Ethylenediaminetetraacetic acid	EDTA

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1.0 CHAPTER ONE: INTRODUCTION

1.1 Cancers and treatments

Cancer is still one of the leading causes of death around the world. The incidence of cancer and cancer related deaths are increasing worldwide surpassed only by heart related diseases. An estimated figure of over 1.6 million people diagnosed with cancer was expected in 2015 with over 589,430 cancer related deaths in the US thus accounting for 1 in every 4 deaths (Siegel et al., 2015). Lung, breast, colorectal, stomach and liver cancers remain the leading causes of death largely due to smoking, alcohol consumption, fat rich diet and less exercise (Cancer Research UK, 2014). Furthermore, epidemiological studies have demonstrated that types of cancer are largely influenced by culture (Kolonel et al., 2004). For instance, there is a higher prevalence of lung, colon, prostate and breast cancers in Western countries compared to Eastern countries. Cancers of the head, neck and cervix are more prevalent in India, whereas, stomach cancer is the most common in Japan (Aggarwal and Shishodia, 2006). The advents of modern treatments that include chemotherapy, radiotherapy and surgery have definitely improved the care of cancer patients. However, high cost of treatment, advanced cancer metastasis, non-selective toxicity, resistance to synthetic drugs and undesirable side effects highlight the limitations of these treatments. Therefore, it is urgently needed for a more efficient and cost-effective cure.

1.2 Plants serve as natural anticancer agents

Over the years, a consensus amongst the scientific community began to emerge on the possible role of diet in the prevention of cancer. This perception has received great attention even amongst consumers on the high prospect of consumptions of vitamins, vegetables, fruits and other natural plant compounds which could potentially provide

an alternative towards the prevention and treatment of diseases (Cai et al., 2004). Indeed, several epidemiological studies have indicated a lower incidence of cancer in populations that consume fruits and vegetables rich food. An extensive review of 22 animal and 206 human epidemiological studies had shown an inverse relationship between consumption of fruits and vegetables and risk for various types of cancer (stomach, oesophagus, lung, oral cavity, endometrium, pancreas and colon) (Steinmetz and Potter, 1996). Medicinal plants have also played a vital role in cancer treatment, especially in the developing and underdeveloped parts of the world and most phytochemicals discovered over the past half century have been used towards treatment of cancer (Balunas and Kinghorn, 2005). In 1950, the U.S. National Cancer Institute (NCI) recognised the potential use of natural products as anticancer agents and since then, there has been a surge of interest in natural product research leading to discovery of new anticancer agents (Cragg and Newman, 2005). About 40% of available anticancer drugs between 1940 and 2002 are natural products while an additional 8% are termed as natural product mimics (Balunas and Kinghorn, 2005). Several natural products isolated from plants have been successfully used as anticancer agents and the prominent examples are the vinca alkaloids, vincristine and vinblastine isolated from *Catharanthus roseus* used against lung, breast, leukemia, germ-cell and renal cancers (Rocha et al., 2001).

1.2.1 *Ficus spp* and *Tabernaemontana corymbosa*

Ficus spp exemplifies an excellent source of phytochemicals that have widespread distribution across Asia and Africa with applications in traditional medicine and serves as food. Historically, the use of *Ficus spp* in health improvement could be traced back to ten thousand years (Shi et al., 2011). Traditionally, parts of the fig tree

which include the root, stem, bark, fruit and latex have been used in treatment of different types of ailment including antitumor (Hashemi et al., 2011; Khodarahmi et al., 2011; Lansky et al., 2008; Shi et al., 2011). Pharmacological studies have shown that the fruit, stem, leaf and latex of *Ficus spp* contain the active ingredient triterpenoids which have cytotoxic activity (Khodarahmi et al., 2011; Lansky et al., 2008). In fact, recent studies have also demonstrated that *Ficus spp* are applicable as adjunct cytotoxic regimens (Nugroho et al., 2013, 2012). Three *Ficus spp* namely *F. hispida*, *F. fistulosa* and *F. schwarzii* were selected for this study. The selection of *F. hispida* and *F. fistulosa* was based on data gathered from previous pharmacological studies indicating good cytotoxic potencies of these plants on a broad range of cancer cells *in vitro* (Pratumvinit et al., 2009; Subramaniam et al., 2009; Yap et al., 2015; Zhang et al., 2002). In contrast, there are no pharmacological studies on *F. schwarzii*. Nonetheless, *F. schwarzii* was selected because studies on phytochemistry have demonstrated that *Ficus spp* are repository to potential cytotoxic phytochemicals. Therefore, the present study would provide insightful information on bioactivity of *F. schwarzii*.

Similarly, *Tabernaemontana corymbosa* is another plant with long history of medicinal uses across Asia for treatment of various diseases including syphilis, fractures and tumors amongst others (Hasan et al., 2012; Van Beek et al., 1984; Wiart, 2006). Phytochemical studies on different parts of the plant revealed a pool of novel indole and bisindole alkaloids and some possess interesting anticancer activity. Initial cytotoxic screenings showed that derivatives of jerantinine (A-G) exhibited the most potent cytotoxic effect in reversing vincristine resistance in human oral KB cancer cells (Lim et al., 2008). Recently, limited mechanistic studies had revealed the

potential mechanism mediating the cytotoxic effects of jerantinine derivatives (A and E), however, similar toxic effect was also evidenced on normal cells (Frei et al., 2013; Raja et al., 2014).

1.2.2 Tocotrienols

Vitamin E, a typical example of nutritious phytochemical derived from non-medicinal plants, is often affected by the need for high-dose delivery and toxicity. Vitamin E represents a group of isomers comprising of tocopherols and tocotrienols that possess potent therapeutic potentials against diseases including cancer. Since the discovery of vitamin E in 1922, several *in vitro*, *in vivo* and human supplementation studies have focused on its therapeutic potentials. However, despite the abundance of literature of vitamin E, only 1% research was conducted on tocotrienols comparing to tocopherols. This represents a major void in the research that necessitates further studies considering that the biological function of vitamin E analogues are not the same (Wong and Radhakrishnan, 2012). There have been well documented *in vitro* studies with certain degree of success about the effects of vitamin E isomers on cancers. However, the same success has not been recorded in intervention studies of vitamin E supplementation in humans. This lack of success has been further complicated with adverse side effects of high dosage such as an increased risk of heart failure and hemorrhagic stroke observed in the HOPE-TOO (Heart outcomes prevention evaluation-the ongoing outcome) trial (Bennett et al., 2012). In addition, low therapeutic dose has limited the potential of oral tocotrienols and this has been attributed to the high-dose delivery that overloads the transport protein subsequently resulting in metabolic degradation (Sylvester et al., 2010). Thus far, combinatorial treatments with adjuvant originated from nutritional and medicinal sources that would

minimize the dose and improve potency of tocotrienols have been recommended as a possible alternative (Constantinou et al., 2008; Sylvester et al., 2010).

1.3 Research problems and rationales of study

Cancer incidence increases dramatically and current treatments are associated with undesirable side effects. Cancer treatment using plant-derived natural products or mimics of natural products such as vinca alkaloids have shown promise. However, as with most drugs, administration of high dosage is often required for potent activity. This results in acquired drug resistance, non-selective toxicity to non-cancerous cells and possible metabolic degradation thereby limiting the availability of therapeutic dose. Indeed, as shown in previous studies, alkaloids derived from *Ficus spp* such as hispiloscine and jerantinines A and B (isolated from *Tabernaemontana corymbosa*) demonstrated similar non-selective toxicity to both cancer and non-cancerous cells *in vitro* (Raja et al., 2014; Yap et al., 2015). Similarly, tocotrienols represent the most potent cytotoxic vitamin E isomers that have demonstrated promising anticancer properties. However, the potential successful application of tocotrienols as cancer therapeutic agents has been limited by problems that include the following;

a) The need for delivery of high dosage especially via oral route of administration.

b) Low bioavailability of therapeutic dose due to the overloading of the transport protein by high doses and subsequent metabolic degradation of tocotrienols.

c) Potential side effects such as increased risk of heart failure and hemorrhagic stroke as evident in the HOPE-TOO trial (Bennett et al., 2012) .

Combinatorial treatment approaches resulting in either synergistic or additive pharmacological interactions are continuously been explored to develop cytotoxic

regimens with improved potency to overcome drug resistance and also minimize non-selective toxicity to non-cancerous cells (Boik, 2001; Lim et al., 2013). The concept of drug combinations is to maximize potency of chemotherapeutic agents especially against drug resistance. The drug combinations can be between direct and indirect acting drugs that can result in sequential, concurrent or concerted synergistic pharmacological interactions (Boik, 2001). Indeed recent studies have suggested drug combinations at lower dosages as potential alternative to circumvent limitations associated with single high-dose tocotrienols(Shirode and Sylvester, 2010). Drug combinations at lower dosages can minimize toxicity of single high doses whilst improving potency via multi-target pathways that results from two or more drug combinations. Although several studies have investigated the synergistic potency of tocotrienols previously, most combinations were conducted at high dosage and the potential toxicity to non-cancerous cells was not properly addressed (Wali and Sylvester, 2007; Yang et al., 2010).

Therefore, in a continual contribution towards developing value-added tocotrienols with an improved anticancer potency and elucidating the mechanisms of action for jerantinines (A and B), the present study was undertaken to investigate the *in vitro* anticancer activities of these tocotrienols, the indole alkaloids jerantinines A and B (isolated from Malaysian *Tabenaemontana corymbosa*), ethanol extracts and alkaloid crude extracts from the leaves and bark of *Ficus sp* (*Ficus hispida, Ficus fistulosa, and Ficus schwarzii*) collected from various locations in Malaysia.

Three different cancer cell lines including brain, lung and colon cancer were selected for this study. The brain (U87MG) cancer cells was selected because only a few

cytotoxic studies have been conducted to date despite the poor survival rate in patients (Lim et al., 2014b; Siegel et al., 2016). In addition, recent studies have shown that tocotrienols can penetrate the blood brain barrier (Kaneai et al., 2016). Likewise, to date there are no reported studies on the potential mechanism jerantinines induced apoptosis on U87MG brain cancer cells. On the other hand, both lung (A549) and colon (HT-29) cells were selected due to the higher incidences. In fact, lung and colon cancers are currently ranked as 1st and 3rd leading causes of cancer related deaths (Siegel et al., 2016). In addition, these cancers offer a potential oral route of administration for chemotherapeutic agents. This would allow for future testing on the potency of successful low-dose combinations via the oral route considering that oral route of administration limits the anticancer potency of high-dose tocotrienols.

1.4 Scope of study

The study enlisted tocotrienols (delta and gamma), extracts from *Ficus spp* and jerantinines (A and B) for the individual and combined treatments to act on three cancer cell lines, namely human lung adenocarcinoma (A549), brain glioblastoma (U87MG) and colorectal adenocarcinoma (HT-29) cells and one normal (non-cancerous) cell line, namely human lung fibroblast (MRC5) cells for a parallel comparison. In this thesis, Chapter Two describes the background knowledge and literature review detailing information on cancers, bioactive compounds and plant extracts selected for this study as well as the apoptotic mechanisms. Chapter Three focuses on the screening for antiproliferative activities of individual and combined treatments as well as quantitative measurement of pharmacological interactions between combined treatments on brain (U87MG), lung (A549) and colon (HT-29) cancers. Following the determination of synergistic growth inhibition, the effects of

potent individual and combined treatments on cellular morphological features and DNA damage patterns were subsequently determined as reported in Chapter Four. Furthermore, investigations on cell cycles, microtubules and the role of caspase enzymes were conducted to learn about the apoptosis phenomenon and presented in Chapter Five. Chapter Six signifies the mechanisms mediating apoptosis induction by individual and combined treatments on brain (U87MG) cancer cells which were elucidated via apoptosis array, enzyme linked immunosorbent assay (ELISA) and Western blotting techniques. Finally, Chapter Seven exemplifies the general discussion of the whole study leading to generation of final research conclusions with limitations of study well-appreciated and future prospects recommended.

1.5 General aim and specific objectives of study

The general aim of this research is to seek for a value added anticancer chemotherapeutic regimen from plant sources via combined treatments which could improve anticancer potency and reduce non-selective toxicity towards normal cells. In order to achieve this general aim, several investigations had been carried out and described in details at separate chapters, i.e. from Chapter Three to Six.

The specific objectives of the study were:

1) To determine the *in vitro* antiproliferative effects of individual and combined treatments of tocotrienols, extracts from *Ficus spp* and jerantinines as well as their pharmacological interactions (Chapter Three).

2) To assess the DNA damage patterns and changes in cellular morphology associated with apoptosis induced by individual and combined treatments of tocotrienols and jerantinines on three cancer cell lines (Chapter Four).

3) To deduce the cell cycle arrest, microtubule integrity and involvement of caspase enzymes in the execution of apoptosis by individual and combined treatments of tocotrienols and jerantinines on three cancer cell lines (Chapter Five).

4) To elucidate the molecular apoptotic mechanisms involving apoptosis related gene expressions triggered by individual and combined treatments of tocotrienols and jerantinines on brain cancer cell line (Chapter Six).

1.6 Experimental hypotheses

Previous ethno-medicinal and pharmacological studies have demonstrated the cytotoxic potentials of *Ficus* species, *Tabernaemontana corymbosa* and palm oil derived tocotrienols(Lansky et al., 2008; Lim et al., 2014b; Qazzaz et al., 2016; Raja et al., 2014). Therefore, it is hypothesized that the individual treatments of tocotrienols, jerantinines (A and B) and extracts (ethanol and alkaloid crude) of *Ficus spp* (*Ficus hispida, Ficus fistulosa,* and *Ficus schwarzii*) shall induce potent antiproliferative effects on lung (A549), brain (U87MG) and colon (HT-29) cancer cells. The ideal combinations at lower dosages of two cytotoxic candidates exhibiting a synergism shall reduce the required potent doses. This would subsequently minimize toxicity to non-cancerous cells and improve the selectivity window between cancer and non-cancerous cells. The combined treatments shall also result in an improved efficacy of apoptosis induction through the activation of multiple pathways as compared to individual treatments.

1.7 Significance of study

The current research activities advance the search for anticancer drug candidates from the nature. This study is impactful as the cancer incident is constantly increasing which necessitates utmost attention of researchers. The qualities of life of the cancer patients have also been jeopardized with the undesirable side effects associated with the current available treatments. The combined treatments may be advantageous in increasing efficacy and effectiveness of chemotherapy. The combined treatments might provide maximum cancer cell cytotoxicity within the range that is tolerable by the patients. This could potentially also minimize the duration of cancer chemotherapy thereby reducing the suffering of patients and the side effects which are often encountered during the chemotherapy process. The use of combined low-dose treatments could also prolong the use of these chemotherapeutic regimens which could potentially reduce the risks of cancer recurrence and drug resistance.

On the other hand, *Tabernaemontana corymbosa* has been placed on the red list under the lower risk category of endangered species by the International Union for Conservation of Nature (World Conservation Monitoring Centre, 1998). Therefore, this study would be significant as it would provide valuable additional knowledge on the pharmacological activities and mechanistic action of jerantinines (A and B) (isolated from *Tabernaemontana corymbosa*) on top of the very limited studies (Qazzaz et al., 2016; Raja et al., 2014). Furthermore, the combinations of delta and gamma tocotrienols at lower doses with jerantinines (A and B) and extracts (ethanol and alkaloid crude) of *Ficus hispida, Ficus fistulosa,* and *Ficus schwarzii* as described in this study were the first of its kind and hence are highly valuable.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Cancer

Cancer is a collection of diseases of more than 100 characterized by uncontrolled proliferation that can lead to undesirable fatal consequences. The incidence of cancer can vary depending on gender, ethnicity and geographical location. Globally, there were over 8.2 million cancer related deaths and over 14 million new cases of cancer in 2012 which are projected to rise by exceeding 70% over the next two decades (American Cancer Society, 2012). Lung (13%), breast (11.9%) and large bowel (9.7%) were the most commonly diagnosed cancers. Whereas, lung (19.4%), liver (9.1%) and stomach cancers (8.8%) were recorded as the most common causes for cancer deaths. Africa, Asia and South America account for 60% of global new cases and 70% of cancer deaths (Stewart and Wild, 2014). In 2016, over 16 million new cases and over five hundred thousand cancer related deaths are expected in the United States (Siegel et al., 2016). Prostate (21%) and breast (29%) cancers are the leading estimated new cases of cancer types in males and females, respectively. Whereas, lung and bronchus are the leading types for estimated cancer related deaths in both males (27%) and females (27%) (American Cancer Society, 2016). Key risk factors related to health and environmental conditions that are avoidable, account for more than 30% of cancer deaths. For instance, tobacco use alone accounts for 20% of cancer death globally. Whereas, HBV (hepatitis B virus) and HPV (human papillomavirus) infections account for 20% cancer deaths especially in low income countries (Stewart and Wild, 2014).

2.1.1 Pathogenesis of cancer

The etiological causes of cancer are classified into three groups that include chemical carcinogens, biological carcinogens and physical carcinogens. These eventually cause damage in genetic materials that is considered as the primary trigger of cancer pathogenesis from normal to tumor cells. Cancers cells cause a range of pathological processes that include inflammation, immune system disruption and invasion, metastasis, angiogenesis and oxidative nitrosative stress (Bukhtoyarov and Samarin, 2015).

Uncontrolled proliferation is one of the pathophysiological mechanisms of cancer. Temporary suspension of anti-tumor activity is a natural physiological condition that allows for repairing tissues that have been damaged due to chemical, physical or biological carcinogens (Martinez and Gordon, 2014; Mosser and Edwards, 2008). However, a myriad of factors that include multiple microdamage often cause prolonged uncontrolled proliferation of tumor cells (Bukhtoyarov and Samarin, 2015). Furthermore, unlike healthy cells, tumor cells are not dependent on exogenous growth factors due to adoption of an autocrine signaling through production of receptor tyrosine kinases that are secreted into extracellular matrix. In addition, tumors alternatively divide by mutating and activating the quiescent Ras oncoprotein which permanently conveys growth signal. Indeed, the Ras oncogene is mutated in about 20% of cancer cells (Whellan et al., 2009). Tumor cells progress by evading apoptotic machinery through mutation of death receptors, gene silencing and increased production of anti-apoptotic factors (Igney and Krammer, 2005). Eventually, tumor cells produce small capillaries (tumor vascularization) through a process termed as neo-angiogenesis. Hypoxia occurs in the event of insufficient

vascularization in tumor cells which triggers the production of pro-angiogenic factors such as fibroblast growth factor (FGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and angiostatin amongst others that promote angiogenesis (Auguste et al., 2005). Invasiveness and metastasis are other important features that distinguish between benign and malignant tumors. Malignant tumors leave primary tumor sites via circulation to invade adjacent tissues to form secondary tumor through a series of steps termed as the metastatic cascade. The secondary tumors proliferate and induce angiogenesis to ensure sufficient vascularization of cancer cells (Leber and Efferth, 2009).

2.1.2 Types of cancer

Globally, cancer is a major public health problem. Although there are over 100 different types of cancers, cancers of the lung, liver, colorectal, stomach, breast, and oesophageal account for the most number of cancer deaths globally. Herein, lung, colon and brain cancers were selected for this study. Both lung and colon cancers have high incidence rate and are the leading causes of cancer related death ranked globally at 1st and 3rd, respectively. On the other hand, brain cancer is the leading cause of cancer death in children and adolescents. In addition few studies have been conducted on the cytotoxic potency of tocotrienols on brain cancer even though studies have shown that tocotrienols can penetrate the blood brain barrier. Similarly, to date there are no reported studies on the cytotoxic potency of jerantinines on brain cancer cells. Finally, the potential oral route of drug administration for these cancers offers an opportunity to determine in the future the potency as well as the susceptibility of combined low-dose treatments to metabolic degradation. This

necessitates investigations on potential future chemotherapeutic regimens against lung, colon and brain cancers.

2.1.2.1 Lung cancer

Lung cancer is one of the most common avoidable cancer diseases worldwide. A rare disease before the 20th century, its incidence has since expanded significantly over the past 10-20 years accounting for the highest mortalities resulting from oncological problems in the 21st century (van Zandwijk, 2001). Non-small cell lung cancers account for about 85% of lung cancers and is characterised by subtypes that include adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma. In the United States, squamous cell carcinoma and adenocarcinoma are the most prevalent types of non-small cell lung cancers in men and women, respectively. According to estimates from the American Cancer Society, in 2015, 221,200 new cases and 158,040 deaths from lung cancer were reported in America (Siegel et al., 2015). There is higher incidence in men and women above 40 years especially in the former thus correlating with the differences in the pattern of tobacco smoking (van Breda et al., 2008). However, there has been an increase in lung cancer deaths in women in developed countries in contrast to observed decrease in fatalities amongst men. This emerging trend has been linked to the increasing prevalence of females smoking cigarette in the United States, Europe and Asia (American Cancer Society, 2012). Furthermore, there are indications of other risk factors such as genetic susceptibility, biologic and hormonal factors by some studies indicating the possible partly or wholly contribution in the regards of tobacco effect in women (Pauk et al., 2005). Family and smoking history have been accepted to play a possible role in lung cancer. In addition, genetically susceptible genes such as CYP1A1 (a cytochrome

P450 family1 member A1 gene), which is common to both lung and breast cancers have also been identified. In addition, the expression of gastrin-releasing peptide receptor (GRPR) as well as the G to T transversion in p53 have been found to be higher in woman lung cancer cases compared to those of males indicating these possible roles towards lung cancer susceptibility in women (Shriver et al., 2000).

2.1.2.2 Brain cancer

Brain tumour is leading cause of cancer related deaths in children, places at the second only to leukaemia, although it only accounts for 50% of frequency of leukaemia (Pfister et al., 2009). In fact, recent data showed that brain cancer has surpassed leukaemia as the leading cause of cancer death among children and adolescents aged 19 years (Siegel et al., 2016). In Britain, around 400 children below the age of 15 years are diagnosed with brain, central nervous system (CNS) and intracranial tumours annually representing 27% and ranked the second most diagnosed childhood cancer (Cancer Research UK, 2014). Epidemiological data in 2008 showed the global age standardised incidence of 3.7 and 2.6 per 100,000 men and women, respectively for primary brain tumour (PBT). Reported approximations of 21,800 individuals in the United States were diagnosed with malignant primary brain tumour in 2008 with Caucasians recording the highest rate (Bondy et al., 2008). In 2011, around 9,400 people were diagnosed with brain and / or CNS, intracranial tumours in the United Kingdom with 57% of the aforementioned diagnosis occurring in people under 65 years. Furthermore, brain cancer is the 15th and 13th leading cause of cancer deaths amongst Taiwanese males and females, respectively with a 1.5 times greater incidence of glioma in the former when compared to the latter (Chiu et al., 2012).

Possible protective roles of female hormones against brain cancer development have been suggested as a probable reason for lower incidence in females. Studies have shown that the higher incidence in females only reaches its maximum around the age of female menopause and subsequently diminishes thereafter (McKinley et al., 2000). Histologically, a population study showed that gliomas accounted for approximately 57% of paediatric brain tumors whereas craniopharyngiomas (4%), pineal tumors (1%), meningiomas (1%), medulloblastomas and other embryonal tumors (26 %) and others (11%) constituted for the remaining 43% (Kaatsch et al., 2001).

2.1.2.3 Colon cancer

Globally, colorectal cancer is ranked the third leading cause of cancer related deaths (Purim et al., 2013). An estimated 136,830 and 50,310 people were reported to be diagnosed or die from colorectal cancer in 2014 (American cancer society, 2014). There are several risk factors to colon cancer that have been classified into modifiable and non-modifiable. Hereditary or family history exemplifies the latter as individuals with family history of colorectal cancer are 2 to 3 times the risk of developing colon cancer. In fact, about 20% of colorectal cancer patients have relatives that were previously diagnosed (Lynch and Chapelle, 2003). On the other hand, behavioural risk activities that include physical activities, diet, smoking, alcohol, overweight and obesity constitute the modifiable risk factors. The survival rate for colorectal cancers varies globally. For instance, 65% of five-year survival rate has been reported in Israel and South Korea in contrast to 20% to 55% recorded in other parts of Asia. Similarly, there is five-year survival rate of 60% to 65% in North America, New

Zealand, Australia and majority of European countries (American Cancer Society, 2015).

2.1.3 Available cancer treatments and limitations

There are different types of cancer treatments that have continuously changed over the past four decades. The most prominent age long treatments include surgery, radiotherapy, and chemotherapy. Surgery is a very effective treatment that has been used prior to the advent of radiotherapy and chemotherapy. As an individual treatment, it is more effective compared to radiotherapy and chemotherapy as it is based on zero-order kinetics thereby kill 100% of excised cells. In addition, surgery is pivotal for improving five-year survival rate of colorectal liver metastases by 30-40% although this therapeutic approach is applicable to only confined metastases that represent 10-15% of cases (Urruticoechea et al., 2010). However, surgery is temporal palliative and accessibility to some parts by surgery is difficult. In addition, surgery is not applicable to sub-clinical metastases and cannot completely eliminate cancer cells.

On the other hand, radiotherapy is an alternative palliative that is used to slow down cancer growth or shrink massive tumors to enable removal by surgery. In fact, over 45% of new cases are expected to receive radiation therapy (Delaney et al., 2005). Unlike surgery, radiotherapy is a curative to early stage cancers of head and neck, pancreas and Hodgkin's disease. In addition, radiotherapy is used in combination with surgery or chemotherapy as a preoperative or intraoperative palliative. However, major limitations include potential damage to organs close to tumor sites, inconvenience of regular hospital visits and inability to completely eradicate cancer cells amongst others (Urruticoechea et al., 2010).

Chemotherapy is the use of drugs at different stages of cancer as a cure, a preventive against cancer spread or palliative. Unlike radiotherapy and surgery, chemotherapy can spread quickly throughout the whole body to kill local cancer, metastasize cancer and restrain tumor growth. A typical example of chemotherapeutic drugs is tamoxifen that has been used as an adjuvant for breast cancer treatment which has resulted in 11% improvement of ten-year survival rate (Shiau et al., 1998). The vast majority of chemotherapeutic drugs currently in clinical use or development are derived from natural products or mimics of natural products (Cragg and Newman, 2005). However, the potential of non-specific killing of cells, acquired drug resistance over time and difficulty of overcoming blood-brain barrier highlight some of the limitations of cancer chemotherapy (Alfarouk et al., 2015). Nonetheless, chemotherapeutic drugs from natural products are continuously being developed. Strategies such as combination therapy between multiple chemotherapeutic agents and targeted delivery using nano-materials are suggested to overcome some of the limitations (Boik, 2001; Sylvester et al., 2010).

2.2 Apoptosis as a natural defense mechanism

Apoptosis or programmed cell death is a highly preserved and regulated mechanism that occurs at developmental stages and aging, playing key roles in regulating physiological growth and tissue homeostasis. The discovery of apoptosis as a mode of cell death was the highlight of cancer research considering its critical role in regulating tumor growth and determining therapeutic response (Fulda and Debatin, 2006a). In addition to apoptosis, there are other forms of cell deaths such as necrosis, autophagy and mitotic catastrophe amongst others (Edinger and Thompson, 2004).

Apoptosis is characterized by morphological features and energy dependent biochemical mechanism. The morphological features of apoptosis are visible under the light microscope. At early stage, apoptosis is characterized by cell shrinkage and nuclear chromatin is condensed into masses that become marginated from the membrane (Saraste and Pulkki, 2000). Thereafter, extensive plasma membrane blebbing occurs and the subsequent separation of cellular fragment into apoptotic bodies. The apoptotic bodies are subsequently phagocytosed by neighboring cells including parenchymal cells and macrophages. Apoptotic cells appear as oval or circular shaped single or small clusters of cells with dark eosinophilic cytoplasm and dense purple nuclear chromatin when examined using histological dyes, namely hematoxylin and eosin (Elmore, 2007). The degradation of DNA by endogenous DNases into double stranded fragments of 180-200 base pair highlights the biochemical hallmark of apoptosis. DNA fragments appear as ladder in electrophoresis whereas cells with fragmented DNA become visible under light microscope when tested with terminal transferase mediated DNA nick end labelling. In addition, the activation of cysteine proteases (caspase) enzymes and the subsequent cleavage of their substrates are a biochemical process that is essential for apoptotic cell death (Hengartner, 2000).

2.2.1 Difference between apoptosis and necrosis

Necrosis represents an undesirable form of cell death in cancer research. Unlike apoptosis, necrosis is an energy independent process that is accompanied by rapid loss of homeostasis resulting in accidental cell death. It is characterized by morphological features that include swelling, formation of distended endoplasmic reticulum, cytoplasmic vacuole and blebs, disrupted membrane organelle and rupture of

mitochondria and ribosomes. Furthermore, unlike apoptosis, necrosis is absence of apoptotic bodies formation and consists of inflammation attributed by subsequent leakage of intracellular contents (Denecker et al., 2001). Nevertheless, there are similarities between apoptosis and necrosis. For instance, mitochondrial membrane permeability present in apoptotic cell death has also been characterized in necrotic cell death. There are similarities on the potential roles of anti-apoptotic proteins such as B-cell lymphoma protein 2 (Bcl2) family proteins and pro-apoptotic proteins including Bax towards inhibition or activation of apoptotic and non-apoptotic cell death. The activation of caspase enzymes ultimately determines phenotypically if cell death is apoptotic or necrotic (Elmore, 2007).

2.2.2 Apoptotic pathways

In anticancer therapy, apoptotic death often involves activation of caspase enzymes that act as mediator of cell death although there is also evidence of caspase independent apoptosis. These processes are mediated via intricate signaling mechanism categorized into extrinsic (death receptor) and intrinsic (mitochondria) pathways.

2.2.2.1 Extrinsic pathway

The extrinsic signaling pathway also known as the death receptor pathway involves transmembrane death receptors belonging to the tumor necrosis factor (TNF) receptor gene superfamily. The TNF has an 80 amino acid rich death domain that plays critical role in transmitting death signal from cell surface to intracellular signaling (Edinger and Thompson, 2004). The best characterized death receptors and their ligands involve in apoptosis include tumor necrosis factor alpha/tumor necrosis factor

receptor 1 (TNF-α/TNFR1), fatty acid synthetase ligand/fatty acid synthetase receptor (FASL/FASR), Apo3 ligand/death receptor 3(Apo3L/DR3), Apo2 ligand/death receptor 3 (Apo2L/DR3), Apo2 ligand/death receptor 5 (Apo2L/DR5) (Rubio-Moscardo et al., 2005). Upon induction of death signal, two of the best understood ligands, TNF-α and FasL bind to the respective receptor thereby recruiting death domains including TNF receptor associated death domain (TRADD) and Fas associated death domain (FADD), respectively. The death domains form death inducing signaling complex (DISC) and subsequently activate caspase 8 via autocatalytic activation. Subsequently, caspase 8 activates executioner caspase 3 to trigger apoptotic cell death. However, this can be inhibited by FLICE inhibitory protein (c-FLIP) that renders the death receptor mediated pathway becoming effective (Fig. 2.1). Alternatively, caspase 8 induces truncation and activation of BH3 interacting death domain agonist (BID) protein that subsequently triggers the mitochondria mediated pathway of apoptosis (Wajant, 2002).

2.2.2.2 Intrinsic pathway

As shown in Fig. 2.1, the intrinsic or mitochondria mediated pathway, caspase activation is accompanied with permeabilization of the mitochondrial membrane. Upon induction of death stimuli and disruption of mitochondrial membrane, intramembrane proteins including cytochrome c, second mitochondrial activator of caspases/direct inhibitor IAP binding protein with low PI (SMAC/DIABLO) and endonuclease G are released into the cytosol to induce apoptosis. Cytochrome c binds to apoptotic protease activating factor (APAF-1) and caspase 9 to form the active apoptosome complex. Eventually, the executioner caspase 3 is recruited by the apoptosome and activated by caspase 9 to trigger key cellular and biochemical events

of apoptosis (Blagosklonny, 2003). Furthermore, additional mitochondrial proteins such as SMAC/DIABLO and high temperature requirement HtrA2/Omi promote mitochondria mediated apoptosis by stopping inhibitors of apoptosis (IAP). A second group of proteins including the apoptosis inducing factor (AIF) and endonuclease G are released by mitochondria and translocated to the nucleus at the later stages of apoptosis (Candé et al., 2002). Both proteins promote apoptosis by causing DNA fragmentation as well as caspase independent apoptosis. The Bcl-2 family proteins are comprised of about 26 pro- and anti-apoptotic proteins that control the mitochondria membrane and the balance between these groups of proteins ultimately determine apoptosis. The pro-apoptotic proteins include Bid, Bad, Bax, Bim, Bik, Bcl-10, Bak and Blk. In addition, the tumor suppressor protein p53 associates with Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) and Bcl2 binding component 3 (Puma) to induce mitochondria mediated apoptosis (Chipuk, 2004). Puma causes conformational change and expression of Bax, cytochrome c release and reduction of mitochondrial membrane potential. Noxa localizes to the mitochondria to interact with anti-apoptotic Bcl2 proteins and induce activation of caspase 9 (Chipuk, 2004).
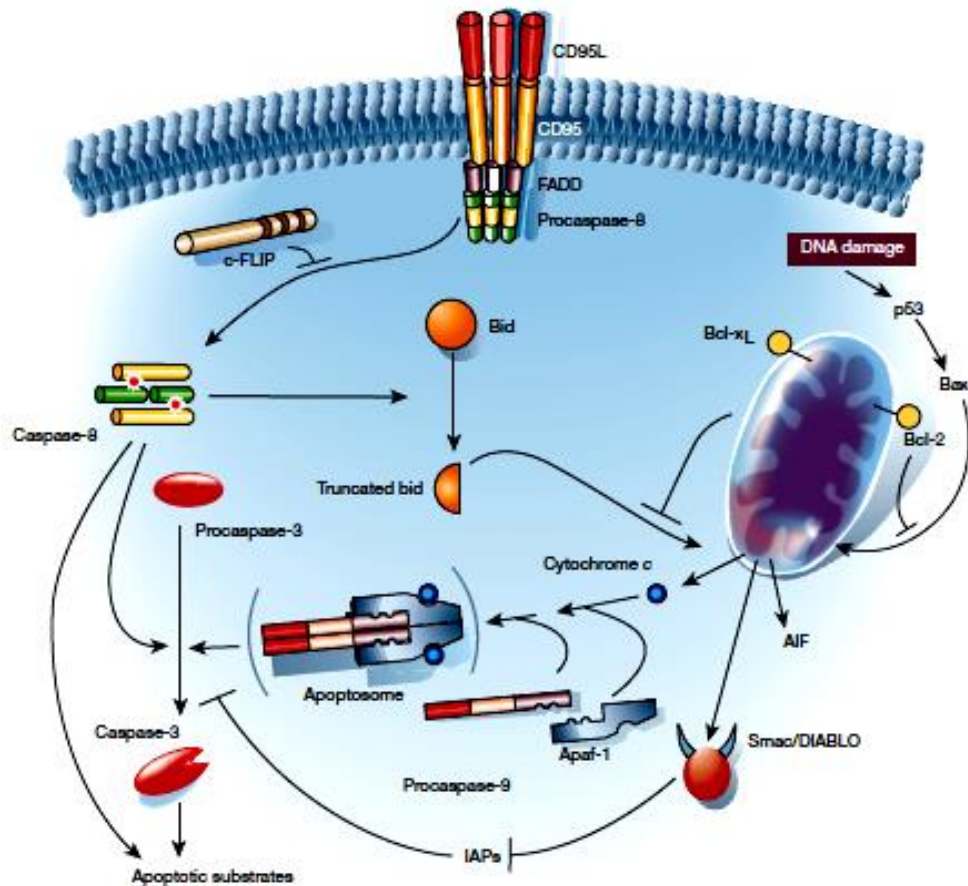
Fig. 2.1: Extrinsic (death receptor) and intrinsic (mitochondria) apoptotic pathways (Hengartner, 2000).

2.3 Plant derived anticancer agents

Mortality and new diagnosed cases of cancer are continuously rising. Some cases can in fact be minimized as shown in the cases of the aforementioned cancers (lung and colon), however, the ineffectiveness of chemoprevention approaches have limited the potent treatment of cancers. Therefore, potential new drugs are continuously under development and many of which are sourced from natural products such as plants. The historical application of plants as remedies for various diseases could be traced back to several decades before the synthetic era with majority of medicines are obtained from barks, roots and leaves (McChesney et al., 2007). A review by Hartwell

in 1982 listed over 3000 plant species which have been used for anticancer treatment although in several instances, the type of cancer is undefined (Cragg and Newman, 2005). An estimate by the World Health Organisation (WHO) showed over 80% of the populace in developing countries rely on traditional medicine for various health needs (Madhuri and Pandey, 2009). In fact, a survey showed that over 60% of cancer patients depended on herbs and vitamins (Unnati et al., 2013). Plants defend themselves from predators and also compete for space against other species through a mechanism that produces toxic metabolites such as tannin, tritepenoids, and alkaloids etc (Rocha et al., 2001) thus making plants interesting sources of novel bioactive compounds for drug development. Bioactive compounds of natural origin constitute over 50% of modern drugs, many of which possess anticancer activity (Unnati et al., 2013). The discovery of the vinca alkaloid family as anticancer agents in the 1950s and subsequent collection and isolation of compounds from plants in temperate regions by the National Cancer Institute (NCI) had led to the discovery of several natural compounds with anticancer activity. Vincristine and vinblastine (vinca alkaloids) isolated from Madagascar periwinkle. *Catharanthus roseus* was the first plant derived anticancer agents to be successfully used in clinical practice to treat different types of cancers such as leukemias, lymphomas, Hodgkins disease, lung and liver cancers (Noble, 1990). Etoposide a semisynthetic compound of epipodophyllotoxin isolated from *Podophyllum* species inhibits topoisomerase II resulting in DNA breaks and has been used in treating lymphomas with even more effective cure rate against testicular cancer when combined with bleomycin (Canel et al., 2000). Furthermore, the discovery of taxanes, paclitaxel and docetaxel with anticancer activity against breast, ovarian, small and non-small lung cancers exemplifies another success in natural product drug discovery. Paclitaxel exerts its

antitumor effect by stabilizing microtubules, resulting in mitotic arrest. Notwithstanding its impressive antitumor activity, taxanes could be associated with severe irreversible toxicity; although these effects are rarely life threatening (Newman et al., 2003).

In addition, there are also plant derived natural compounds undergoing clinical development. Flavopiridol is another interesting plant based anticancer agent currently entering clinical phase. Although a synthetic flavonone, flavopiridol was derived from an alkaloid rohitukine isolated from stems and leaves of *Amoora rohituka* and *Dysoxylum binectariferum.* It blocks the phosphorylation of cyclin dependent kinases (CDK) leading to cell cycle arrest at G_1 or G_2 and growth inhibition against breast and lung carcinoma cell lines (Kelland, 2000). A collaborative effort by the NCI and botanical research institute of South Africa lead to the collection of the plant, *Combretum caffrum* (Combretaceae) and subsequent isolation of ccombretastatins. Combretastatins is considered to be the most cytotoxic phytomolecule isolated thus far (Cirla and Mann, 2003). Combretastatins act as antiangiogenic agent by causing vascular short down and tumor necrosis.

Although these drugs have shown a great potential, however, toxicity and in most cases drug resistance due to the multidrug resistance gene (MDR) especially with regards to microtubule targeting drugs have limited the potency of such chemotherapeutic drugs. In fact, considering the significance in combating cancer, new crops of anti-microtubule drugs are under development and these are mostly synthetic derivatives of natural products such as the vinca alkaloids (Perez, 2009). Multiple drugs combination approach has been attracting interest amongst the

scientific community as an alternative to combat drug resistance and minimize the non-selective toxicity of single high-dose treatments. Indeed as shown by previous studies, natural products or even extracts from plants such as *Ficus spp* can be effectively combined to induce synergistic anticancer effects (Nugroho et al., 2013).

2.3.1 *Ficus spp*: ethnobothany and ethnomedicinal application

Moraceae also commonly known as the mulberry family with flowering plants comprising of forty (40) genus with over a thousand (1000) species, spans across tropical and subtropical regions and to a lesser extent in temperate climates. The genus contains great secondary metabolites thus exhibiting potential biological activities like anticancer, anti-inflammatory, antibacterial and respiratory disorders amongst others. *Ficus spp* possess genus of over 800 species of woody trees, shrubs and vines in the family of Moraceae, which are mostly found in the tropics. The height of the *Ficus* ranges from 30-40 m tall with a stout trunk up to 2 m in diameter. It has a broad oval leaves of 10-35 cm long and 5-15 cm broad and the flowers are pollinated by particular species of fig wasp in a co-evolved relationship. As a moderate sized tree with a height of almost 3m, *Ficus hispida* is native to Asia spanning across India, Sri Lanka, Myanmar, Southern region of China (Howlader and Dey, 2012). Its habitat mostly includes secondary forest river banks with an altitude of 1200m and open lands (Kunwar and Bussmann, 2006). *Ficus schwarzii* Koord is a small dioecious tree of approximately 8-10 m height. The plant often grows as clusters along streams found predominantly in Sarawak, Malaysia with a widespread distribution across Southeast Asia (Harrison, 2014). *Ficus spp* also commonly called figs, are found in Egypt at parks, streets, gardens with fruits from *F. carcia* and *F. sycomorus* have been favourably consumed (Abdel-Hameed, 2009).

Traditionally, the ethnomedicinal application of several members of *Ficus spp* as agents for treatment of various ailments and their consumption as food probably predates cereals, dating back to ten thousand years (Lansky et al., 2008; Shi et al., 2011). A historical perspective of potential activity and application of figs against cancer and related diseases showed that *Ficus carcia* and *Ficus sycomorus* were of most concern as parts including the tree barks, leaf twigs, young shoots, latex and fruits from different stages of ripening (dried or fresh) were used for treatment of tumor and inflammation related diseases (Lansky et al., 2008). Furthermore, fruit and latex of figs were used in combination with other ingredients such as blue flag, barley and fenugreek and administered orally against cancer ailments. Whereas, other parts of fig used for treatment of cancer, tumor and swelling were administered topically regardless of whether the ailment was internal or external. Additionally, three species (*F. benghalensis, F. racemosa and F. religiosa*) have been used in ayurvedic medicine for external treatment of diseases such as eczema, leprosy ulcers and pain (Khan and Balick, 2001). In contrast, there has been little medicinal application of figs in traditional Chinese medicine (TCM) as its fruits are often consumed as food and considered for improving appetite, whereas, its leaves are considered to be slightly poisonous (Lansky et al., 2008).

2.3.1.2 *Ficus spp*: phytochemistry and pharmacological activities

Phytochemical investigations have been conducted on various parts of *Ficus spp* and several studies have shown phenolics as the major phytochemical constituent of many *Ficus spp* (Li et al., 2006; Sandabe et al., 2006; Tuyen et al., 1998). Biological activity has been reported in roots, leaves and fruits of *Ficus hispidia*. These include

antidiarrhoeal (Mandal and Ashok, 2002), antidiabetic, cardioprotective (Shanmugarajan et al., 2008) and antinociceptive and neuropharmacological activities of ethanolic extracts (Fig. 2.2) (Howlader and Dey, 2012). Phytochemical studies have resulted in identification of over 100 compounds; most of which are triterpenoids, phenanthroindolizidine alkaloids, sitosterols amongst others. *Ficus hispida* and *Ficus septica* had substantial amount of phenanthroindolizidine alkaloids (Chiang et al., 2005; Damu et al., 2005; Peraza-Sánchez et al., 2002; Rubnov et al., 2001). Furthermore, cumarins and multiple flavonoids and triterpenoids were identified from roots, stems, leaves and latex of different *Ficus spp* (Chiang and Kuo, 2000, 2002; Li et al., 2006). Studies on biological activities have shown that both crude extracts and pure compounds of *Ficus spp* possess antioxidant and free radical scavenging activities (Abdel-Hameed, 2009; Rawat et al., 2012; Shi et al., 2011; Singh et al., 2011). A review on ethnomedicinal and pharmacognostic properties of *Ficus hispida* showed it possess cardiporotective, antidiarrheal, neuroprotective, hepatoprotective, anti-inflammatory and antineoplastic activities (Safwan et al., 2011). Similarly, species such as *F. fistulosa* has been shown to possess cytotoxic and antimalaria activities (Subramaniam et al., 2009; Zhang et al., 2002). However, to date there are no studies on the bioactivity of *F. schwarzii*.
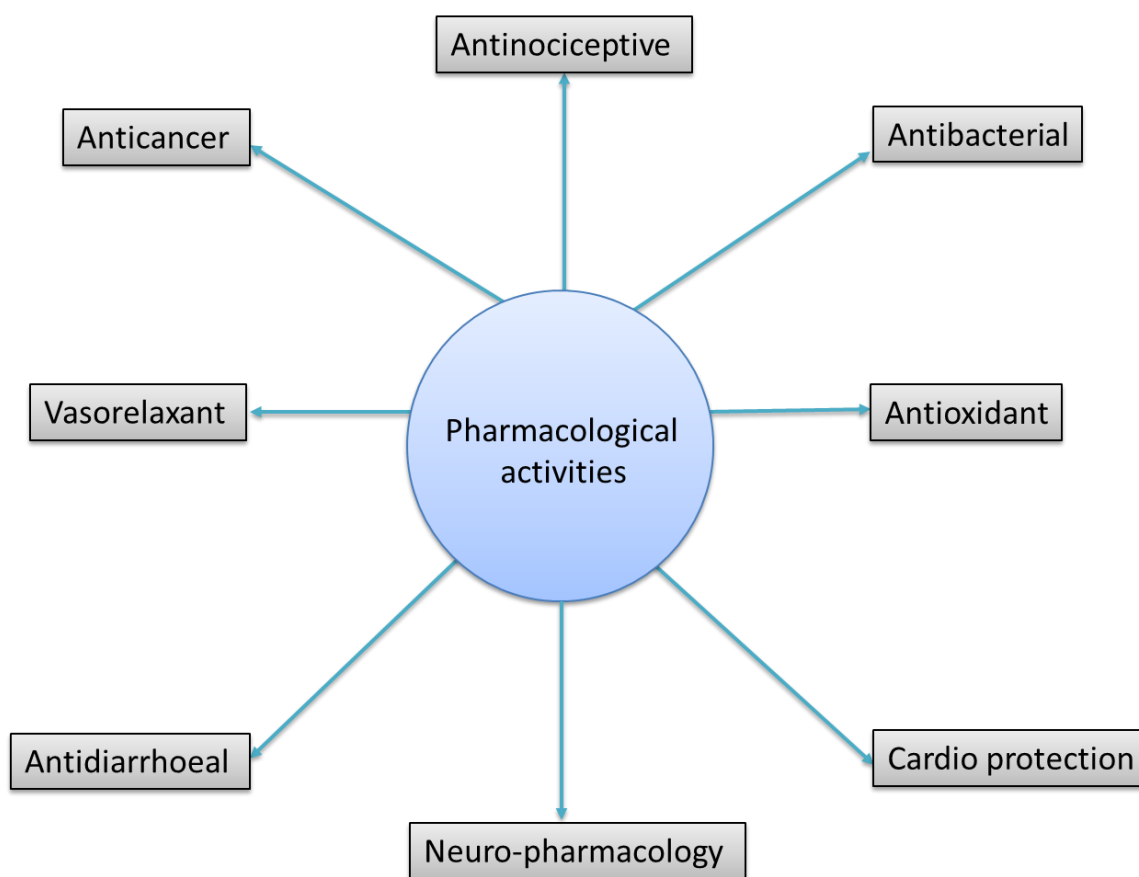
Fig. 2.2: Pharmacological activities of *Ficus* species.

Discovery of the anticancer properties of *Ficus spp* dates back to the 19th century when preliminary investigations by Ullman and colleagues in 1940 showed that latex extracts of *Ficus carcia* inhibited growth of sarcoma with subsequent tumor disappearance in albino rats (Lansky et al., 2008). Over the years, several *Ficus spp* have been extensively investigated on potential anticancer activity leading to isolation and subsequent structural elucidation of a series of compounds. A study investigating the potential anticancer activity of *Ficus hispida* showed that chloroform extract of its twigs and leaves had significant cytotoxicity against human lung (Lu1) and colon (Col2) cancers (Peraza-Sánchez et al., 2002). Furthermore, the fig fruit latex from *Ficus carcia* showed a potent cytotoxicity by inducing apoptosis, blocking DNA synthesis and cell cycle arrest in a range of human cancer cells but only also displayed

a mild effect on normal cells (Wang et al., 2008; Hashemi et al., 2011). Similarly, a mixture of 6-O-acyl-β-D-glucosyl-β-sitosterols isolated from *Ficus carcia* latex and soybeans showed potent cytotoxicity and in *vitro* growth inhibitory effects against burkitt B cell lymphoma, T-cell leukemia, prostate and mammary cancer cells (Rubnov et al., 2001).

In addition to single treatments, extracts and compounds from *Ficus spp* have also been shown to effectively combine with other chemotherapeutic drugs. For instance, a fraction of dichloromethane extract of *Ficus critifolia* combined effectively with vinblastine and daunomycin by enhancing the cytotoxicity of the former and increasing the intracellular concentration of the latter in human sarcoma (MESSA/Dx5) and human chronic myelogenous leukaemia (K562/R7) cells, respectively. These are models of cells that overexpress p-glycoprotein, a major cause of multidrug resistance, thus suggesting the therapeutic potential of *Ficus criticola* in cancer chemotherapy (Simon et al., 2001). In another combinational study, hexane insoluble fraction (HIF) of *Ficus septica* combined with doxorubicin exhibited higher growth inhibitory effect, cell cycle arrest and up regulation of cleaved PARP compared to single treatment in T74D breast cancer cells indicating possible future development of HIF as a co-chemotherapeutic drug (Nugroho et al., 2013).

Pharmacological studies have demonstrated the cytotoxic potency of *Ficus hispida* as demonstrated in the previous literature; however, there are no studies on *Ficus schwarzii* and a few studies on Vietnamese and Singaporean *Ficus fistulosa* yielded non-cytotoxic alkaloids with antifungal and antimalarial properties (Subramaniam et al., 2009; Zhang et al., 2002). More importantly, there is a potential of *Ficus spp* to

act as adjuvant for combined treatments thus further justifies the need to investigate the cytotoxic potency of the aforementioned species (*fistulosa, hispida* and *schwarzii*) individually and in combination with other pytochemical agents as potential future therapeutic agents.

2.3.2 *Tabernaemontana corymbosa*: ethnobotany and ethnomedicinal application

The genus *Tabernaemontana* also has vast medicinal application, exemplifying an excellent source of bioactive phytochemicals and thus has been of research interest for phytochemists especially from Asia. There are about 110 species of the genus *Tabernaemontana* of the Apocynaceae plant family native to tropical, subtropical regions and West African forest, with a widespread distribution across Asia, Africa and South America (Kuete et al., 2010; Lim et al., 2008; Low et al., 2010; Pratchayasakul et al., 2008). *Tabernaemontana* constitutes amongst the sixteen species of the sub family Plumerioideae which is known to be rich in structurally novel indole and bisindole alkaloids (Kam et al., 1999; Kam and Sim, 2003; Lim et al., 2008). Medicinal application of genus *Tabernaemontana* in Thai, Ayurvedic and Chinese traditional medicine includes treatment of ailments such as fever, pain dysentery etc (Van Beek et al., 1984). According to ethnobotanical data on the uses of 75 *Tabernaemontana* species, up to 78% was for medicinal purpose, whereas, 22% was for non-medicinal purpose. The species were mostly used to treat diseases, classified as antimicrobial, antiparasitic and analgesic in Africa, whereas, antimicrobial, central nervous system (CNS), febrifuge, antitumor, and analgesic were mostly treated in Americas, Asia/Australia and Pacific regions (Fig. 2.3).
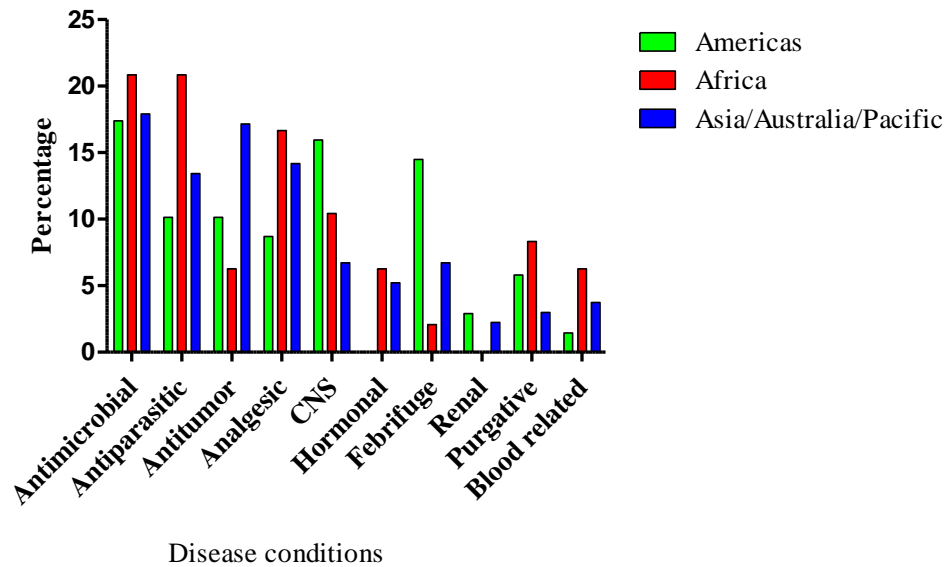
Fig. 2.3: Disease conditions treated by *Tabernomantana* species in traditional medicine at different regions (Van Beek et al., 1984).

One of the interesting species is the Malayan *Tabernaemontana corymbosa*. Its roots and bark are used in making arrow poison in Thailand while the latex is used by Malaysians to treat syphilitic ulceration (Table 2.1). Sap from the leaves is used to treat sores while pounded roots are used against orchitis (Van Beek et al., 1984).The leaves and bark are also used to treat fracture in Southern region of China (Wiart, 2006). In Bangladesh, the leaves are used to treat tumors, whereas, the roots are crushed and the extracted juice used to treat jaundice (Hasan et al., 2012; Rahmatullah et al., 2010).

Table 2.1: Summarized bioactivities, medicinal and non-medicinal uses of different parts of *Tabernaemontana corymbosa*.

Bioactivities			Medicinal Uses			Non-medicinal Uses	
Parts of Plant	**Phytochemical Constituents**	**Pharmacological Activities**	**Parts of Plant**	**Preparation Methods**	**Disease Conditions**	**Parts of Plant**	**Uses**
Leaf, stem bark, twigs and whole plant	Alkaloids	• Anticancer • Vasorelaxation	Leaf	Sap and poultice	• Tumor • Sore • Fractures • Postnatal recovery	Root	• Arrow Poison
Stem bark	Alkaloid crude extract	• Antimicrobial • Antifungal • Anticancer	Bark	Decoction, boiled juice and infusions	• Syphilis • Ulceration, fractures		
Leaf, seed, stem, flower and root	Methanol extract	• Antiviral • Antinematodal Antihelmintic • Analgesic	Root	Pounded, decoction and crushed root juice	• Syphilis • Fever • Jaundice • Postnatal recovery	Latex	• Birdlime
			Unspecified	Boiled juice and paste	• Fever • Orchitis		

2.3.2.1 Phytochemistry and pharmacological activities

As shown in Table 2.1, the medicinal history of *T. corymbosa* prompted pharmacological studies that reveal several bioactivities including antioxidant, antimalarial, vasorelaxant, antiviral, antinematodal, antihelmentic and cytotoxicity amongst others (Alen et al., 2000; Das and Chowdhury, 2015; Nge et al., 2014; Rizwana et al., 2010; Taher et al., 2011; Zulkefli et al., 2013). Over the past 20 years studies, phytochemistry of *T.corymbosa* has led to isolation and structural elucidation of novel structured compounds comprising mostly of indoles and bisindole alkaloids. Studies have also shown that the types of alkaloids produced by *T. corymbosa* depended on the time of collection and location of plants. For instance, *T. corymbosa* collected from two different locations in Malaysia produced several different alkaloids (Lim et al., 2015). As shown in Table 2.2, these alkaloids demonstrated interesting bioactivities most notably is cytotoxicity and the reversal of vincristine resistance in human drug resistant carcinoma (KB) cells. As demonstrated by previous studies, the aspidosperma type alkaloids jerantinine derivatives (Fig 2.4) are the most potent cytotoxic alkaloids isolated from *T. corymbosa* with growth inhibitory concentrations (IC_{50} <1 µg/ml) against drug sensitive and vincristine resistant KB cells (Lim et al., 2008). Although jerantinine derivatives exhibited the highest potency, thus far fewer studies have been conducted on the potential mechanism of action on jerantinine A and E derivatives only. Indeed, preliminary study on synthetic jerantinine E showed disruption of microtubule networks as possible mechanism of action (Frei et al., 2013). Further mechanistic study on jerantinine A revealed inhibition of tubulin polymerization, microtubule disruption and mitotic arrest as mechanism of action. However, similar toxic effects were also evidenced against non-cancerous cells treated with jerantinine A (Raja et al., 2014). Recent mechanistic

study further confirmed jerantinine B as a microtubule targeting agent (MTA) and unlike the an established MTA, vincristine, jerantinine B induced potent cytotoxic effects against variant vincristine resistant (VR-HCT 116) cells that express p-glycoprotein with over 300-fold resistance to vincristine (Qazzaz et al., 2016). This suggests an alternative mechanism of action to circumvent the efflux effects of the p-glycoprotein. To date, MTAs are under different stages of clinical trials although toxicity to non-cancerous cells and susceptibility to p-glycoprotein induced multidrug resistance limit the efficacy of these drugs. This has prompted the development of new crop of MTAs with improved potency, some of which are synthetic derivatives of the vinca alkaloids (Perez, 2009). The non-susceptibility of jerantinine to p-glycoprotein further justifies the need to understand the mechanism of action against broad range of cancer cells. Furthermore, the toxicity to non-cancerous cells as demonstrated in previous studies could be minimized via combinational treatments at low dosage and more efficient targeted-delivery systems.

Jerantinine A $R^1 = H_2$, $R^2 = H$, $\Delta^{14,15}$
Jerantinine C $R^1 = O$, $R^2 = H$, $\Delta^{14,15}$
Jerantinine E $R^1 = H_2$, $R^2 = H$

Jerantinine B $R^1 = H_2$, $R^2 = H$
Jerantinine D $R^1 = O$, $R^2 = H$

Jerantinine F
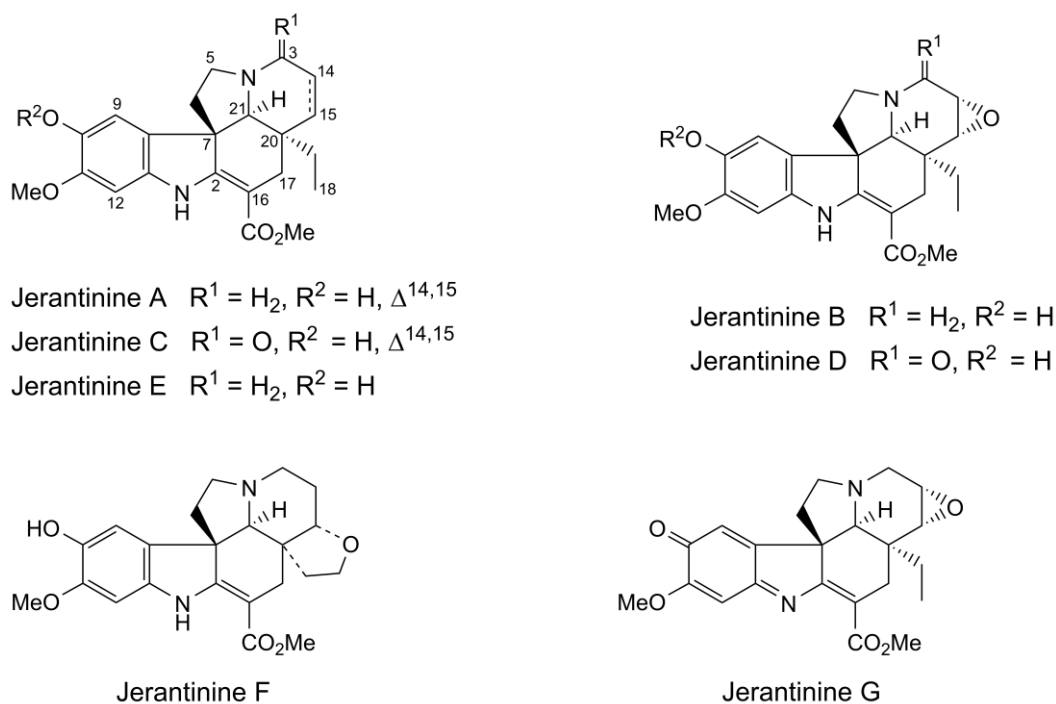
Jerantinine G

Fig. 2.4: Structure of jerantinines (A-G) (Lim et al., 2008)

Table 2.2: Cytotoxic alkaloids isolated from whole plant, leaf, stem bark and twig parts of *Tabernaemontana corymbosa.*

No.	Parts of Plant	Location of Collection	Types	Alkaloids	IC$_{50}$ (µM) on Cell Types							Ref.
					KB/S	KB/VJ300	KB/VJ300[ab]	Lung	Liver	Breast	Colon	
1	Leaf	Tekam Forest, Pahang Malaysia	Aspidosperma indole	Jerantinine A	1.99	1.73	-	-	-	-	0.43 (VR-HCT-116)	(Lim et al., 2008)
				Jerantinine B	1.11	0.95	-	-	-	-	0.49 (VR-HCT-116)	(Lim et al., 2008)
				Jerantinine C	0.81	1.54	-	-	-	-	-	
				Jerantinine D	0.68	0.95	-	-	-	-	-	
				Jerantinine E	2.55	2.03	-	-	-	-	-	
				Jerantinine F	12.80	12.30						
				Jerantinine A-acetate	1.04	0.83	-	-	-	-	-	
				Jerantinine B-acetate	0.70	0.75	-	-	-	-	-	
				10-*O*-methyl jerantinine A	12.0	13.6	-	-	-	-	-	
				10-*O*-methyl jerantinine B	7.36	10.70	-	-	-	-	-	
2	Stem bark	Tekam Forest, Pahang, Malaysia	Hexacyclic indole	Conolutinine	NA	NA	51.25[a]	-	-	-	-	(Lim et al., 2009)
3	Stem bark	Malaysia	Indole	Lirofolines A	NA	NA	10.45[a]	-	-	-	-	(Low et al., 2010)
				Lirofolines B	NA	NA	21.11[a]	-	-	-	-	
4	Whole plant	Yunnan province, China	Indole	Ervachinine E	-	-	-	11.22 (A549)	13.61 (SMMC-7721)	14.44 (MCF-7)	14.70 (SW480)	Guo et al., 2012)
5	Bark	Johor, Malaysia	Pentacyclic indole	Voatinggine	>80.00	-	-	-	-	-	-	(Nge et al., 2013)
				Tabertinggine	>58.00	-	30.94[a]	-	-	-	-	
6	Leaf	Yunnan province, China	Indole	Tabercarpamine A	-	-	-	-	3.31 (HepG2)	8.54 (MCF-	-	(Ma et al.,

No	Part	Location	Type	Compound								Reference
									6.76 (SMMC-7721)	7)		2014a)
7	Leaf	Yunnan province, China	Monoterpene indole	Bistabercarpamine A	-	-	-	-	38.14 (HepG2)	-	-	(Ma et al., 2014b)
8	Stem bark	Johor, Malaysia	Indole	Criofolinine	>60.00	>60.00	-	>60.00 (A549)	-	-	>60.00 (HCT-116)	(Nge et al., 2014)
				Vernavosine	>60.00	-	-	>60.00 (A549)			>60.00 (HCT-116)	
9	Stem bark	Tekam Forest, Pahang, Malaysia	Indole	Vincamajicine	>70.00	>70.00	2.62[a]	-	-	-	-	(K.-H. Lim et al., 2015)
				Cononusine	>70.00	>70.00	>70.00[a]	-	-	-	-	
				Ervaluteine	>70.00	>70.00	53.20[a]	-	-	-	-	
				Tacamonidine	>70.00	>70.00	>70.00[a]	-	-	-	-	
10	Leaf	Malaysia	Vobasine-iboga bisindoles	Conodiparine A	25.59	17.99	1.93[b]	-	-	-	-	(Kam et al., 1998)
				Conodiparine B	27.69	19.98	3.26[b]	-	-	-	-	
				Conodiparine C	28.58	22.69	7.48[b]	-	-	-	-	
				Conodiparine D	24.84	18.16	6.14[b]	-	-	-	-	
11	Whole plant	Yunnan province, China	Vobasinyl–ibogan bisindole	Ervachinine A	-	-	-	2.86 (A549)	3.35 (SMMC-7721)	3.20 (MCF-7)	2.39 (SW480)	Guo et al., 2012
				Ervachinine B	-	-	-	15.98 (A549)	11.40 (SMMC-7721)	15.17 (MCF-7)	15.33 (SW480)	
				Ervachinine C	-	-	-	0.84 (A549)	3.46 (SMMC-7721)	3.25 (MCF-7)	3.66 (SW480)	
				Ervachinine –D	-	-	-	3.10 (A549)	4.63 (SMMC-7721)	11.76 (MCF-7)	3.63 (SW480)	
12	Stem bark	Taiping, Perak, Malaysia	Indole Bisindole	Vobasidine A	15.48	>70.00	>70.00[a]	-	-	-	-	(Sim et al., 2014)
				Vobasidine B	53.67	>70.00	>70.00[a]	-	-	-	-	
				Vobasidine C	15.53	>70.00	26.54[a]	-	-	-	-	
				16-epivobasine	15.90	62.47	38.33[a]	-	-	-	-	
				Taipinisine	44.22	>70.00	18.57[a]	-	-	-	-	
				Tabernaemontanine	>70.00	>70.00	>70.00[a]	-	-	-	-	
				Dreagamine	>70.00	>70.00	>70.00[a]	-	-	-	-	
				Vobasine	>70.00	>70.00	>70.00[a]	-	-	-	-	
				Vobasenal	>70.00	>70.00	>70.00[a]	-	-	-	-	
				16-epivobasenal	14.48	>70.00	>70.00[a]	-	-	-	-	
13	Stem bark	Tekam Forest, Pahang, Malaysia	Vobasinyl-iboga isindole	Ervatensine A	1.39	1.21	0.98[a]	-	-	-	-	(K.-H. Lim et
				Ervatensine B	0.95	1.11	1.20[a]	-	-	-	-	

al., 2015)

14	Leaf and twig	Xishuangbanna, Yunnan, China	Vobasinyl-ibogan binsindole	Tabercorine A	-	-	-	4.14 (A549)	3.53 (SMMC-7721)	8.10 (MCF-7)	9.24 (SW480)	(Zhang et al., 2015)
				Tabercorine B	-	-	-	10.34 (A549)	13.31 (SMMC-7721)	4.70 (MCF-7)	16.93 (SW480)	
				Tabercorine C	-	-	-	33.16 (A549)	31.52 (SMMC-7721)	>40.00 (MCF-7)	>40.00 (SW480)	

Cell lines: KB/S = vincristine-sensitive human oral epidermoid; KB/VJ300 = vincristine-resistant human oral epidermoid; SMMC-7721 = human hepatocellular carcinoma; HepG2 = human hepatocellular liver carcinoma; HL-60 = human myeloid leukemia; A549 = human lung adenocarcinoma; MCF-7 = human breast adenocarcinoma; SW480 = human colon adenocarcinoma; HCT-116 = human colorectal carcinoma; VR-HCT-116 = vincristine-resistant human colorectal carcinoma. KB/VJ300: [a] = with addition of vincristine (0.12μM) as adjunct treatment; [b] = with addition of vincristine (0.30μM) as adjunct treatment. NA = no activity. (-) = not reported.
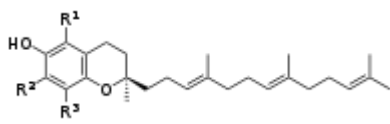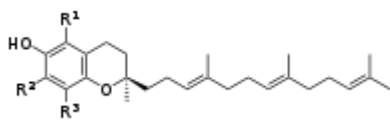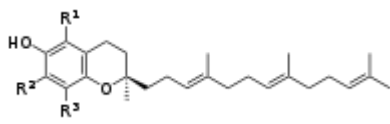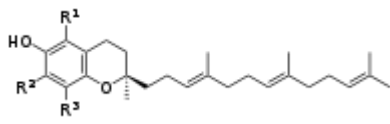
2.3.3 Vitamin E: Sources, structure and bioavailability

Vitamin E was first discovered in 1922 by Evans and Bishop as a necessary dietary factor for reproduction in rats. It is an essential fat soluble vitamin which contains natural sources of tocopherol and tocotrienol with an antioxidant property that has made it an ideal and popular supplement. Natural vitamin E has eight isomers known as alpha (α)-, beta (β)-, gamma (γ)- and delta (δ)-tocopherols and alpha-, beta-, gamma-, and delta-tocotrienols. Since the discovery of vitamin E, major research has focused on tocopherols representing an almost 99% research. However, there has been a dramatic change on the direction of vitamin E research, where research on bioactivity of tocotrienols has gained substantial recognition since the year 2000 representing a two third of available PubMed literature (Sen et al., 2007).

Both tocotrienols and tocopherols are synthesized as natural sources in plants. Tocopherols occur exclusively in plant leaves and seeds of most monocot plants. On the other hand, tocotrienols are present in seed endosperm of most monocots and few dicots such as tobacco. Cereals such as barley, wheat, and rice also contain a decent amount of tocotrienols. Palm oil represents an excellent source of tocotrienols with a significant vitamin E distribution of 70% tocotrienols compared to 30% for tocopherols (Sundram et al., 1989). Structurally, tocopherols and tocotrienols are similar yet only differentiated by the presence of double bond in the side chain (Bardhan et al., 2011). Tocopherols have a trimethyltridecyl tail with three chiral centres at carbons 2', 4' and 8' occurring naturally in the RRR configuration whereas tocotrienols have one chiral centre and three unsaturated bonds in the carbon side chain at positions 3', 7', 11' (Schneider, 2005). The unique structure of tocotrienols

with the presence of three double bonds (Table 2.3) gives it a better mobility and penetration through lipid membrane of various target organs (Wong and Radhakrishnan, 2012). The level of electron unsaturation in tocotrienols gives it a superior antioxidant activity and reversal of lipid peroxidation compared to tocopherols (Tan and Houston, 2005).

Table 2.3: Structure of tocotrienols.

Types	R1	R2	R3	STRUCTURE
Alpha-Tocotrienol	Me	Me	Me	
Beta-Tocotrienol	Me	H	Me	
Gamma-Tocotrienol	H	Me	Me	
Delta-Tocotrienol	H	H	Me	

Tocotrienols are characterised by unsaturated side chain: Sourced from (Zingg, 2007). Tocopherols are absorbed by the intestinal mucosa and incorporated into chylomicron particles where they are transported to the liver tissue via the lymphatic system. Alpha-tocopherol transport protein (alpha-TTP) mediates the transfer of alpha-tocopherol in the liver to a very low density lipoprotein (VLDL) where it is subsequently secreted into circulation into peripheral tissues. The preferential transportation of alpha-tocopherol is due to its high affinity for alpha-TTP. However, other isoforms of vitamin E, beta-, gamma- and delta-tocopherols have low affinity for alpha-TTP resulting in rapid metabolism in the liver, hence, only small portions

are found in blood and tissues and most of them are excreted in the faeces (Ju et al., 2010). In addition, due to its low affinity to transport protein, excess tocotrienols undergo β-oxidation and subsequent conversion to α,γ,δ-carboxyethylhydroxychroman metabolites which are excreted through the urine (Schneider, 2005). High concentration of tocotrienols would interfere with cellular reactions as studies have shown that the four tocotrienols exert biological activities at nanomolar concentration (Zingg, 2007). Although studies on transportation of vitamin E over the last two decades had focused on α-TTP which has an 8.5-fold lower binding affinity for alpha-tocotrienol compared to alpha-tocopherol, the extent of its effect on delivery of orally supplemented tocotrienol is yet to be determined. More recent animal and human studies have shown an increase delivery of oral tocotrienols independent of alpha-TTP indicating the possibility of other transport mechanisms (Khanna et al., 2005; Sylvester et al., 2010). For instance, animal studies showed that TTP-knockout mice suffered from alpha-tocopherol deficiency even in the presence of supplemented alpha-tocopherol (Brigelius-flohé, 2009). In contrast, oral supplementation of alpha-tocotrienol in TTP-knockout mouse restored fertility indicating possible alternative mechanisms of its delivery to tissues (Khanna et al., 2005).

2.3.3.1 Pharmacological activities of tocopherols and tocotrienols

Several vitamin E isomers have been shown to poses anticancer activities and initial studies showed that alpha-tocopherol was potent in suppressing carcinogen induced cancer in mice and especially when the dietary supplement was given in combination with selenium. However, inconsistent results were observed in epidemiological and subsequent *in vivo* and *in vitro* studies further confirmed that alpha-tocopherol has

little or no anticancer effect (Gould et al., 1991; Sylvester et al., 2005). The anticancer effects of vitamin E are mediated via induction of apoptosis which often involves activation of defective intracellular signal pathways and enzymes present in cancer cells. Despite having the strongest antioxidant activity, available literatures have shown that alpha-tocopherol is a weak pro-apoptotic inducer compared to other vitamin E isomers such as gamma-tocopherols and tocotrienols thus suggesting that apoptotic properties are not related to antioxidant properties (Constantinou et al., 2008 ; Sylvester et al., 2001). Alpha-tocopherol failed to induce any form of cell death in a range of colon cancer cell lines, whereas, a significant induction of apoptosis was observed when cells were treated with gamma-tocopherols (Campbell et al., 2006). In contrast, alpha-tocopherol blocked cell cycle at late G1 phase in smooth muscle at a concentration of 10-50μM (Constantinou et al., 2008).

On the other hand, gamma-tocopherol which is the most abundant form of vitamin E in American diet with low plasma and tissue concentration and a mean serum concentration of 5.74 μM (Ford et al., 2006) has more potent and effective pro-apoptotic activity than alpha-tocopherol (Campbell et al., 2003). A combination of alpha-tocopherol and gamma-tocopherol induced apoptosis only in androgen-sensitive LNCaP prostate cancer cells via the mitochondria pathway (Jiang et al., 2004). In addition, gamma tocopherol also inhibits cell cycle progression which was associated with down regulation of cyclin D1 and cyclin E (Gysin et al., 2002).

In contrast to tocopherols, studies have demonstrated that tocotrienols exhibit more effective and potent anticancer effects even at lower concentrations (Kline et al., 2004). Anticancer potential of tocotrienols was first discovered when studies

investigated the role of high dietary fat intake on the development of carcinogen induced mammary cancer in rats. Although all fat diets regardless of the formulation, stimulated tumor development, it was observed that palm oil diet suppressed tumor development indicating the anti-tumor potential of palm oil rich diet (Sundram et al., 1989). The potential role of tocotrienol diet against cancer was further confirmed when palm oil diet devoid of tocotrienol promoted mammary tumor in rats (Nesaretnam, 2008; Sylvester et al., 2010). *In vitro* studies have shown that tocotrienol rich fraction (TRF) obtained from palm oil (80% tocotrienols and 20% tocopherols) exhibited antiproliferative potencies and induced cell death in a dose dependent mammary tumor cells (Srivastava and Gupta, 2006). Further studies conducted to determine the most potent vitamin E isomers present in TRF by comparing the two subclasses of vitamin E showed that tocotrienols had significantly more antiproliferation and induced cell death at micro molar concentrations with minimal toxicity to normal cells compared to tocopherols (Constantinou et al., 2008).

The pathway and intracellular mechanisms for mediation of apoptosis by tocotrienol isomers differ depending on the types of cancer. For instance, gamma-tocotrienol induced apoptosis in neoplastic mammary epithelial cells by activating caspase 8 and 3 which was subsequently blocked when combined with specific caspase inhibitors strongly suggesting that apoptosis was associated with the death receptor signalling pathway (Shah et al., 2003). Further studies by Shah and Sylvester, (2005) confirmed the above suggestion by showing that treatment with cytotoxic doses of gamma-tocotrienol did not disrupt mitochondrial membrane potential or release of cytochrome c to the cytoplasm. In addition, there was an observed increase in the ratio of anti-apoptotic proteins compared to pro-apoptotic proteins; an indication of

enhanced mitochondrial membrane stability (Shah and Sylvester, 2005). In contrast, gamma-tocotrienols caused disruption of mitochondrial membrane in human breast cancer with concomitant release of cytochrome c to the cytoplasm in a mechanism independent of caspase 9 activation or altered BcL2 levels (Takahashi and Loo, 2004). Similarly, Agarwal et al., (2004) showed that increase in Bax/BcL2 ratio, release of cytochrome c and caspase 9 activation were associated with tocotrienol mitochondria stress mediated apoptosis in human colon carcinoma cells.

Unlike drugs that regulate or act on single target, tocotrienols induce apoptosis regulating signalling molecules which are vital to cancer progression. The transcriptional factor, nuclear factor-kappa B (NF-kB) and signal transducer and activator of transcription 3 (STAT3) also plays vital role in cancer progression as chemoresistance and radioresistance have been related to activated STAT3 in collaboration with other factors such as NF-kB, hypoxia inducible Factor (HIF)-1 and peroxisome proliferator-activated receptor. *In vitro* studies demonstrated that gamma-tocotrienol blocked STAT3 activation in multiple myeloma cells by inhibiting the activation of Src kinase, Janus Kinase (JAK)-1 and JAK-2 (Kannappan et al., 2010a). Similarly, combined gamma-tocotrienol and tyrosine inhibitors (erlotinib and gefitinib) inhibited growth of anchorage-independent cells with a reduction in the levels of phosphorylated PDK-1, AKT, STAT3 and STAT5 in murine mammary tumor cells (Bachawal et al., 2010). Furthermore, studies showed that gamma-tocotrienol induced apoptosis in neuroplasmic +SA mammary epithelial cells by suppression of receptor tyrosine kinase (ErbB3) tyrosine phosphorylation and down regulation of PI3K/PDK-1/AKT mitogenic signalling (Samant and Sylvester, 2006). Whilst studies have demonstrated the potency of tocotrienols, fewer studies have been

conducted to understand the mechanism of action of delta-, alpha- and beta-tocotrienol isomers compared to gamma-tocotrienols. Furthermore, even least studies have been conducted on lung and brain cancers (Lim et al., 2014a; Lim et al., 2014b). Despite the remarkable therapeutic potency, the potential clinical application of tocotrienols as anticancer agents has been limited by the need to use high dosage. High doses of tocotrienols overload the TPP resulting in metabolic degradation and subsequent reduction in available therapeutic doses (Shirode and Sylvester, 2010). Enhanced delivery mechanism using nano-particles and synergistic drug combinations with other chemotherapeutic agents are under investigation as alternative way to reduce dosage and improved potency of tocotrienols (Constantinou et al., 2008; Shirode and Sylvester, 2010)

2.4 Combined treatments using tocotrienols

Synergism is another area of anticancer research that has recently attracted more interest. The prospect of combining more than one compound with anticancer effects seems promising with the possibilities of anticancer activities at sub-effective doses, thus reducing the risk and adverse side effects commonly posed by other chemotherapeutic agents (Boik, 2001). Drug synergism has continuously been explored as potential alternative to circumventing limitations of tocotrienols and in the last 20 years, studies have been conducted investigating the synergistic effect of tocotrienols combined with other natural compounds against cancer cells. Indeed, some combined treatments demonstrated lower toxicity towards non-cancerous cells as well as dose reduction. For instance, combined treatment of sesamin with gamma-tocotrienol at sub-effective doses caused reduction of required potent doses and induced synergistic antiproliferative effects on neuroplastic mouse and human

mammary cancer cells. The combined treatments had little or no effects on normal cells. However, subsequent studies revealed the effects were rather cytostatic and sesamin did not prevent metabolic degradation of gamma-tocotrienol (Akl et al., 2013; Akl et al., 2012). In addition, the combined treatment of statin and gamma-tocotrienol caused reduction of required potent doses and induced potent cytotoxic effects on +SA mammary tumor cells. Toxicity has limited the clinical use of statin. Therefore, dose reduction of statin following combined treatments with gamma-tocotrienol suggested a potential future use of statin while avoiding the undesirable myotoxicity associated with statin chemotherapy (Wali and Sylvester, 2007; Wali et al., 2009). However, it is noteworthy to mention that the studies did not investigate the potential toxicity of the combined treatments towards non-cancerous cells.

On the other hand, in some studies although the combined treatment with tocotrienol induced potent cytotoxicity, the combinations were at high dosage and the potential toxicity towards non-cancerous cells was not determined. For instance, combined treatment of gamma-tocotrienol with atorvastatin and celecoxib synergistically inhibited growth of HT29 and HCT116 human colon cancer cells with induction of G0/G1 cell cycle arrest and apoptosis (Yang et al., 2010). Similarly, combined treatment of gamma-tocotrienol with statin (Tuerdi et al., 2013) and 6-gingerol (Yusof et al., 2015) induced potent cytotoxic effects on human malignant mesothelioma and human colorectal cancer cells, respectively. However, the combinations in these studies were conducted at high doses and hence did not address the problems of metabolic degradation associated with high-dose tocotrienols. Again, the potential toxicity of these high-dose combinations towards non-cancerous cells was not reported.

3.0 CHAPTER THREE

In-vitro anti-proliferative effects of individual and combined treatments of tocotrienols, jerantinines (A and B) and extracts of *Ficus* species

3.1 Introduction

Tocotrienols are a group of vitamin E isomers that have demonstrated potent cytotoxic effects against cancer cells *in vitro*. However as reviewed in literatures from Chapter Two, such potency has been limited especially in orally delivered tocotrienols due to high dosage associated toxicity and metabolic degradation which results in low availability of therapeutic dose. Thus, combined treatments have been suggested as a potential alternative to circumvent such limitations. Similarly, indole alkaloids jerantinines (A-G) isolated from *Tabernaemontana corymbosa* have been shown to possess potent cytotoxic effects with a potential of reversing vincristine drug resistance in cancer cells (Lim et al., 2008). However despite this remarkable potency, not many mechanistic studies have been conducted on jerantinines (A, B and E) (Frei et al., 2013; Qazzaz et al., 2016; Raja et al., 2014). In addition, the studies have also demonstrated that jerantinine derivatives induce toxicity to non-cancerous cells. As such it was imperative to further study the cytotoxic effects of jerantinines to elucidate the mechanism of action and also adopt a combinatory treatment approach that would reduce the dosage and potentially minimize toxicity to non-cancerous cells.

On the other hand, *Ficus* species have a well-documented history of use for anticancer treatment in traditional medicine (Lansky et al., 2008). The ethno-medicinal uses have been supported by pharmacological data that demonstrated the cytotoxic potency of *Ficus* species. In fact recent literatures have suggested that *Ficus* species can be used

as an adjunct treatment (Nugroho et al., 2013, 2012) thus, making suitable for combined treatments with tocotrienols that could potentially improve potency and minimize required dosage and toxicity to non-cancerous cell lines. Three different plant species were employed in the study, namely as *F. fistulosa*, *F. schwarzii* and *F. hispida* collected from different locations in Malaysia. *F. fistulosa* and *F. hispida* were selected based the respective medicinal and pharmacological history as indicated in different literatures. Whereas, *F. schwarzii* was chosen because thus far no pharmacological activity of this plant was conducted, hence the present study would provide new and valuable information. The leaf and bark of plants were extracted by ethanol to obtain the crude ethanol extract that was subsequently subjected to acid-base extraction for collection of alkaloid crude extract. The comparison between ethanol and alkaloid crude extracts would allow determining if the observed antiproliferative effects are attributed by the presence of alkaloids or non-alkaloids in the extracts. Three different cancer cell lines representing brain, lung and colon cancer were chosen. The brain (U87MG) cancer cells was selected because only a few cytotoxic studies have been conducted to date despite the poor survival rate in patients (Lim et al., 2014b; Siegel et al., 2016) whereas, both lung (A549) and colon (HT-29) cells were selected due to the higher incidences and the potential oral route of administration for chemotherapeutic agents, respectively. On the other hand, as a control measure normal lung fibroblast (MRC5) cells and commercial chemotherapeutic agent vinblastine were selected as negative and positive controls, respectively for comparison purpose.

The distinct characteristics in cancer cells are uncontrolled proliferation. Therefore, cell proliferation assay was employed as the first line investigation for cancer drug

discovery. For the determination of antiproliferative potency the neutral red uptake assay was adopted. The assay is based on the lysosomal capacity of viable cells to take up the neutral red dye hence it offers greater sensitivity with less interference compared to unstable reagents used in other tests such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In addition, a recent study demonstrated that vitamin E isomers reduced MTT in the absence of cells (Lim et al., 2015). For the combined treatments, a fixed sub-effective (IC_{20}) dose of plant extracts or jerantinines (A and B) with a dose range of tocotrienols was adopted to allow determination for possible reduction in the potent doses and subsequent synergistic effects via combination index and dose reduction index methods.

The specific objectives of this chapter are:

i) To determine the antiproliferative potency (IC_{50}) of tocotrienols (delta and gamma), jerantinines (A and B) and extracts of leaf and bark of *F. hispida*, *F. fistulosa* and *F. schwarzii* on U87MG, A549 and HT-29 cancer cells.

ii) To determine the required potent doses in combined treatments of the potent extracts of *Ficus* species, jerantinines (A and B) with the low-dose delta and gamma tocotrienols.

iii) To determine the type of pharmacological interaction (combination index) between the combined treatments as well as the fold reduction (dose reduction index) in the required potent doses.

3.2 Materials and Methods

3.2.1 Chemical reagent/kits and cell cultureware

The following reagents and kits listed in Table 3.1 were used to conduct the experiments.

Table 3.1: List of chemical reagents, kits and glassware used and the respective manufacturers.

S/no	Chemical reagents/kits/lab wares	Manufacturer
1	RPMI 1640, trypsin/EDTA, 0.5%-trypan blue stain solution, minimum essential medium (MEM) with Earle's salts, L-Glutamine and non-essential amino acids, phosphate buffer saline (D-PBS) (-) without Ca^{2+} and Mg^{2+}	Nacalai Tesque, Japan
2	Penicillin/streptomycin	GIBCO, USA
3	Fetal bovine serum	JR Scientific, USA
4	Neutral red dye, dimethyl sulfoxide (DMSO)	Sigma Aldrich, USA
5	Acetic Acid	Amresco, USA
6	Ethanol	Merck, USA
8	28% ammonia solution	Reindmann Schmide, Switzerland
9	Tataric acid, chloroform	Fisher Scientific, USA
10	Vitamin E isomers	Davos Life Science Ltd Pte, Singapore
11	96-well plates	SPL Life Sciences, Korea

Jerantinines (A and B) were isolated from leaf alkaloid crude extract of *Tabernaemontana corymbosa* as previously described (Lim et al., 2008) and supplied in kind by Prof. Toh-Seok Kam of University of Malaya.

3.2.2 Plant collection and extraction

The plants, namely *Ficus fistulosa* Reinw ex. *Blume, Ficus hispida* and *Ficus schwarzii* Koord used for anticancer studies were collected from various locations in

Malaysia and identified by Dr Yong Kien Thai (Institute of Biological Sciences, University of Malaya). The bark and leaves of *F. fistulosa, F. hispida, F. schwarzii* were collected, tagged and assigned specimen vouchers as UNMC 68 (KLU 47988), UNMC 77 (KLU 48172) and UNMC87 (KLU 48174), respectively (Table 3.2). The tagged plants were subsequently deposited at the herbarium of University of Malaya.

Table 3.2: Summary on scientific names, specimen, collection and extraction details of the collected plant species under this study.

Plant code	Plant name	Date of collection	Place of collection	Plant Part Used	Type of extract used
UNMC 68 (KLU 47988)	*Ficus fistulosa* Reinw ex. Blume	08/06/11	Near Semenyih Dam, Selangor	Bark, leaves	Ethanol, alkaloid crude
UNMC 77 (KLU 48172)	*Ficus hispida*	24/07/13	Jalan Broga, Semenyih, Selangor	Bark, leaves	Ethanol, alkaloid crude
UNMC 87 (KLU 48174)	*Ficus schwarzii* Koord	24/07/13	Jalan Gombak, Selangor	Bark, leaves	Ethanol, alkaloid crude

The leaves and bark of the plants under investigation were cut into smaller pieces and air dried. The crude ethanol extract of the plants was prepared by soaking in distilled ethanol for three days with continuous collection until all extracts were collected. The crude ethanol extract was subjected to evaporation until dryness using a rotary evaporator. To further obtain the alkaloid crude extracts, the ethanol extracts were acidified with 3% tartaric acid (in dH_2O), filtered and basified to pH 10 with 28% ammonia solution and chloroform was subsequently added to obtain an aqueous layer which was collected using a separating funnel. Distilled water was used to wash three times to remove excess ammonia and sodium anhydrase was added to dry up any water, and subsequently filtered. Thereafter, the mixtures were evaporated to dryness to obtain the alkaloid crude extracts and were stored at -20°C until use.

3.2.3 Cell lines and culture conditions

All cell lines were purchased from American Type culture collection (ATCC, USA). Human lung adenocarcinoma (A549), normal human lung fibroblast (MRC5) and human colorectal adenocarcinoma (HT-29) cells were cultured in RPMI 1640 with L-glutamine and non-essential amino acids supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin mix solution. Human brain glioblastoma (U87MG) cells were cultured in minimum essential medium (MEM) with Earle's salt, sodium pyruvate and supplemented with 1% penicillin/streptomycin mix solution and 10% FBS. Cells were grown in an incubator (Binder, Germany) with a humidified condition of 5% CO_2 at $37^{o}C$. Medium was changed at least 3 times a week and only viable cells with at least 95% confluency were seeded for neutral red up take assay for single and combined low dose treatments.

3.2.4 Experimental design

One gram of delta or gamma tocotrienol was dissolved in 40 ml DMSO to give a final stock of 25 mg/ml. A working concentration of 1mg/ml was prepared and thereafter subsequent dilutions in a range of 0.01 μg/ml to 24 μg/ml were prepared. Similarly, 1 mg of jerantinines (A and B) or vinblastine (control drug) was dissolved in 40 μl DMSO and 960 μl dH_2O for a stock concentration of 1 mg/ml. For *Ficus* species, 10 mg was dissolved in 1ml DMSO and a working concentration of 1 mg/ml was subsequently prepared. For single treatments of plant extracts, a dose range of 0.1 μg/ml – 120 μg/ml was prepared against cancer cell lines. On the other hand, a dose range of 0.1 μg/ml – 300 μg/ml and 0.1 μg/ml – 40 μg/ml for *Ficus* species and pure compounds (jerantinines A and B), respectively were tested against normal MRC5 cells.

For combined treatments, a sub-effective dose (IC_{20}) of jerantinines (A and B) and alkaloid crude extracts of leaf and bark *F. fistulosa* and *F. schwarzii* was combined with a dose range of 0.01 μg/ml to 24 μg/ml for tocotrienols (delta and gamma) to screen against normal and cancer cells. The potent concentration that resulted in 50% growth inhibition (IC_{50}) was subsequently determined.

3.2.5 Cell viability studies

For assessment of cell viability and subsequent determination of IC_{50}, the neutral red uptake cell viability assay was adopted and all reagents were prepared as previously described (Repetto et al., 2008). A total of 5 x 10^3 cells were seeded in a 96-well plate and incubated under cell culture conditions for 24 h to facilitate attachment. Thereafter, the cells were placed into different groups (treated and untreated) and the medium was replaced with 200 μl treatment medium without FBS for 72 h under cell culture conditions (treatment group). Whereas, plain medium containing an equivalent amount of DMSO was added to the untreated group (negative control). The neutral red medium was prepared 24 h prior to the the assay and incubated in cell culture condition. The neutral red medium was centrifuged at 1,800 rpm for 10 min to separate un-dissolved crystals prior to use. The treatment and plain media were discarded after 72 h and each well was rinsed with 150 μl PBS after which 100 μl of the neutral red medium was added and incubated for 2 h under cell culture conditions. Finally, after removal of the neural red medium the plates were washed with 150 μl PBS. De-stain solution in a volume of 150 μl was added to extract the absorbed neutral red dye and the absorbance was measured at 540nm after shaking for 10 min using the Varioskan multiplate reader (Thermo Scientific, USA)

3.2.6 Determination of synergism and dose reduction

For combined treatments, sub effective doses of IC_{20} of potent plant extracts and pure compounds (Jerantinines A and B) were combined with doses of gamma and delta tocotrienols and the combined low-dose treatments that induced 50% growth inhibition (IC_{50}) were subsequently determined using the GraphPad prism 5 software. The combination index (CI) and dose reduction index (DRI) were determined as described by Wali and Sylvester, (2007). Combination index (CI) was used to quantitatively measure the pharmacological interaction between combined treatments of tocotrienols with plant extracts or jerantinine (A and B). A CI value <1 indicates synergistic effect whereas values >1 or =1 represent antagonistic or additive pharmacological interaction, respectively between combined treatments. The combination index (CI) was quantified using the following formula:

$$CI = (Xc/X + Tc/T)$$

X represents the concentrations of plant extracts or jerantinines (A and B), whereas, T represents the concentration of delta or gamma tocotrienol that inhibited 50% cell growth. Similarly, Xc represents the IC_{20} doses of plant extracts or jerantinines (A and B), whilst, Tc represents the low doses of delta or gamma tocotrienol that induced 50% growth inhibition following combined treatments. Dose reduction Index (DRI) is a representation of fold decrease of combined treatments that resulted in 50% growth inhibition. It was calculated as DRI= (X/Xc or T/Tc) for plant extracts or jerantinines (A and B) and tocotrienols, respectively.

3.2.7 Statistical analysis

The IC_{50} for the tested compounds was determined by the non-linear regression curve fit using the GraphPad prism 5 software (GraphPad software Inc, USA). Each sample

was done in triplicates at three independent experiments and results were expressed as Mean ± SEM. One way ANOVA using the Dunnet T-test was used to compare the treated and untreated groups. P values <0.001 (***), P <0.01 (**) and P <0.5 (*) were considered statistically significant.

3.3. Results

3.3.1 Observation of dose dependent growth inhibition on cancer cells by single treatments

Results from acid-base extraction showed that *Ficus hispida* (leaf) had the highest crude alkaloid content compared to *F. fistulosa* and *F. schwarzii* (Table 3.3). Alkaloids were found with tending to accumulate more in leaves compared to bark although the bark of *F. fistulosa* had higher crude alkaloid content than the leaves. Following extraction, A549, U87MG, HT-29 and MRC5 cells were subjected to 72 h treatment with ethanol and alkaloid crude extracts of leaves and bark from *F. hispida, F. fistuosa and F. schwarzii*, the antiproliferative potencies and respective IC_{50} values were evaluated and are summarized in Table 3.4. Of all the plant extracts, only 4 extracts, 5 extracts, and 1 extract had potent antiproliferative effects with IC_{50} values below 20 µg/ml (bolded in Table 3.4) on U87MG, HT-29 and A549 cells, respectively. The strongest antiproliferative activity observed was for the alkaloid crude extracts of *F. fistulosa* leaf and bark (IC_{50}= 0.96 - 2.10 µg/ml) on HT-29 and U87MG cells (Table 3.4). Ethanol and alkaloid extracts of leaves from *F. hispida* (FHL ET and FHL ALK) inhibited proliferation of U87MG cells whereas only alkaloid extracts of the bark (FHB ALK) was toxic to HT-29 cells although a similar effect was observed on non-cancerous MRC5 cells (Table 3.4) (For histogram, see appendix 1a,b). Ethanol extracts of *F. schwarzii* bark (FSB ET) had growth inhibitory effects against A549 with $IC_{50} < 30$ µg/ml. Similar growth inhibitory effects were evident on HT29 cells treated with alkaloid extracts from bark and leaves of *F. schwarzii* (FSB ALK and FSL ALK) with IC_{50} values below 20 µg/ml. The antiproliferative potency of the plant extracts are ranked in a descending order as *Ficus fistulosa > Ficus schwarzii > Ficus hispida*.

Table 3.3: Yield of crude alkaloid extracts of *Ficus spp.*

s/no	Plant species	Plant part	Yield of alkaloid extract (g/kg)
1	*Ficus fistulosa* (FF)	Leaf (L)	0.28
		Bark (B)	0.37
2	*Ficus hispida* (FH)	Leaf (L)	0.56
		Bark (B)	022
3	*Ficus schwarzii* (FS)	Leaf (L)	0.22
		Bark (B)	0.07

Table 3.4: Antiproliferative effects (IC_{50}) of plant extracts of *Ficus* species tested on U87MG, A549, HT-29 and MRC5 cells (Mean ± SEM).

s/no	Plant	Part of plant	Type of extract	U87MG (µg/ml)	A549 (µg/ml)	HT-29 (µg/ml)	MRC 5 (µg/ml)
1	FF	B	ET	21.62±1.15	49.61±1.18	32.08±1.19	30.95±1.12
			ALK	**2.10±1.34**	44.35±1.18	**2.00±1.23**	18.90±1.36
2	FF	L	ET	66.02±1.21	46.95±1.08	27.73±1.11	29.32±1.14
			ALK	**0.96±1.12**	46.81±1.25	**1.92±1.30**	12.47±2.79
3	FH	B	ET	88.01±1.14	61.41±1.07	57.09±1.18	72.20±1.14
			ALK	31.24±1.26	52.98±1.08	**15.14±1.21**	19.94±1.16
4	FH	L	ET	**11.98±2.28**	40.82±1.22	43.90±1.21	18.39±1.31
			ALK	**17.49±1.25**	49.58±1.12	20.71±1.31	11.27±1.78
5	FS	B	ET	84.38±1.17	**19.21±1.16**	>120±1.24	61.60±1.17
			ALK	107.90±1.16	44.83±1.13	**14.49±1.20**	>120±1.14
6	FS	L	ET	35.31 ± 1.31	73.71±1.09	29.00±1.19	>120±2.29
			ALK	29.39 ± 1.25	>120±1.15	**8.79±1.20**	63.54±1.23

ET = ethanol extract, ALK = alkaloid crude extract, FF = *Ficus fistulosa*, FH = *Ficus hispida, FS = Ficus scharzii*, B = bark, L= leaf. Potent extracts with $IC_{50} < 20$ µg/ml are bolded.

In addition to the crude extracts, vitamin E isomers (delta and gamma tocotrienols) and jerantinines (A and B) were screened for antiproliferative activity. Following 72 h treatment, a dose dependent growth inhibition was evident and the IC_{50} values of jerantinines A and B against U87MG, HT-29 and A549 cells tested were below the 4 µg/ml (Fig. 3.1 c d, 3.2 c d, 3.3 c, d). However, similar toxicity of jerantinines (A and B) was evidenced on non-cancerous MRC5 cells too (Table 3.5). In contrast, although

tocotrienols demonstrated dose dependent growth inhibition (Fig. 3.1 a b, 3.2 a b, 3.3 a b), only gamma and delta tocotrienols had IC_{50} < 4 μg/ml against U87MG cells compared to the higher IC_{50} values of gamma and delta tocotrienols against A549 and HT-29 cells (Table 3.5). Furthermore, delta and gamma tocotrienols demonstrated good selectivity window in exhibiting lower toxicity towards normal lung fibroblast cells (MRC5) compared to U87MG and HT-29 cells. However, delta and gamma tocotrienols induced similar toxic effects on A549 and MRC5 cells demonstrating poor selectivity window. Control drug vinblastine demonstrated the highest toxicity towards both normal and cancer cells. In term of antiproliferative potency based on this study delta tocotrienol was better than gamma tocotrienol.

Table 3.5: Antiproliferative effects (IC_{50}) of delta and gamma tocotrienols, jerantinine (A and B) and vinblastine (Mean ± SEM) on U87MG, A549, HT-29 and MRC5 cells.

s/no	Compound	U87MG	A549	HT-29	MRC 5
		IC_{50} (μg/ml) (Mean ± SEM)			
1	Delta Tocotrienol (δ-T3)	**3.12 ± 1.26**	12.40 ± 1.09	5.71 ±1.23	15.71 ± 1.19
2	Gamma Tocotrienol (γ-T3)	**3.17 ± 1.29**	16.36 ± 1.07	6.14 ± 1.15	14.20 ± 0.08
3	Jerantinine A (JA)	**0.62 ± 1.19**	**1.42 ± 1.31**	**1.74 ± 1.24**	0.97 ± 0.11
4	Jerantinine B (JB)	**0.74 ± 0.04**	**1.48 ± 1.14**	**0.58 ± 0.18**	2.83 ± 0.38
5	Vinblastine	**0.04 ± 0.14**	**0.03 ± 0.16**	**0.71 ± 0.12**	0.26 ± 0.47

IC_{50} values of tocotrienols (delta and gamma), Jerantinines (A and B) and vinblastine below 4 μg/ml on cancer cells are bolded.
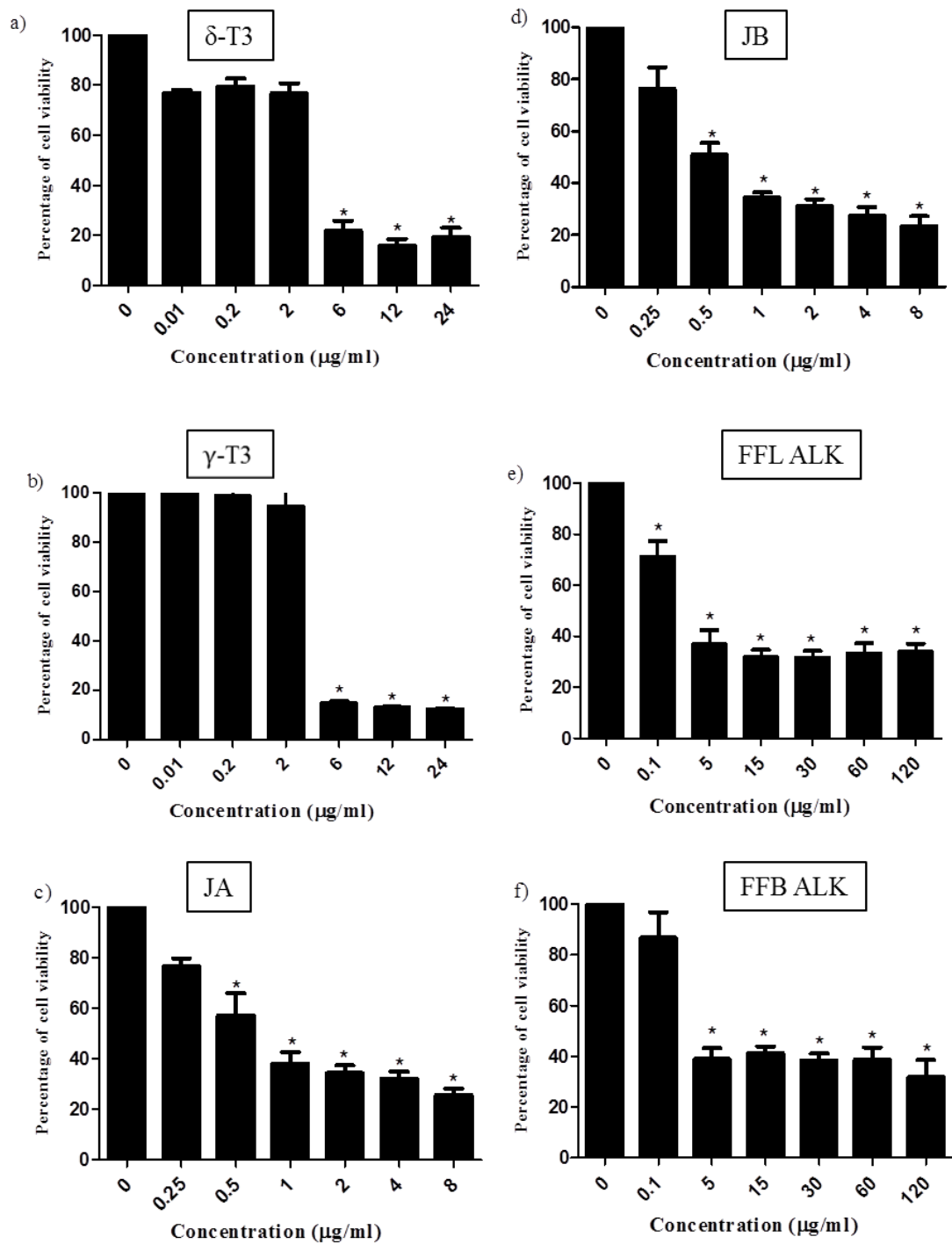
Fig 3.1: Antiproliferative profiles of selected individual treatments with potent dose < 20 µg/ml on U87MG brain cancer cells. a) Delta tocotrienol, b) gamma tocotrienol, c) jerantinine A, d) jerantinine B, e) *F. fistulosa* Leaf (ALK) and f) *F. fistulosa* Bark (ALK). U87MG cells were exposed to treatments for 72 h and the cell viability was determined using the neutral red uptake assay as described in methods. Results are expressed in Mean ± SEM. N=9 *P<0.001.
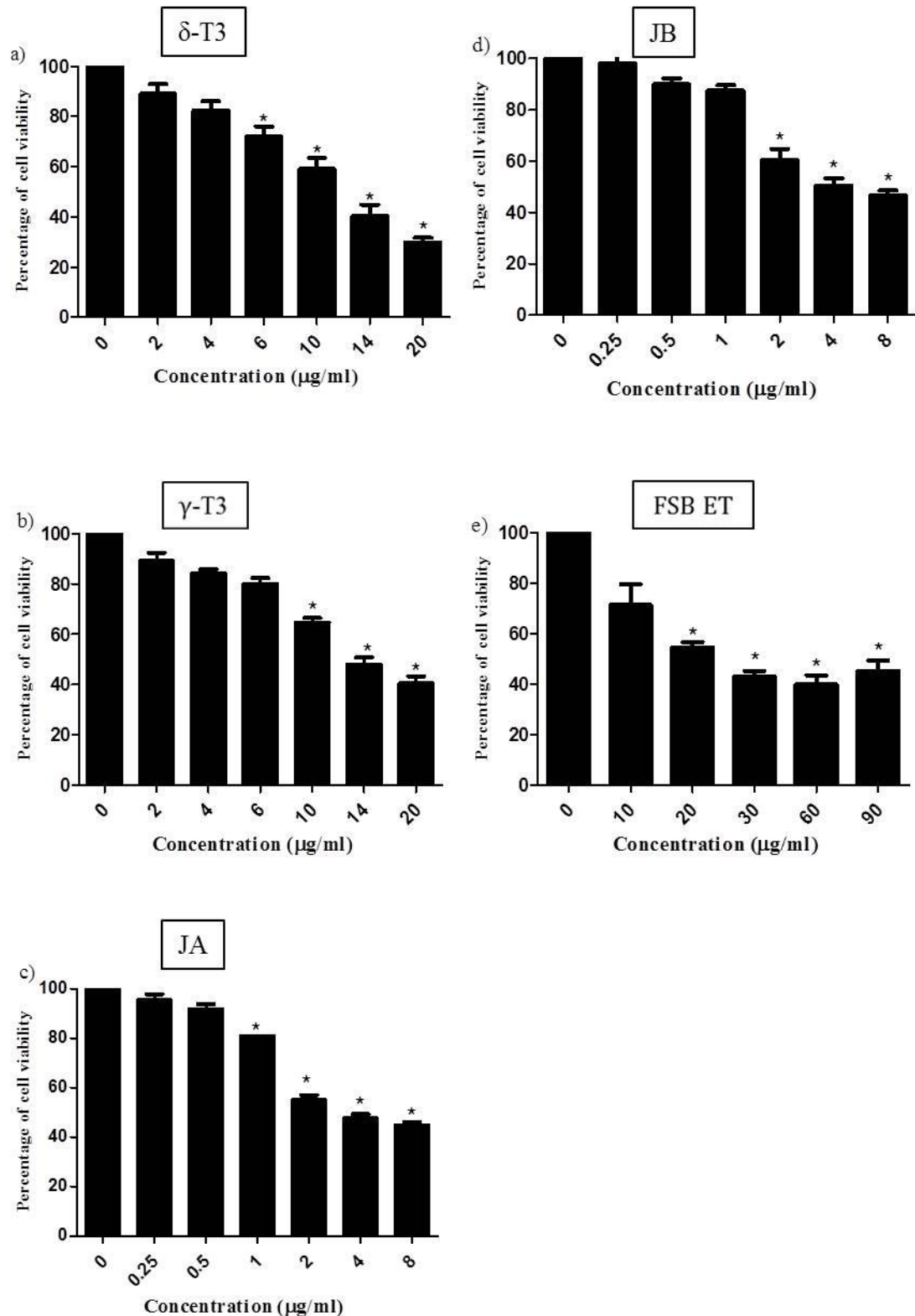
Fig 3.2: Antiproliferative profiles of selected individual treatments with potent dose < 20 µg/ml on A549 lung cancer cells a) Delta tocotrienol, b) gamma tocotrienol, c) jerantinine A, d) jerantinine B, e) *F. schwarzii* bark (ET). A549 cells were exposed to treatments for 72 h and the cell viability was determined using the neutral red uptake assay as described in methods. Results are expressed in Mean ± SEM. N=9 *P<0.001.
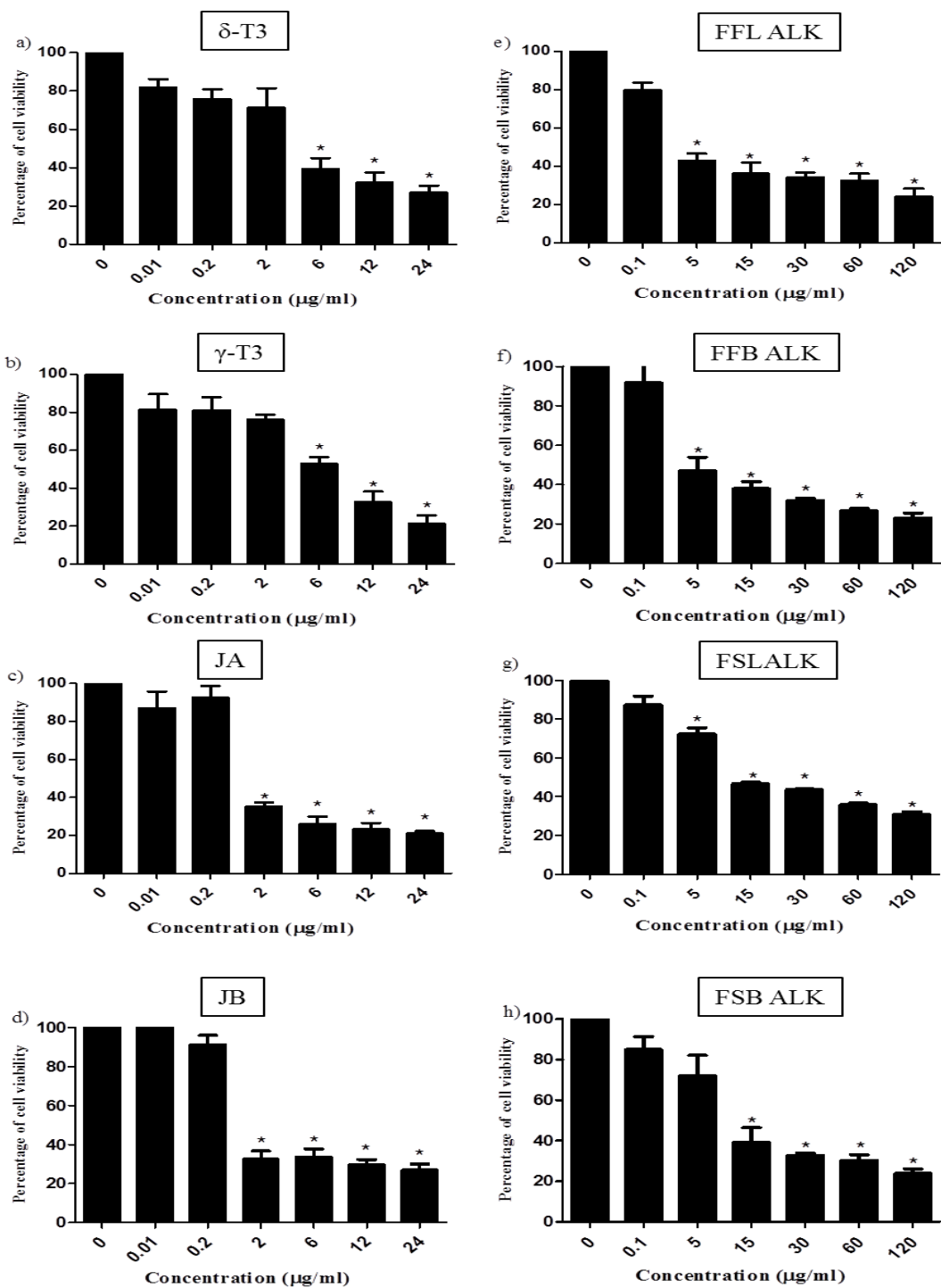
Fig 3.3: Antiproliferative profiles of selected individual treatments with potent dose < 20 µg/ml on HT-29 colon cancer cells. a) Delta tocotrienols, b) gamma tocotrienols, c) jerantinine A, d) jerantinine B, e) *F. fistulosa* leaf (ALK), f) *F. fistulosa* bark (ALK), g) *F. schwarzii* leaf (ALK), h) *F. schwarzii* bark (ALK). HT-29 cells were exposed to treatments for 72 h and the cell viability was determined using the neutral red uptake assay as described in methods. Results are expressed in Mean ± SEM. N=9 *P<0.001.

3.3.2 Induction of synergistic growth inhibition on cancer cells by combined treatments

Guided by antiproliferative results, the sub-effective dose (IC_{20}) of potent *Ficus* extracts and jerantinines A and B ($1C_{50} < 20$ µg/ml and < 4 µg/ml, respectively) were combined with a dose range of delta and gamma tocotrienols. This was aimed at improving the potency and efficacy of gamma and delta tocotrienols and on the other hand minimizes the toxicity to normal cells. The combined treatments of delta and gamma tocotrienols with alkaloid crude extracts of *F. fistulosa* leaf (FFL ALK) and bark (FFB ALK) induced strong synergistic antiproliferative effect on U87MG cells (CI = 0.28 - 0.29) (Fig. 3.4 c, g and 3.5 c, g). The combinatory approach caused up to 4-fold and 34.7-fold reduction in required potent doses for extracts and tocotrienols, respectively thereby minimizing toxicity to MRC5 cells (Table 3.6) (appendix 1b). Furthermore, a moderate synergistic antiproliferative effect was evident for combined treatment of delta or gamma tocotrienol with alkaloid extracts of leaf (FFL ALK) and bark (FFB ALK) of *F. fistulosa* against HT-29 cells (Table 3.8). However, a poor selectivity window with toxicity imposed towards MRC5 cells was also evident. In contrast, a weak synergistic effect and antagonistic effect were observed when alkaloid crude extracts of bark (FSB ALK) and leaf (FSL ALK) of *F. schwarzii* were combined with gamma tocotrienol against HT-29 cells, respectively (Fig. 3.8 k, i and 3.9 k, i). Similarly, as shown in Table 3.8 and isobologram (Fig. 3.9 e, f), the combined treatment delta tocotrienol with alkaloid crude extracts from leaf (FSL ALK) and bark (FSB ALK) of *F. schwarzii* induced an antagonistic effect on HT-29 cells.

Isobologram analysis revealed that the combined treatment of delta tocotrienol with IC_{20} dose of jerantinine A induced an antagonistic effect on U87MG cells (Fig 3.5). In

contrast, a dose dependent synergistic antiproliferative effect was evident on U87MG cells receiving combined treatment of delta tocotrienol with IC_{20} dose of jerantinine B and the combined treatment of gamma tocotrienol with IC_{20} dose of jerantinines A and B (Fig 3.4, 3.5). This caused up to 2.5-fold and 4-fold reductions in potent doses of tocotrienols (delta and gamma) and jerantinines (A and B), respectively (Table 3.6). On the other hand, the combination of jerantinine A or B with delta or gamma tocotrienol induced synergistic antiproliferative effect against A549 cells that resulted to almost 5-fold decrease in tocotrienol doses (Fig. 3.6, 3.7). However, a poor selectivity window between A549 and MRC5 cells was also evident in addition to combined IC_{50} values above 4 µg/ml (Table 3.7). Nonetheless, on a more positive note combined treatment of Jerantinine A with delta tocotrienol against A549 cells demonstrated an improved selectivity window compared to single delta tocotrienol dose with IC_{50} below 4 µg/ml (Table 3.7). Similarly, as shown in Table 3.8, the combined treatments of low-dose tocotrienols (delta and gamma) with jerantinines (A and B) induced dose dependent synergistic antiproliferative effect on HT-29 cells (Fig. 3.8, 3.9). This caused up to 2.7- fold and 4-fold reductions for jerantinines (A and B) and tocotrienols (delta and gamma), respectively (Table 3.8).

Table 3.6 Combinational index (CI) and dose reduction index (DRI) values for combined treatments of tocotrienols (delta and gamma) with *F. fistulosa* extracts and jerantinines (A and B) on U87MG and MRC5 cells.

s/no	Treatments	Combined Low-Dose Treatments (µg/ml)		Combination Index (CI)	Dose Reduction Index (DRI)		Low-Dose Tocotrienol on MRC5 Cells
			U87MG				
		Low-dose T3	IC_{20} Dose of J/Ficus extract		T3	J/Ficus extract	
				δ-T3			
1	JA	2.52±1.19	0.16±0.29	1.10	1.24	3.88	13.71±1.12
2	JB	1.83±1.11	0.19±0.27	0.85	1.70	3.89	6.77±1.67
3	FFL ALK	0.11±0.22	0.24±0.28	0.29	28.36	4.00	2.23±1.44
4	FFB ALK	0.09±0.24	0.53±0.34	0.28	34.67	3.96	4.68±1.17
				γ-T3			
5	JA	1.29±1.26	0.16±0.29	0.67	2.46	3.88	6.55±1.13
6	JB	1.61±1.38	0.19±0.27	0.77	1.99	3.89	7.70±1.13
7	FFL ALK	0.11±0.19	0.24±0.28	0.29	28.82	4.00	1.44±1.48
8	FFB ALK	0.12±0.20	0.53±0.34	0.29	26.42	3.96	3.07±1.12

J = Jerantinine, JA = Jerantinine A, JB = Jerantinine B, δ-T3 = delta tocotrienol, γ-T3 = gamma tocotrienol, T3 = Tocotrienol, FFL = *Ficus fistulosa* leaf, FFB = *Ficus fistulosa* bark, ALK = alkaloid extract. CI values show < 1 (synergism), > 1 (antagonism) and = 1 (additive) pharmacological interactions between combined treatments. Whereas, DRI represents the fold decrease in potent dose required to induce 50% growth inhibition following the combined treatments.

Table 3.7 Combinational index (CI) and dose reduction index (DRI) values for combined treatments of tocotrienols (delta and gamma) with jerantinines (A and B) on A549 and MRC5 cells.

s/no	Treatments	Combined Low-Dose Treatments (µg/ml)		Combination Index (CI)	Dose Reduction Index (DRI)		Low-Dose Tocotrienol on MRC5 Cells
			A549				
		Low-dose T3	IC_{20} Dose of J/Ficus extract		T3	J	
				δ-T3			
1	JA	2.77±1.09	0.35±0.32	0.47	4.48	4.06	5.57±1.22
2	JB	3.89±1.11	0.37±0.29	0.56	3.19	4.00	4.14±1.19
				γ-T3			
5	JA	4.95±1.07	0.35±0.32	0.55	3.31	4.06	5.90±1.11
6	JB	5.27±1.13	0.37±0.29	0.57	3.10	4.00	5.80±1.18

J = Jerantinine, JA = Jerantinine A, JB = Jerantinine B, δ-T3 = delta tocotrienol, γ-T3 = gamma tocotrienol, T3 = Tocotrienol. CI values show < 1 (synergism), > 1 (antagonism) and = 1 (additive) pharmacological interactions between combined treatments. Whereas, DRI represents the fold decrease in potent dose required to induce 50% growth inhibition following the combined treatments.

Table 3.8 Combinational index (CI) and dose reduction index (DRI) values for combined treatments of tocotrienols (delta and gamma) with *F. fistulosa*, *F. schwarzii* extracts and jerantinines (A and B) on HT-29 and MRC5 cells.

s/no	Treatments	Combined Low-Dose Treatments (μg/ml)		Combination Index (CI)	Dose Reduction Index (DRI)		Low-Dose Tocotrienol on MRC5 cells
		Low-dose T3	*IC$_{20}$ Dose of J/Ficus extract*		*T3*	*J/Ficus extract*	
				HT-29			
				δ-T3			
1	JA	2.12±1.49	0.44±0.28	0.62	2.69	3.95	4.68±1.29
2	JB	2.99±1.18	0.15±0.05	0.77	1.91	3.87	9.99±1.53
3	FFL ALK	2.10±0.16	0.50±0.33	0.63	2.72	3.84	0.92±0.22
4	FFB ALK	2.62±0.12	0.50±0.31	0.71	2.17	4.00	4.40±1.41
5	FSL ALK	5.43±1.29	2.20±0.31	1.20	1.05	4.00	4.32±1.17
6	FSB ALK	4.42±1.26	3.60±0.30	1.02	1.29	4.02	5.66±1.14
				γ-T3			
7	JA	3.70±1.59	0.44±0.28	0.60	1.66	3.95	4.50±1.15
8	JB	4.37±1.66	0.15±0.05	0.96	1.41	3.87	8.50±1.15
9	FFL ALK	2.45±0.18	0.50±0.33	0.66	2.51	3.84	0.72±0.26
10	FFB ALK	2.67±0.19	0.50±0.31	0.68	2.30	4.00	4.82±1.32
11	FSL ALK	8.29±1.20	2.20±0.31	1.60	-	4.00	4.67±1.26
12	FSB ALK	4.48±1.31	3.60±0.30	0.98	1.37	4.02	4.31±1.28

J = Jerantinine, JA = Jerantinine A, JB = Jerantinine B, δ-T3 = delta tocotrienol, γ-T3 = gamma tocotrienol, T3 = Tocotrienol, FFL = *Ficus fistulosa* leaf, FFB = *Ficus fistulosa* bark, FSL = *Ficus schwarzii* leaf, FSB = *Ficus schwarzii* bark, ALK = alkaloid extract. CI values show < 1 (synergism), > 1 (antagonism) and = 1 (additive) pharmacological interactions between combined treatments. Whereas, DRI represents the fold decrease in potent dose required to induce 50% growth inhibition following the combined treatments.
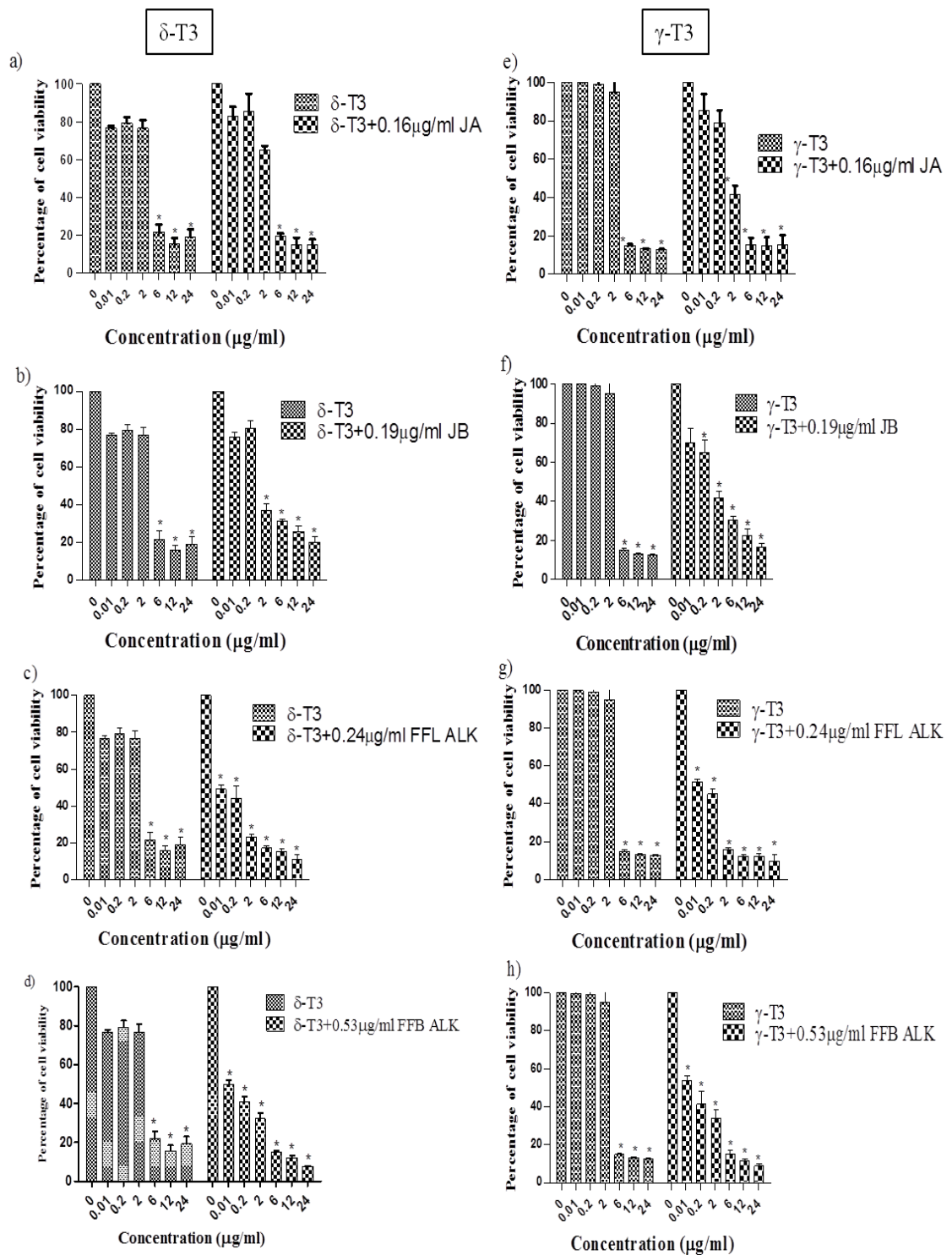
Fig 3.4: Antiproliferative profiles of combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of *F. fistulosa* extracts and jerantinines A and B on U87MG cells. U87MG cells were exposed to treatments for 72 h and the cell viability was determined using the neutral red uptake assay as described in methods. Results are presented as Mean ± SEM. N=9 *P<0.001. NB: JA= jerantinine A, JB= jerantinine B, FF (B), (L) = *F. fistulosa* (bark), (Leaf), ALK= alkaloid crude extract.
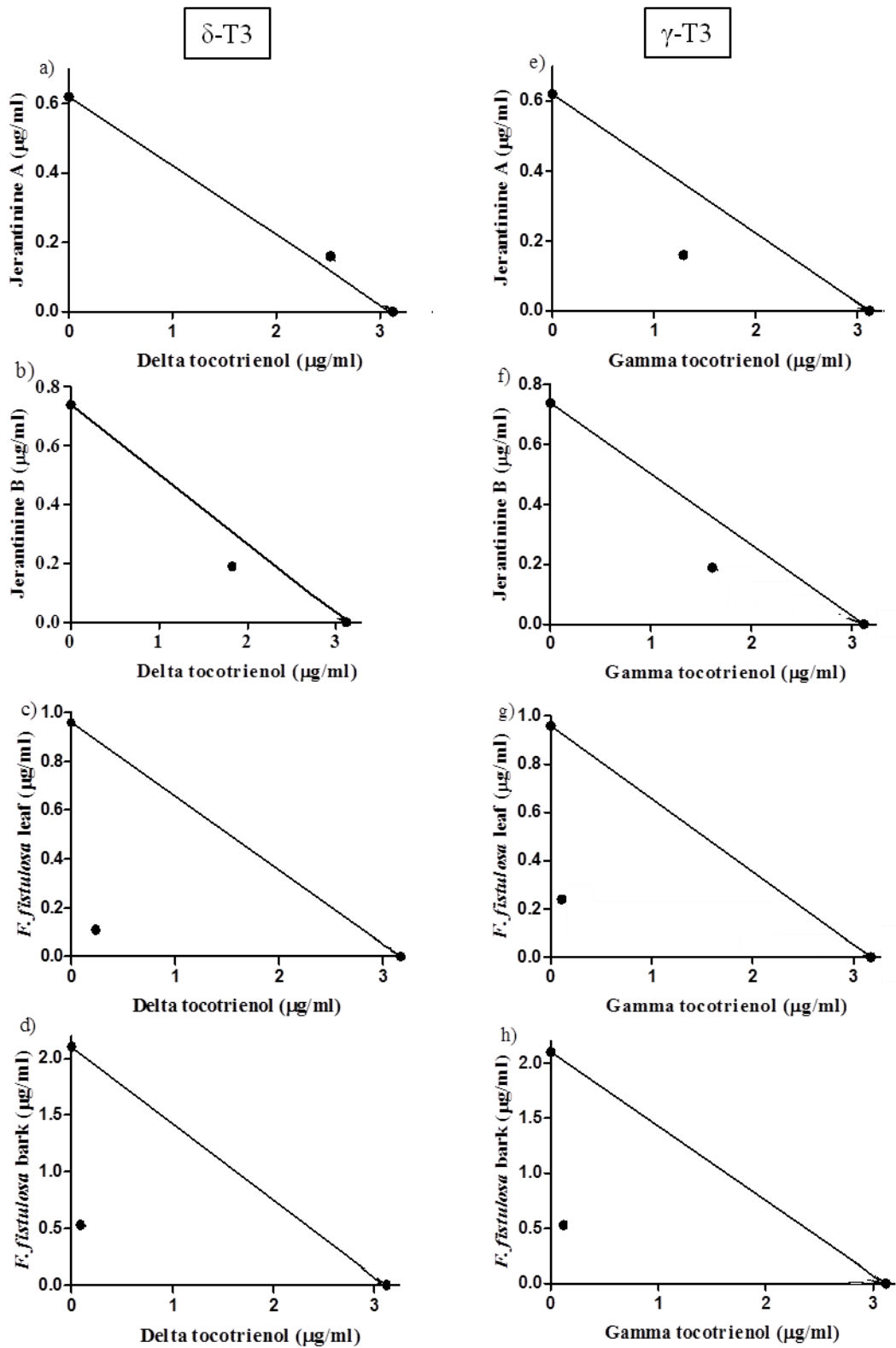
Fig 3.5: Isobologram profiles of U87MG cells receiving combined low-dose of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) or alkaloid extracts form leaves and bark of *F. fistulosa*. Data point below, above and on top of the diagonal line indicated synergism, antagonism and additive pharmacological interactions between combined treatments, respectively. All combined treatments except for combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine A induced synergistic effect on U87MG cells.
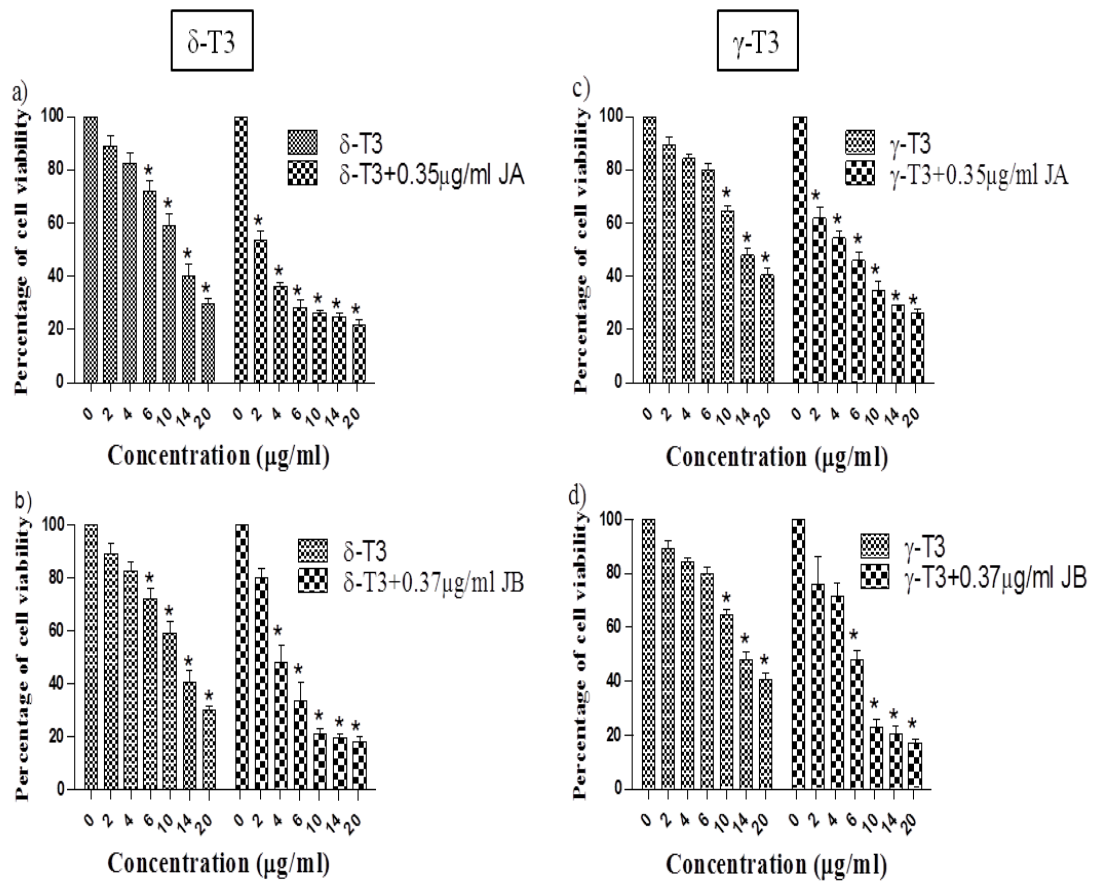
Fig 3.6: Antiproliferative profiles of combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) on A549 cells. A549 cells were exposed to treatments for 72 h and the cell viability was determined using the neutral red uptake assay as described in methods. Results are presented as Mean ± SEM. N=9 *P<0.001. NB: JA= jerantinine A, JB= jerantinine B. Individual and combined low-dose treatments induced dose dependent growth inhibition.
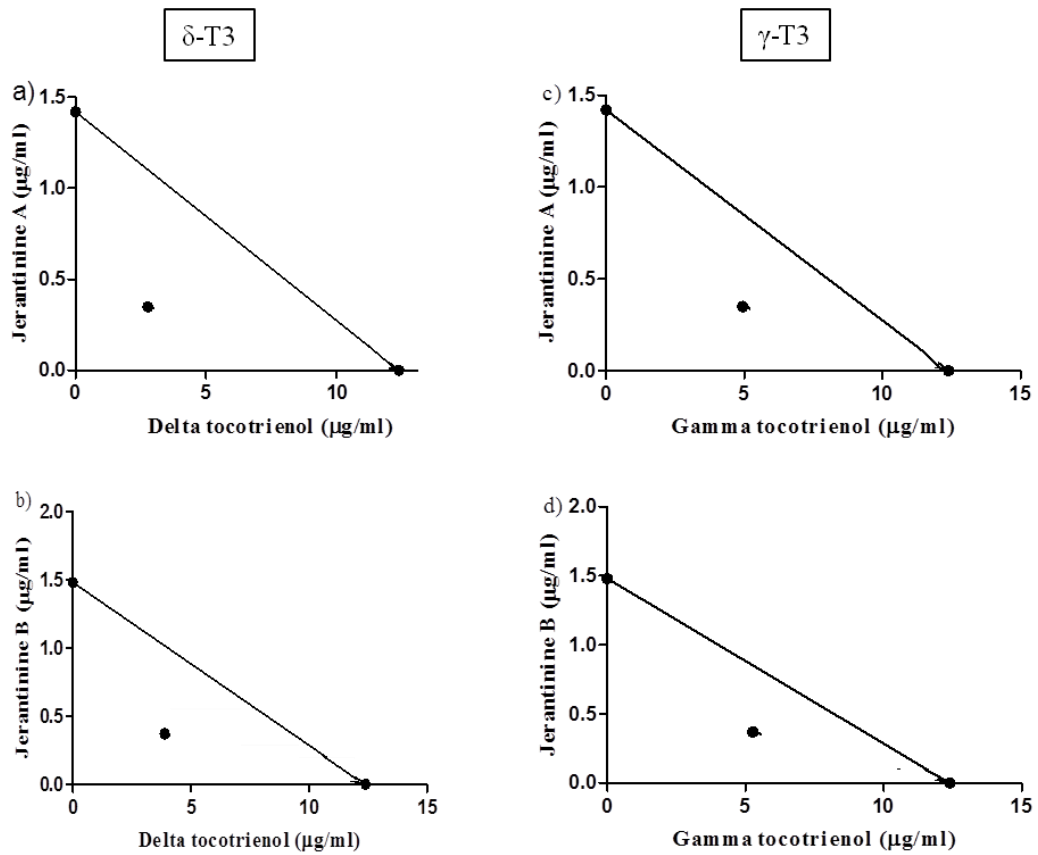
Fig 3.7: Isobologram profiles of A549 cells receiving combined low-dose of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B). Data point below, above and on top of the diagonal line indicate synergism, antagonism and additive pharmacological interactions between combined treatments, respectively. Combined treatment of low-dose tocotrienol (delta and gamma) with IC_{20} dose of jerantinines A and B induced synergistic effect on A549 cells.
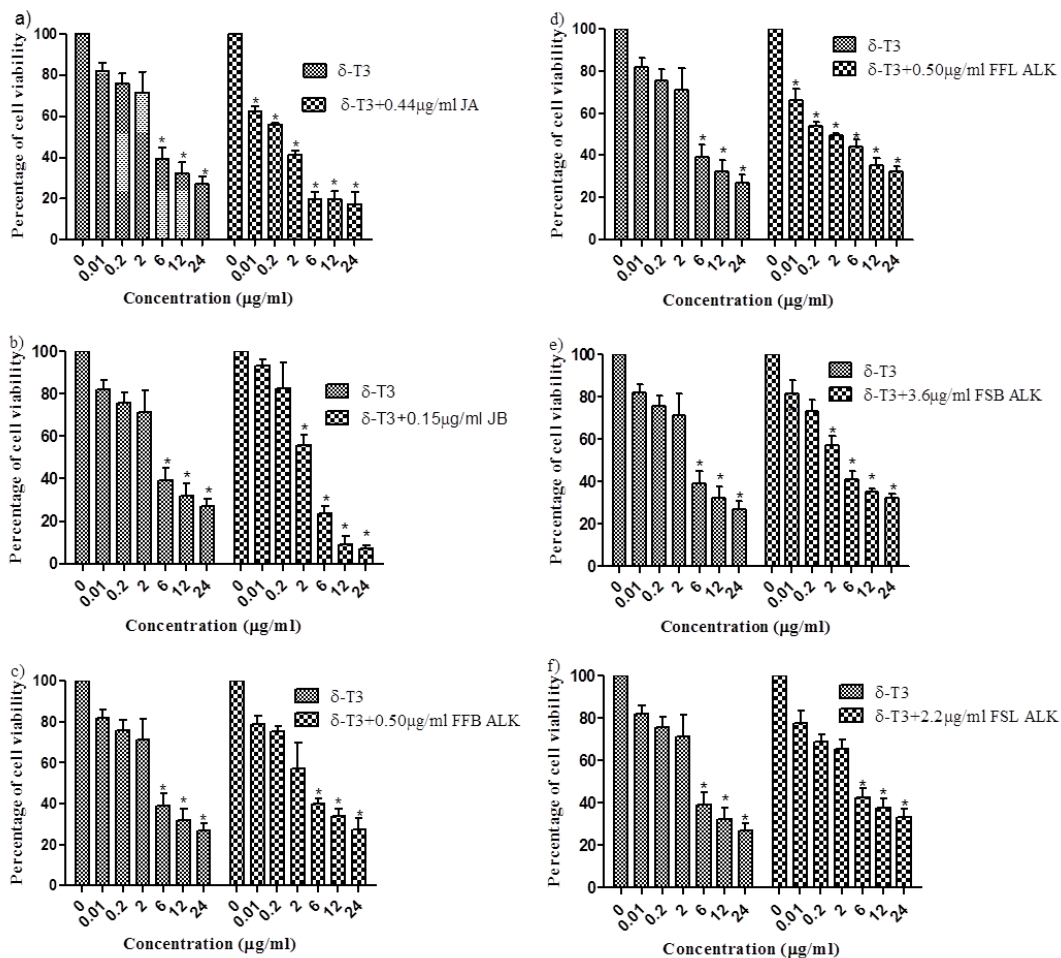
δ-T3
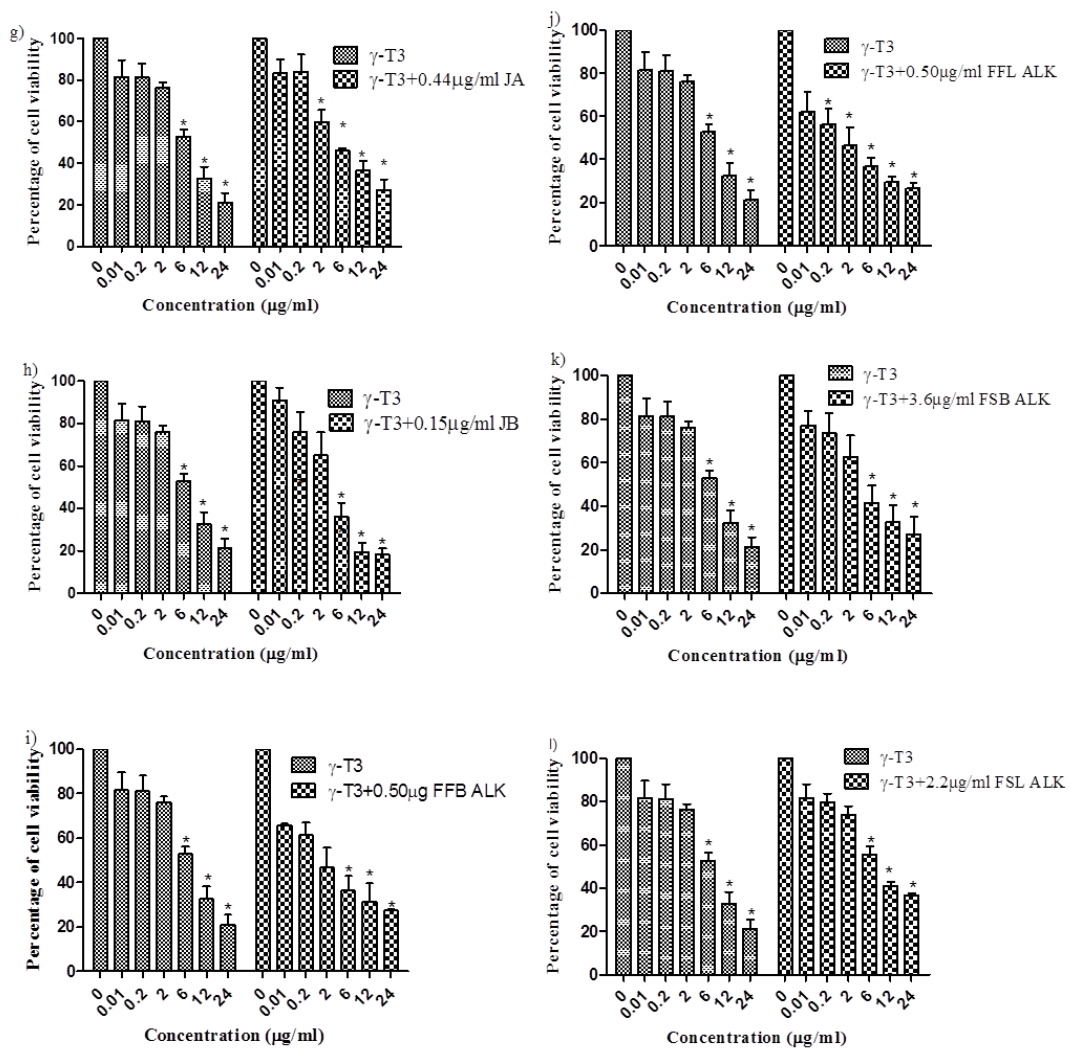
Fig 3.8: Antiproliferative profiles of combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of *F. fistulosa*, *F. schwarzii* and jerantinines (A and B) on HT-29 cells. HT-29 cells were exposed to treatments for 72 h and the cell viability was determined using the neutral red uptake assay as described in methods. Results are presented as Mean $\pm$ SEM. N=9 *P<0.001. NB: JA= jerantinine A, JB= jerantinine B, FF (B), (L) = *F. fistulosa* (bark), (Leaf), FSB (B), (L) = *F. schwarzii* (bark), (leaf), ALK= alkaloid crude extracts. Both individual IC_{50} doses of tocotrienols (delta and gamma tocotrienols) and combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} dose of jerantinines (A and B) and extracts of *F. fistulosa* and *F. schwarzii* induced dose dependent growth inhibition.
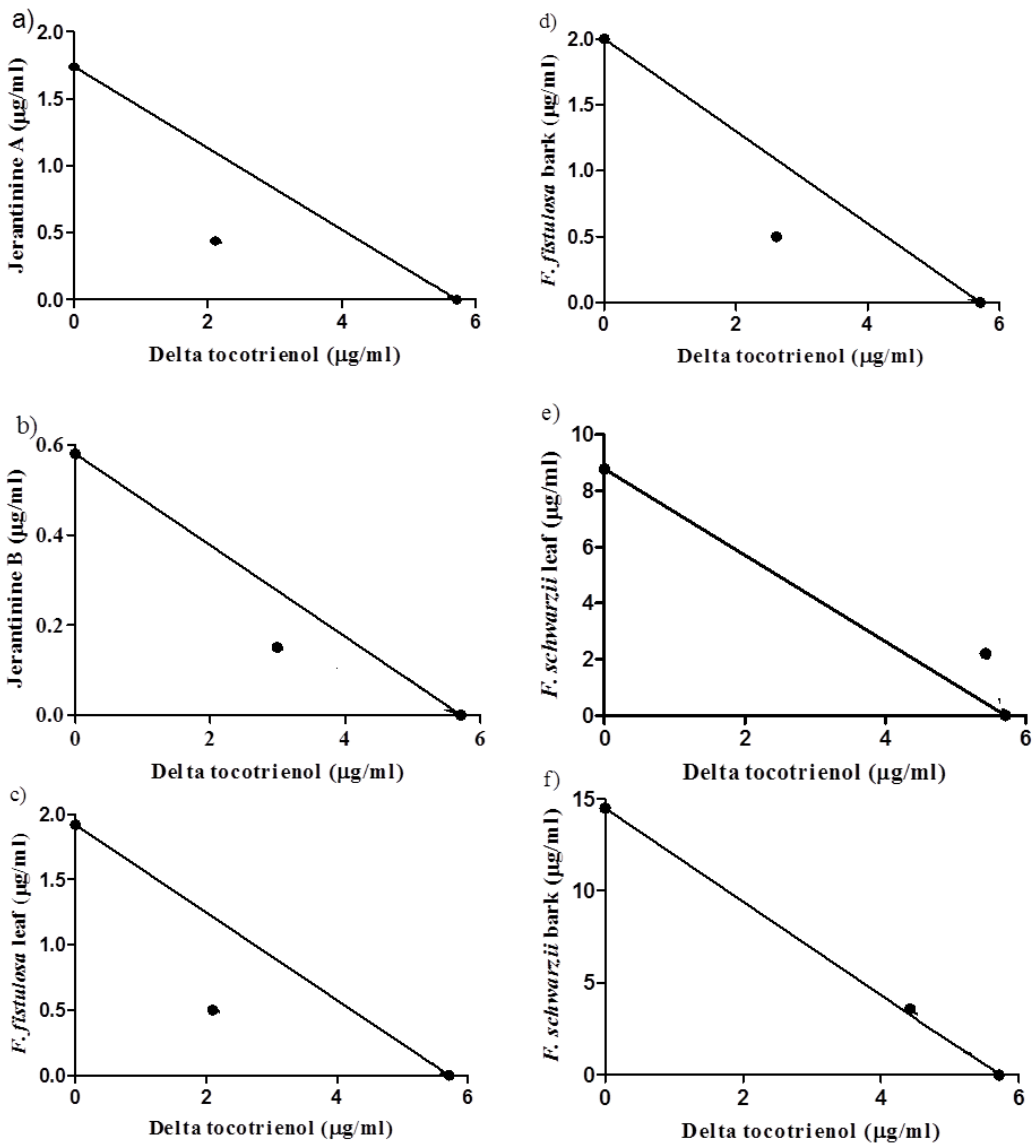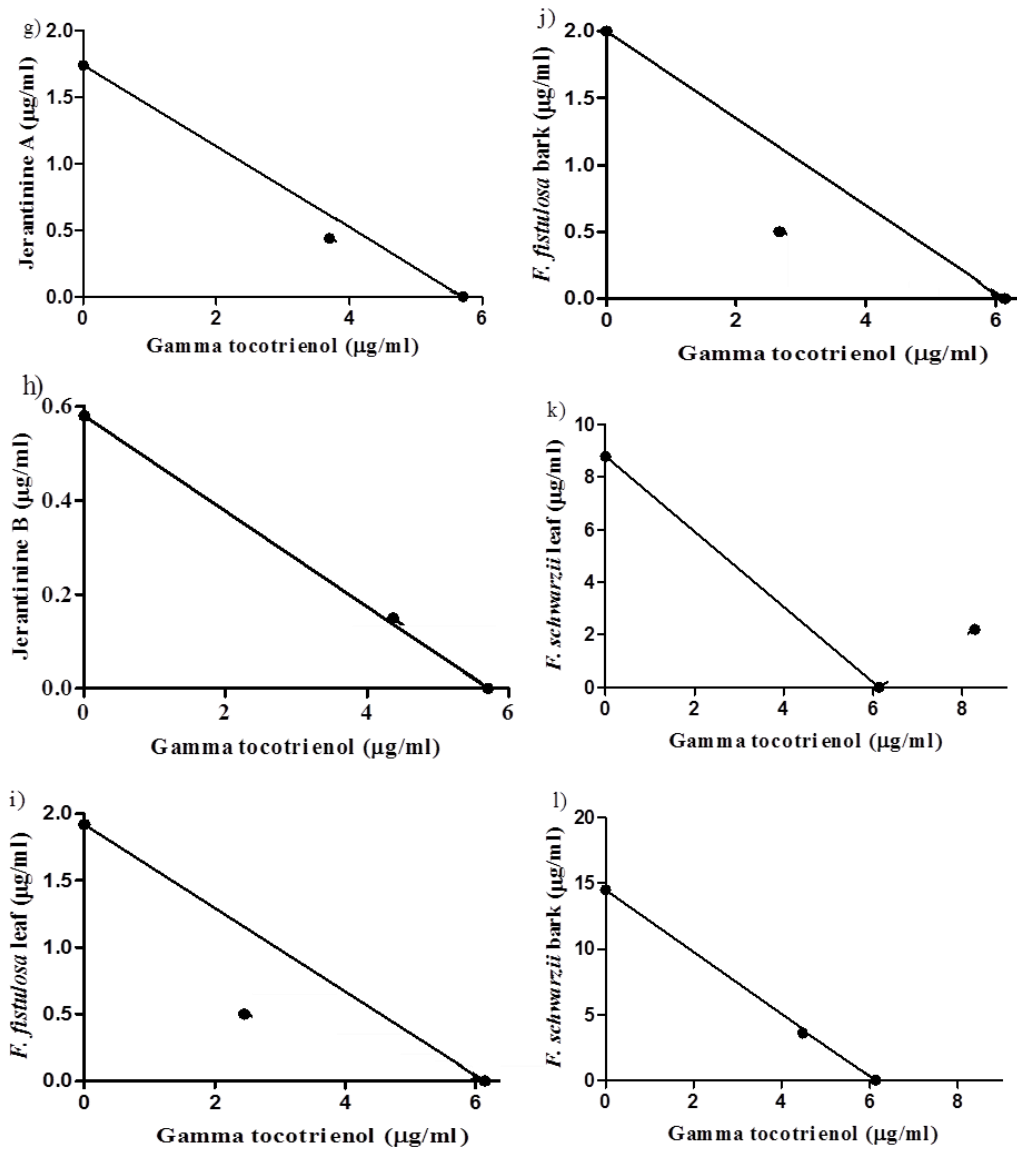
Fig 3.9: Isobologram profiles of HT-29 cells receiving combined low-dose of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) or alkaloid extracts form leaves and bark of *F. fistulosa* and *F. schwarzii*. Data point below, above and on top of the diagonal line indicated synergism, antagonism and additive pharmacological interaction between combined treatments, respectively. Combined treatment of tocotrienols with IC_{20} dose of jerantinines induced synergistic effect. Combined treatment of low-dose tocotrienol (delta and gamma) with IC_{20} dose of *F.schwarzii* leaf induced antagonistic effect on HT-29 cells.

3.4 Discussion

The flora across Asia and specifically Malaysia is hugely endowed with plants possessing potential bioactivities. The Moreacea plant family constitutes decent proportion and in fact over 40 different species are found in Malaysia. Thus, in search for new plant alkaloids with antiproliferative activity, three different *Ficus* species collected from various locations in Malaysia were selected based on published literatures that indicated the presence of bioactive compounds amongst *Ficus species.* Indeed, previous pharmacological study on constituents of chloroform extracts of a mixture of twigs and leaves of *F. hispida* collected from Chiang Rai in Thailand showed a cytotoxic activity against human Lu1 lung cancer (IC_{50} = 0.5 µg/ml) and Col2 colon cancer (IC_{50} = 0.1 µgml) (Peraza-Sánchez et al., 2002). In contrast, results obtained in this study showed that alkaloid extract of *Ficus hispida* leaves did not induce potent growth inhibition on A549 lung cancer cells with IC_{50} > 40 µg/ml although improved antiproliferative activity was observed on U87MG brain cancer cells and HT-29 colon cancer cells (IC_{50} > 20 µg/ml). The combination of twigs and leaves as reported in the previous study may have resulted in synergistic effect of constituents of the chloroform extracts compared to that of leaves only in the present study. Hence, this probably accounted for the observed difference on the level of cytotoxicity to different types of lung cancer cells.

In addition*, F. fistulosa* demonstrated a potent antiproliferative effect on U87MG and HT-29 cells with IC_{50} < 20 µg/ml and causing mild toxicity towards non-cancerous MRC5 cells thus conforming to the potency standard for crude extracts as established by the national cancer institute (NCI) (Suffness and Pezzuto, 1990). However, this is

contrary to previous studies that demonstrated non cytotoxic potency of crude and purified alkaloids of *F. fistulosa* species from Vietnam and Singapore (Zhang et al., 2002; Subramaniam et al., 2009). Location of plant as well as time of collection may affect the availability of metabolites as these secondary metabolites are produced by plants in response to environmental conditions such as stress, competition for space and protection etc. (Rocha et al., 2001). This accounts for the different potencies exhibited by *Ficus* species investigated in the present study which could be ranked in descending order as *F. fistulosa > F. schwarzii > F. hispida*. Furthermore, the greater potency demonstrated by the crude alkaloid extracts compared to ethanol extracts especially with *F. fistulosa* and *F. schwarzii* indicates that such antiproliferative effects are due to alkaloids present in the crude mixture rather than other phytochemicals contained in the ethanol extracts. In addition, the observed antiproliferative effects of *F. schwarzii* on A549 and HT-29 cells which to date there are no reported studies on its bioactivity provides new information for potential future uses as source of new cytotoxic alkaloids.

Similarly, treatment of cells with jerantinine A and B demonstrated dose-dependent antiproliferative effects against A549, HT-29, and U87MG cells in agreement with previously published study on the potency of jerantinine derivatives (Frei et al., 2013). However, toxicity to normal cells was evident which is in agreement to previously published study on jerantinines (Qazzaz et al., 2016; Raja et al., 2014). On the other hand, a dose dependent growth inhibition was evident on U87MG, A549 and HT-29 cells treated with tocotrienols. In fact, preferential toxicity towards cancer cells was only evident on U87MG and HT-29 cells receiving delta and gamma tocotrienols. Based on

this study, the potency of delta tocotrienol is higher than that of gamma corroborating to previous studies (Bardhan et al., 2011). Furthermore the study provides additional information on the antiproliferative potency of tocotrienols especially delta isomer on brain and lung cancers which were scarcely reported compared to gamma tocotrienol (Lim et al., 2014a, 2014b; Yano et al., 2005).

Undoubtedly, tocotrienols possess remarkable anticancer properties as demonstrated by previous studies (Aggarwal and Nesaretnam, 2012; Wada, 2012). However, metabolic degradation resulting from high dose delivery and subsequent over load of transport protein for tocotrienol has continuously limited the level of therapeutic doses. Thus far, studies and reviews have suggested synergism that would minimize dosage, toxicity and improve potency in addition to nano-deliveries as a potential alternative (Constantinou et al., 2008; Sylvester et al., 2010). Indeed studies have been conducted demonstrating the synergistic potency of tocotrienols (Akl et al., 2012; Tuerdi et al., 2013), however in some instances, the potential toxicity of such combinations to non-cancerous cells were not determined (Wali and Sylvester, 2007). Similarly, a study by Hafid et al., (2010) showed that tocotrienols are also good adjuvants for development of cancer vaccines.

Herein, the combined studies demonstrated the synergistic potency of delta and gamma tocotrienols. Interestingly, combined treatments with alkaloids extracts of bark and leaf of *Ficus fistulosa* demonstrated the strongest synergistic effects against U87MG cells with reduction of up to 35 and 29 folds for delta and gamma tocotrienols, respectively (Table 3.6). Such remarkable reduction clearly resulted in the nontoxic effects evidenced

on non-cancerous MRC5 cells in comparison to U87MG cancer cells. However, though synergistic effects were also evident against HT-29 cells, there was poor selectivity window between cancerous and non-cancerous cells treated with combined doses of *F. fistulosa* and delta or gamma tocotrienol. This indicated that the observed synergistic effect preceded by mild toxicity to normal cells was cell specific. Besides, this might also be attributed by the combined synergistic effects of several alkaloids present in the crude alkaloid extracts. In contrast, combined treatments with *F. schwarzii* caused an additive or antagonistic effect against HT-29 cells with toxic effects to normal cancer cells. Nevertheless, this demonstrates the antiproliferative and synergistic potencies of alkaloid extracts from *Ficus* species which may vary depending on species as demonstrated by previous studies. For instance, a combined treatment of hexane insoluble fraction of *Ficus septica* with doxorubicin increased the incidence of apoptosis and also caused a shift from G2/M to G1 phase cell cycle arrest. Although the study demonstrated the potential application of *F. septica* as adjunct treatment however, the potential toxicity of such high dose combinations to non-cancerous cells as well as the type of pharmacological interaction between *F. septica* and doxorubucin was not reported (Nugroho et al., 2013). In contrast a synergistic pharmacological interaction was reported for combined sub-effective doses of doxorubicin and ethyl acetate extract of *F. septica* against T47D human breast cancer cells (Nugroho et al., 2012). However, unlike the present study, the potential toxicity of such combinations to non-cancerous cells was also not investigated.

Recent studies have demonstrated that the cytotoxic potency of Jerantinine derivatives is mediated via inhibition of microtubules (Frei et al., 2013; Raja et al., 2014). Microtubule inhibiting drugs such as the vinca alkaloids are currently under clinical use as anticancer chemotherapeutic agents. However, susceptibility to p-glyprotein multidrug resistance and toxicity to the peripheral nervous system has limited the potency of these classes of drugs. This has prompted the development of more efficient synthetic analogues (Canta et al., 2009). Indeed, control drug vinblastine in the present study exhibited the highest toxicity to non-cancerous MRC5 cells compared to jerantinines A and B which conforms to the aforementioned suggestion on the undesirable toxicity of tubulin inhibiting drugs. The potential of combination of two drugs as hybrid drugs with at least one a tubulin inhibiting drug has been suggested as an alternative to enhancing activity and improving clinical outcome of tubulin targeting drugs (Breen and Walsh, 2010). In the present study, the combined low dose treatment of delta or gamma tocotrienol with IC_{20} dose jerantinine showed up to 5-fold selective toxicity towards U87MG cells compared to MRC5 cells (Table 3.6). Similarly, up to 2-fold selectivity was also evident for combined treatments except for gamma tocotrienol with jerantinine A towards HT-29 cells compared to MRC5 cells. In contrast, only combined treatments of delta tocotrienol with jerantinine A showed at least 2-fold selective toxicity towards A549 cells compared to MRC5 cells (Table 3.7). This demonstrates the synergistic potency of jerantinines A and B in combination with delta or gamma tocotrienol did occur and more importantly toxicity towards non-cancerous MRC5 cells had been minimized. The induction of such antiproliferative effects by tocotrienol and jerantinines (A and B) is potentiated via different mechanisms further justifies the present study. Certainly, these potentially

varied mechanisms would provide an alternative for more potent multi-targeted cancer killing and probably account for the observed potent effects herein at low dosage of tocotrienol and jerantinines (A and B).

3.5. Conclusion

The study demonstrated the antiprofilrative potencies of *Ficus* species on A549, HT-29 and U87MG cancer cells which can be ranked in a descending order as *Ficus fistulosa > Ficus schwarzii > Ficus hispida* in terms of superior potency. Similarly, tocotrienols (delta and gamma) and jerantinines (A and B) induced dose dependent growth inhibition on A549, HT-29 and U87MG cells although similar toxicity was evidenced for jerantinines (A and B) towards MRC5 cells. The combined low-dose treatments of tocotrienols with jerantinines (A and B) or alkaloid extracts of *F. fistulosa* caused reduction in required potent dosage and subsequently minimizing toxicity to non-cancerous cells. Combination treatments have been reported as alternatives to circumvent limitation of high dose tocotrienols and minimize the undesirable toxicity of tubulin targeting drugs. Thus, it is interesting to further investigate and elucidate the mechanism mediating the antiproliferative effects of combined low-dose treatment of tocotrienols with jerantinines (A and B) or alkaloid extracts of *F. fistulosa*.

4.0 CHAPTER FOUR

Induction of DNA damage and manifestation of morphological features of apoptosis by individual and combined treatments of tocotrienols (delta and gamma) and jerantinines (A and B)

4.1 Introduction

Cell viability study from Chapter Three confirmed the antiprofilerative potency of individual and combined low-dose treatments of tocotrienols (delta and gamma), jerantinines (A and B) and alkaloid crude extracts of leaves and bark of *F. fistulosa* and *F.* schwarzii thus necessitating further investigations on programmed cell death (PCD) or apoptosis. Apoptosis is one of the many models of programmed cell death in addition to other proposed alternative models such as autophagy, paraptosis and mitotic catastrophe. Apoptosis is a key regulator of physiologic growth and homeostasis and hence cell death by apoptosis has been recognised as crucial to antitumor growth which determines the response to chemotherapeutic agents (Fulda and Debatin, 2006b). Morphologically, apoptosis is characterised by features including nuclear chromatin condensation and fragmentation, shrinkage and disassembly into apoptotic bodies that are eventually phargocytosed. In addition to morphological features, apoptosis is also characterised by biochemical hallmarks that include internucleosomal DNA fragmentation and caspase enzymes activity. This is contrary to undesirable necrotic cell death characterised by swelling of organelles and early plasma membrane rupture thereby inducing inflammatory response (Saraste and Pulkki, 2000). Therefore it is imperative to determine the type of cell death induced by individual and combined low-dose treatments.

For evaluation of possible changes in cell morphology, histochemical (haematoxylin and eosin) and fluorescence (acridine orange and propidium iodide) staining techniques were adopted. Haematoxylin and eosin (H&E) stains would allow for extensive evaluation of morphological features of apoptotic cell death to distinguish from necrosis. Cells undergoing apoptosis upon H&E staining appear as single or cluster of cells with dark eosinphillic cytoplasm, dense nuclear chromatin fragments, extensive membrane blebbing and apoptotic body formation. Similarly, acridine and propidium (AO/PI) staining was adopted to further corroborate the data from H&E and potentially determine the different stages of apoptosis. Cells stained green indicate early stage of apoptosis whereas cells stained yellow or orange indicate late stage of apoptosis. Cells undergoing early stage apoptosis are AO positive and stain green. Whereas, cells at the late stage of apoptosis are permeable to both AO and PI and therefore appear as yellow or orange stained cells.

On the other hand, oxidative DNA damage is a biochemical hallmark of apoptosis that results from degradation of DNA at internucleosomal regions by DNases characterised by double stranded DNA fragments of 180 – 200 base pairs (Saraste and Pulkki, 2000). Therefore, the single gel electrophoresis assay (COMET) was adopted for investigation on DNA damage pattern as an additional diagnostic tool for apoptosis. The COMET assay is based on the ability of negatively charged DNA fragments in single cells to migrate through low melting agarose in response to and electric field (Kumaravel et al., 2009). Alkali COMET assays detects single stranded breaks whereas, COMET assay

done under neural conditions allows for detection of double stranded breaks independent of single stranded breaks (Olive and Banáth, 2006). This technique was chosen because unlike the DNA laddering technique that detects only DNA fragments, the COMET assay is able to obtain more details and allows for the detection of both single and double stranded DNA breaks. The comparison on the changes in cell morphology and DNA damage pattern induced by vinblastine (positive control) and treatments (individual and combined low-dose) would allow the possibility to predict a hypothetical clue of apoptotic mechanism in U87MG, A549 and HT-29 cells. This study signified the absolute determination of potential apoptosis in comparison to uncontrolled necrosis based on morphological and DNA damage assessments.

Guided by data gathered from cell viability studies in Chapter Three, the following treatments listed in Table 4.2 were selected for further assessment of morphological features of apoptosis and DNA damage patterns. For single treatments studies only purified alkaloids, jerantinines A and B and tocotrienol isomers (delta and gamma) were selected for further mechanistic studies. Although alkaloid crude extracts of *Ficus fistulosa* demonstrated strong synergistic antiproliferative effects however such effects could be as a result of multiple bioactive compounds present in the crude extracts. Furthermore, unlike pure compounds, crude extracts can trigger both single stranded and double stranded DNA breaks as demonstrated by previous studies on extracts of *Acalypha wilkesiana* (Lim et al., 2013, 2011). Hence, this would not allow for an absolute determination of the type of cell death induced by alkaloid crude extract of *Ficus fistulosa* based on morphology and DNA assessments. On the other hand, combined low-

dose of jerantinines (A and B) and tocotrienols (delta and gamma) that induced synergistic effects with IC_{50} values below 4 µg/ml and demonstrated at least 2-fold selective toxicity towards cancer cells compared to normal cells were also selected for further mechanistic studies.

The specific objectives of this chapter are:

i) To assess changes in cell morphology for features indicative of apoptotic cell death on U87MG, A549 and HT-29 cells induced by individual and combined low-dose treatments.

ii) To determine the pattern of DNA damage induced by individual and combined low-dose treatments.

4.2 Materials and Methods

4.2.1 Chemical reagent/kits and cell cultureware

The following reagents and kits listed in Table 4.1 were used to conduct the experiments.

Table 4.1: List of chemical reagents, kits and glassware used and the respective manufacturers.

S/no	Chemical reagents/kits/lab wares	Manufacturer
1	Haematoxylin, eosin Y powder, per-mount	Sigma, USA
2	Methanol, absolute ethanol	Systems, USA
3	Ethylene glycol, sodium iodate and ammonium aluminium sulphate, xyelene, acridine orange, propidium iodide	Nacalai Tesque, Japan
4	Glacial acetic acid	Amresco, USA
5	Fluorogel mounting medium	Genetex, USA
6	Oxiselect STA 351 Comet assay kit	Cell Biolabs, USA

Table 4.2: Compounds and their concentrations selected in individual and combined low-dose treatments for morphological and DNA damage evaluation studies.

s/no	Compounds	Cancer cells		
		Single treatments (µg/ml)		
		U87MG	A549	HT29
1	Delta tocotrienol (δ-T3)	3.17	12.40	5.71
2	Gamma tocotrienol (γ-T3)	3.12	-	-
3	Jerantinine A (JA)	0.62	1.42	1.74
4	Jerantinine B (JB)	0.74	1.48	0.58
5	Vinblastine	0.04	0.03	0.71
		Combined treatments (µg/ml)		
6	Delta tocotrienol + jerantinine A (δ-T3+ JA)	-	2.77 + 0.35	2.12 + 0.44
7	Delta tocotrienol + jerantinine B (δ-T3+ JB)	1.83 + 0.19	-	2.99 + 0.15
8	Gamma tocotrienol + jerantinine A (γ-T3 + JA)	1.29 + 0.16	-	-
9	Gamma tocotrienol + jerantinine A (γ-T3 + JB)	1.61 + 0.19	-	-

4.2.2 Haematoxylin and eosin (H&E) staining

A total of 1 x 10^4 U87MG, A549 and HT-29 cells were seeded in 2-well chamber slides and incubated overnight to facilitate attachment. Thereafter, the medium was replaced with treatment medium containing individual IC_{50} doses or combined low-dose tocotrienols (delta and gamma) with IC_{20} dose of jerantinines (A and B) for respective cell lines as shown in Table 4.2. Whereas, vinblastine treated and untreated cells containing plain medium with equivalent amount of DMSO served as positive and negative controls, respectively. The treated cells were incubated in cell culture conditions for 24 h prior to staining. For H&E staining (For recipe see appendix 6a, b), cells were fixed and rehydrated in decreasing ethanol concentrations (100% (3x), 95% (3x), and 70% (1x)) for 3min each, after the final step, the slides were rinsed in distilled water for 3

min. Rehydrated slides were stained in gills (I) haematoxylin for 3min and subsequently washed in 95% ethanol for 1 min. The slides were stained with alcoholic eosin for 2 min and then dehydrated in decreasing ethanol concentrations (95% (2x) and 100% (2x)) for 1 min each. The slides were finally cleared with two changes of xylene for 1min, mounted with per-mount and viewed under the Nikon 80i eclipse microscope (Nikon, Japan) at 40x magnification.

4.2.3 Acridine orange and propidium iodide (AO/PI) Staining

A total of 5×10^3 U87MG, A549 and HT-29 cells were seeded in 2-well chamber slides and incubated for 24 h to facilitate attachment. Thereafter, cells were treated with individual IC_{50} doses or combined low-dose tocotrienols (delta and gamma) with IC_{20} dose of jerantinines (A and B) as shown in Table 4.2. Similarly, vinblastine treated and untreated cells containing plain media with equivalent amount of DMSO served as positive and negative controls, respectively. Following 24 h treatment, the cells were fixed with -20°C methanol for 10 min, rinsed three times with PBS and stained with a mixture of acridine orange (10 µg/ml) and propidium iodide (10 µg/ml) in ratio 1:1. Slides were mounted with fluorogel mounting medium and morphological images were captured using the Nikon Ti eclipse confocal microscope (Nikon, Japan) at 40x magnification.

4.2.4 Single cell gel electrophoresis (COMET)

COMET assay was conducted according to previously described protocol (Lim et al., 2011). A total of 5×10^3 U87MG, A549 and HT-29 cells were treated with individual

IC$_{50}$ doses or combined low-dose tocotrienols (delta and gamma) with IC$_{20}$ dose of jerantinines (A and B) as shown in Table 4.2. Cells treated with vinblastine or plain media with DMSO served as positive and negative controls, respectively. After 72 h treatment, the cells were detached and centrifuged at 2000rpm for 5min. Subsequently, 200 µl of low melting agarose was mixed with 20 µl of cell pellets in PBS and 75 µl of the mixture was spread gently on the COMET slides. The slides were laid horizontally in the dark at 4°C for 30 min to solidify. The slides were then lysed in pre-chilled 1x lysis buffer for 60 min at 4°C and further incubated horizontally in a container containing cold alkaline solution for 30 min at 4°C in the dark. Thereafter, neutral and alkaline COMET assays were conducted to investigate for double strand breaks (DSBs) and single strand breaks (SSBs), respectively. For neutral COMET assay, the slides were placed in horizontal electrophoresis chamber containing cold 1 x TBE buffer and voltage of 20V was applied for 45 min. For alkaline COMET assay, the horizontal chamber was filled with cold alkaline electrophoresis solution (300 mM NaOH, pH >13, 1 mM EDTA) and voltage of 20V was applied for 30 min. After electrophoresis, the slides were rinsed in three changes of cold distilled water for 2 min and then placed horizontally in ice cold 70% ethanol for 5 min. Finally, the slides were air-dried and stained with 100 µl of vista green dye for 15 min. Images were captured using Nikon AZ100 fluorescence microscope (Nikon, Japan) at 20x magnification. For quantification of DNA damage, the DNA COMET profiles were analysed using the OpenComet software according to previously described protocol (Gyori et al., 2014). A total of 200 cells were analysed from two independent experiments and results were presented as Mean ± SD fold change of percent DNA in the tail compared to untreated controls.

4.2.5 Statistical analysis

Statistical analysis was conducted using one way ANOVA to compare between treated and untreated groups. P values <0.001 (***), P <0.01 (**) and P <0.5 (*) were considered statistically significant.

4.3 Results

4.3.1. Morphological changes on U87MG, A549 and HT-29 cells treated with individual and combined low-dose treatments

4.3.2. Hematoxylin and eosin staining

Following 24 h treatment of U87MG cells with IC_{50} doses of jerantinines A and B, morphological features of apoptosis including nuclear chromatin condensation and fragmentation, cytoplasmic extension and formation of multinucleated bodies were evident (Fig. 4.1 d, e). In addition, U87MG cells treated with IC_{50} doses of delta and gamma tocotrienols showed evidence of condensed nuclear chromatin and cell shrinkage (Fig. 4.1 b, c), whereas, apoptotic bodies were observed in cells treated with control drug vinblastine (Fig. 4.1 f). Similarly, A549 cells subjected to 24 h treatments with individual IC_{50} doses of jerantinines A and B or vinblastine showed clear evidence of membrane blebbing, nuclear chromatin condensation and cell shrinkage (Fig. 4.2 c, d, e) compared to untreated cells (Fig. 4.2a). Apoptotic cells appeared as dense bodies with a circular or oval structure. HT-29 cells treated with jerantinine A or B showed massive membrane blebbing, nuclear chromatin condensation and apoptotic bodies (Fig. 4.3 c, d) compared to untreated cells (Fig. 4.3 a). Circular shaped shrinking cells with condensed nuclear chromatin morphologies were also observed in HT-29 cells treated with delta tocotrienol and vinblastine (Fig. 4.3 b, e).
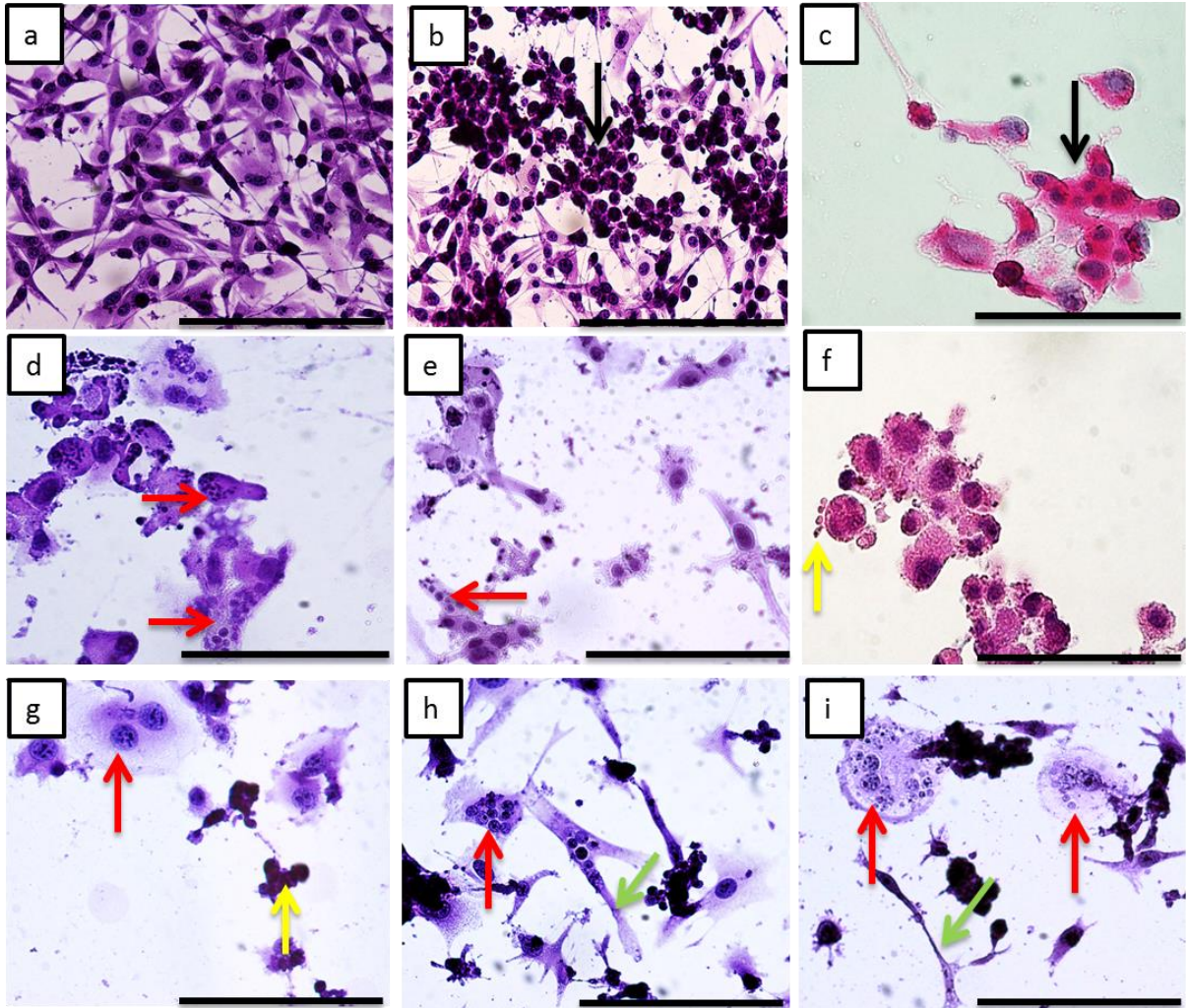
Fig 4.1: Representative H&E images of U87MG cells treated for 24 h. a) Untreated, b) delta tocotrienol, c) gamma tocotrienol, d) jerantinine A, e) jerantinine B, f) vinblastine, g) gamma tocotrienol + jerantinine A, h) delta tocotrienol + jerantinine B, i) gamma tocotrienol + jerantinine B. Morphological features of apoptosis including nuclear chromatin condensation (black arrows), multinucleated bodies (red arrows), cytoplasmic extension (green arrows) apoptotic bodies (yellow arrow) and cell shrinkage were evident. Scale bar represents 50 µm.
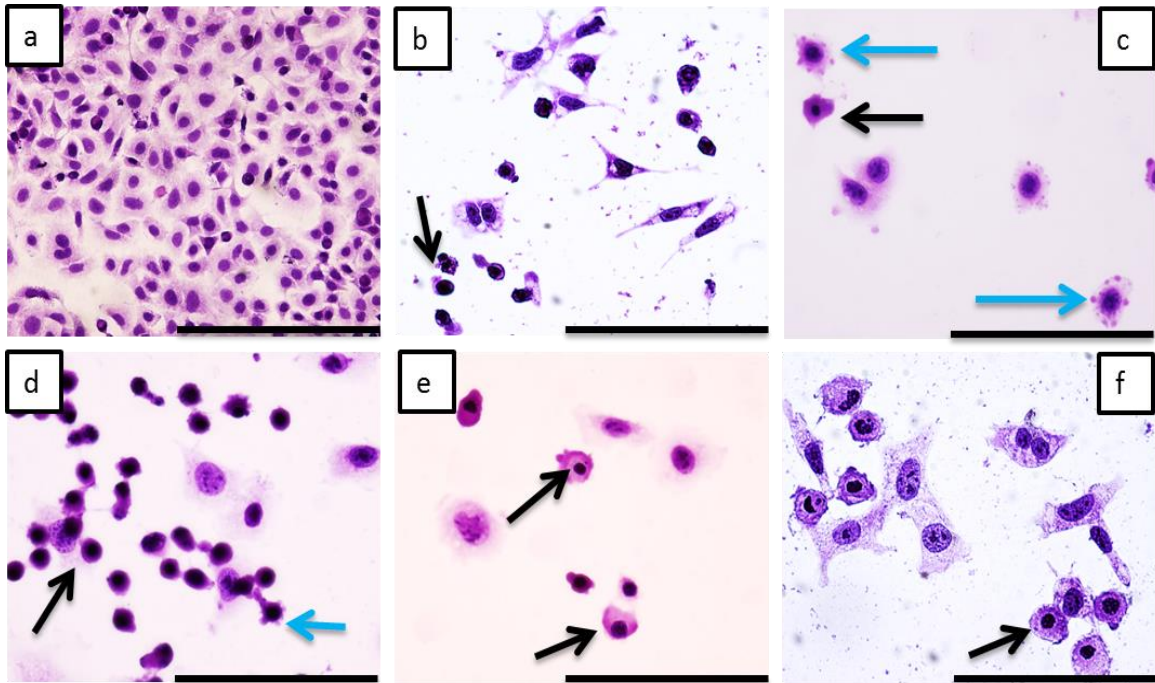
Fig 4.2: Representative H&E images of A549 cells treated for 24 h. a) Untreated, b) delta tocotrienol, c) jerantinine A, d) jerantinine B, e) vinblastine, f) jerantinine A + delta tocotrienol. Morphological features of apoptosis including membrane blebbing (blue arrows), nuclear chromatin condensation and cell shrinkage (black arrows) were evident. Scale bar represents 50 μm.
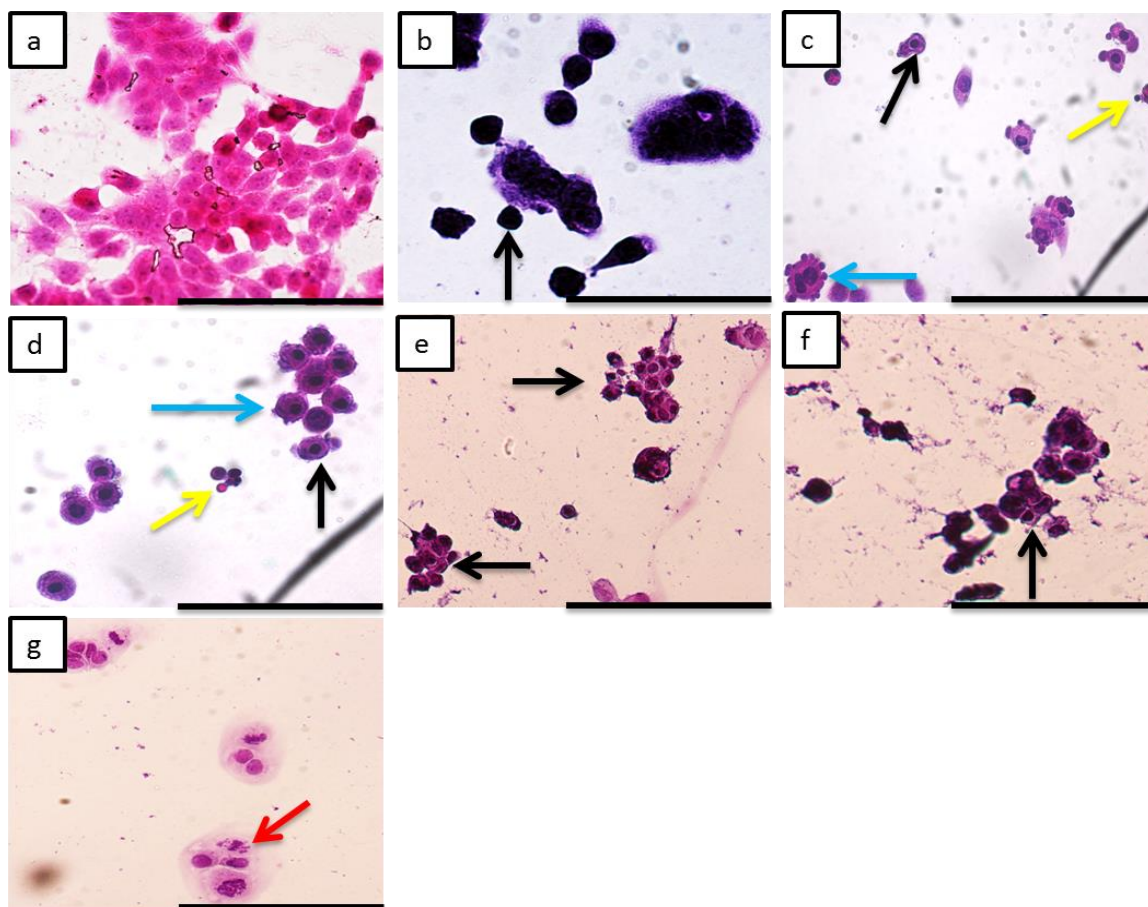
Fig 4.3: Representative H&E images of HT-29 cells treated for 24 h. a) Untreated, b) delta tocotrienol, c) jerantinine A, d) jerantinine B, e) vinblastine, f) delta tocotrienol + jerantinine A, g) delta tocotrienol + jerantinine B. Morphological features of apoptosis including membrane blebbing (blue arrows), nuclear chromatin condensation and cell shrinkage (black arrows), nuclear chromatin fragmentation (red arrow) and apoptotic bodies (yellow arrows) were evident. Scale bar represents 50 μm.

For combined studies, U87MG cells were treated with combined low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) for 24 h and subsequent evaluation of morphological changes revealed features of apoptosis including multinucleated bodies, nuclear chromatin condensation and cytoplasmic extension (Fig. 4.1 g, h, i). These features were similar to those observed in single treatments of tocotrienols (Fig. 4.1b, c) thus indicating potential synergism of the combined drugs at lower dose with an effect of the same multitude as that of single high dose. Similarly,

A549 and HT-29 cells treated with combined low-dose delta tocotrienol with IC_{20} doses of jerantinines (A and B) showed morphologies similar to single high-dose tocotrienol including circular or oval shaped cells with condensed nuclear chromatin and minimal membrane blebs as observed in (Fig. 4.2 f) and (Fig. 4.3 f, g), respectively.

4.3.3. Acrindine orange and propidium iodide staining (AO/PI)

Guided by H&E staining, further fluorescence staining was conducted to further corroborate evidence of morphological features of apoptosis. The massive multinucleated bodies observed in H&E stained U87MG cells treated with jerantinines A and B were also evident in fluorescence stained cells (Fig. 4.4), including minor nuclear chromatin fragmentation and condensation. Live cells or cells at early stage of apoptosis are impermeable to PI staining which are contrary dying cells or cells at the late stage of apoptosis. Cells at late stage of apoptosis loose membrane integrity and are permeable to PI thereby producing yellow or orange stained cell morphology when counter stained with AO/PI. Interestingly, massive staining of U87MG cells by PI and the orange colour stained cells observed in merged images of AO and PI further suggest late stage of apoptosis. Similar features were also observed in cells treated with vinblastine (Fig. 4.4). Similar massive multi nucleated bodies and nuclear fragmentation were observed in U87MG cells treated with combined low-dose gamma tocotrienol with IC_{20} dose of jerantinine A and combined low-dose delta tocotrienol with IC_{20} dose of jerantinine B (Fig. 4.4). The orange coloured stained cells in merged images of AO and PI indicating the late stage of apoptosis. In contrast, only minor nuclear chromatin condensation and fragmentation were observed in U87MG cells treated with single IC_{50} doses of delta or

gamma tocotrienol (Fig. 4.4) thus further supporting the proposition that apoptosis was still at its early stage. Furthermore, this indicates better and more potent effect of combined low-dose treatments as well as improved potency of tocotrienols compared to single IC_{50} doses of tocotrienols.

On the other hand, apoptotic features including nuclear chromatin condensation and membrane blebbing were evident in A549 cells treated with IC_{50} doses of jerantinines A and B (Fig. 4.5). While, only nuclear chromatin condensation was observed in cells treated with IC_{50} doses of control drug, vinblastine, delta tocotrienol and combined low-dose delta tocotrienol with IC_{20} dose of jerantinine A (Fig. 4.5). Interestingly, unlike U87MG cells, minimal staining with PI was observed in A549 cells where cells were stained green in merged images of AO and PI. In addition to the indication of early apoptotic stage, this also demonstrates that apoptosis occurred at an earlier stage in U87MG cells compared to A549 cells treated with jerantinines A and B or the combined low-dose treatments with delta and gamma tocotrienols.

Furthermore, massive membrane blebs with condensed nuclear chromatin were observed in circular and oval shaped HT-29 cells treated with IC_{50} doses of jerantinines A and B and control drug vinblastine for 24 h (Fig. 4.6). HT-29 cells treated with jerantinine B appeared to be at the later stage of apoptosis and thus the yellow colour was seen in merged images of AO and PI compared to HT-29 cells receiving jerantinine A. In contrast, only minimal nuclear fragmentation was evident in HT-29 cells treated with IC_{50} dose of delta tocotrienol (Fig. 4.6). Nevertheless, on a more positive note, nuclear

chromatin condensation and nuclear fragmentation were evident in HT-29 cells treated with combined low-dose delta tocotrienol with IC_{20} doses of jerantinines A or B (Fig. 4.6) indicating much improved potency compared to single high-dose delta tocotrienol.

	AO	PI	MERGED
Untreated			
δ-tocotrienol			
γ-tocotrienol			
Jerantinine A			
Jerantinine B			
Jerantinine A + γ-tocotrienol			
Jerantinine B + δ-tocotrienol			
Jerantinine B + γ-tocotrienol			
Vinblastine			

113
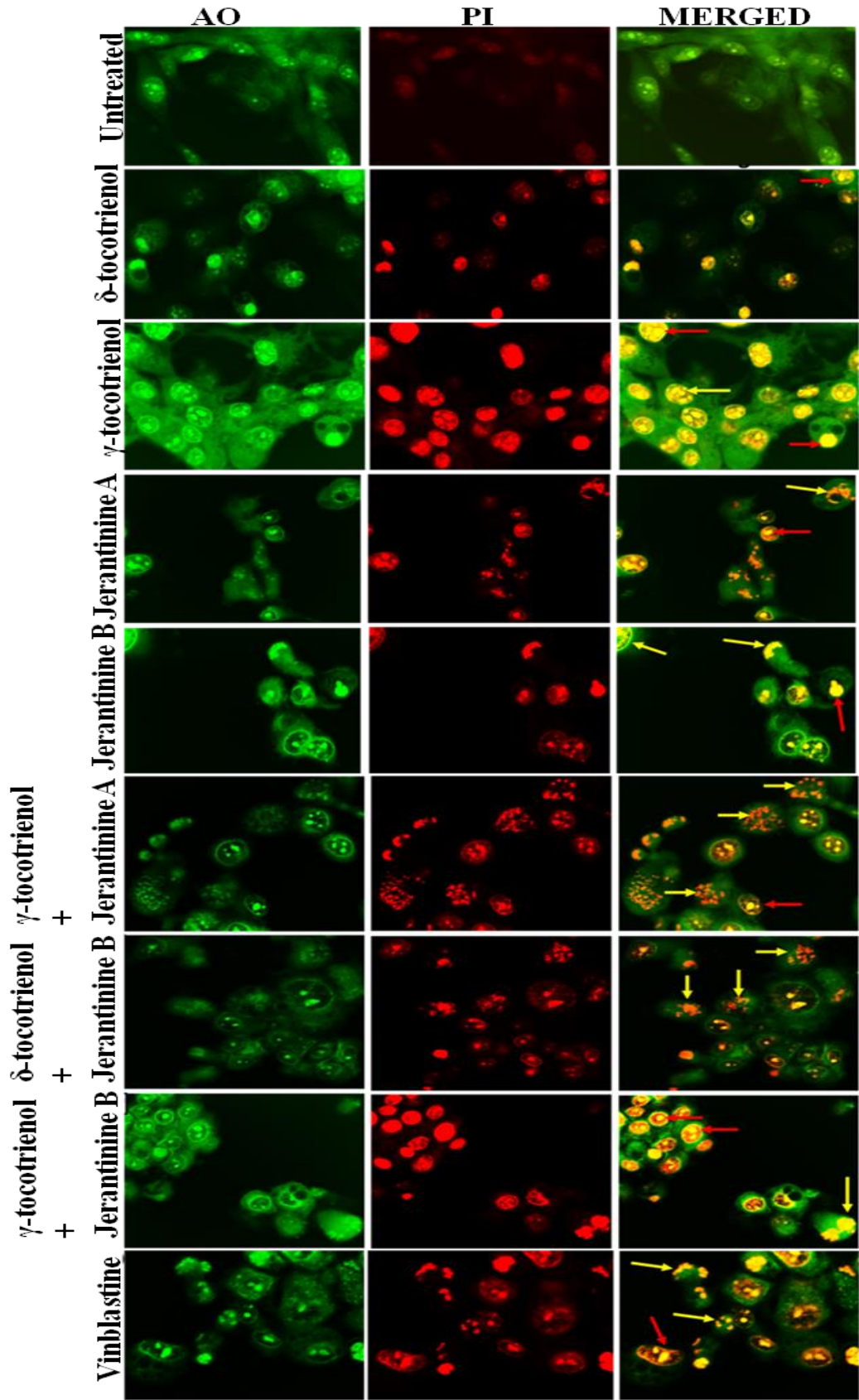
Fig 4.4: Representative AO/PI images of U87MG cells treated for 24 h with individual and combined low-dose tocotrienols (delta and gamma) with IC_{20} dose of jerantinines (A and B). U87MG cells treated with vinblastine and plain media containing DMSO served as positive and negative controls, respectively. Morphological features of apoptosis including nuclear chromatin condensation (red arrows) and nuclear chromatin fragmentation (yellow arrows) were evident. Images were captured at 40X magnification and merged using the NIS-Elements software under Nikon Ti eclipse confocal microscope observation.

	AO	PI	MERGED
Untreated			
δ-tocotrienol			
Jerantinine A			
Jerantinine B			
δ-tocotrienol + Jerantinine A			
Vinblastine			

115
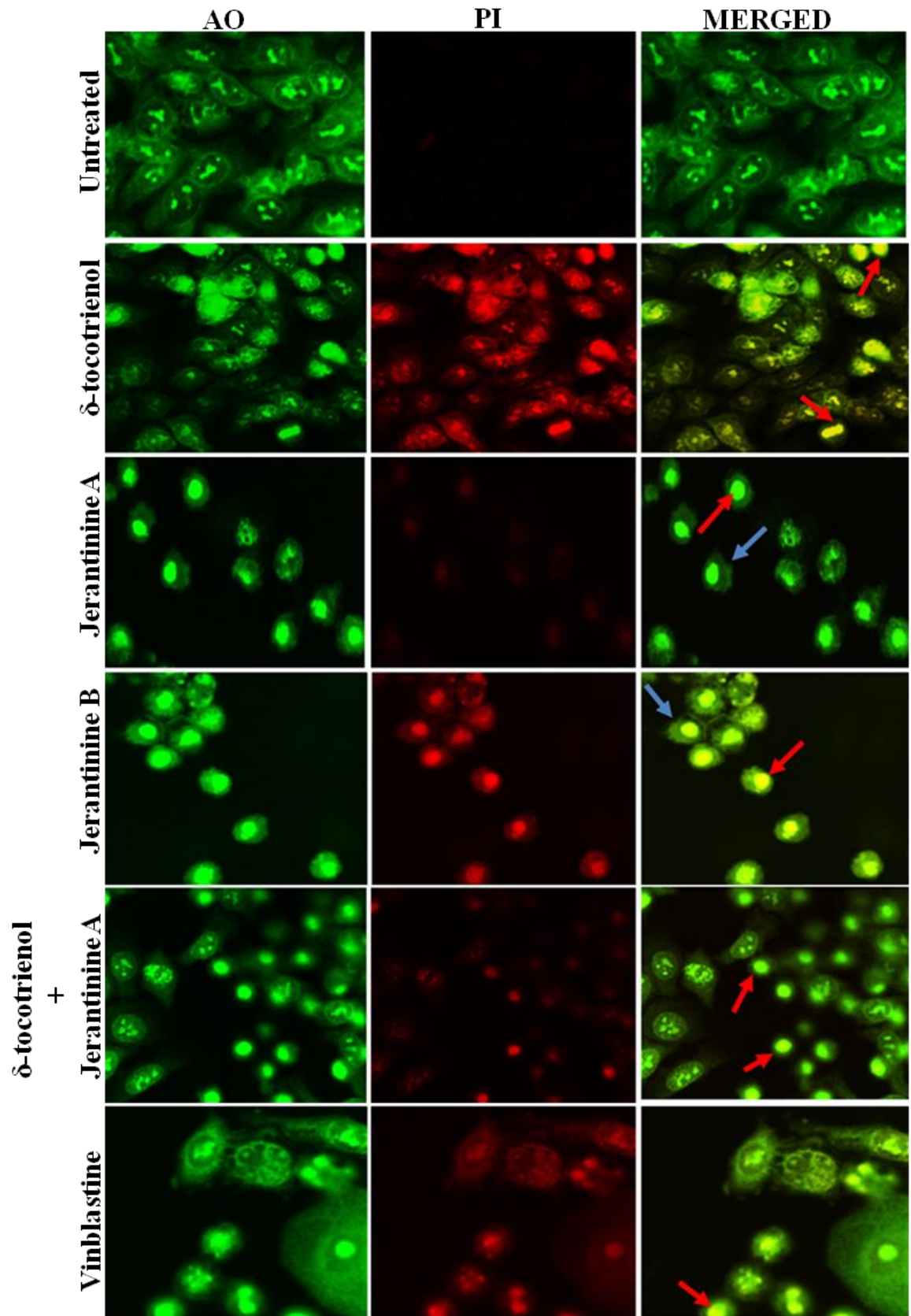
Fig 4.5: Representative AO/PI images of A549 cells treated for 24 h with individual and combined low-dose delta tocotrienol with IC_{20} dose of jerantinine A. A549 cells treated with vinblastine and plain media containing DMSO served as positive and negative controls, respectively. Morphological features of apoptosis including nuclear chromatin condensation (red arrows) and membrane blebbing (blue arrows) were evident. Images were captured at 40X magnification and merged using the NIS-Elements software under Nikon Ti eclipse confocal microscope observation.
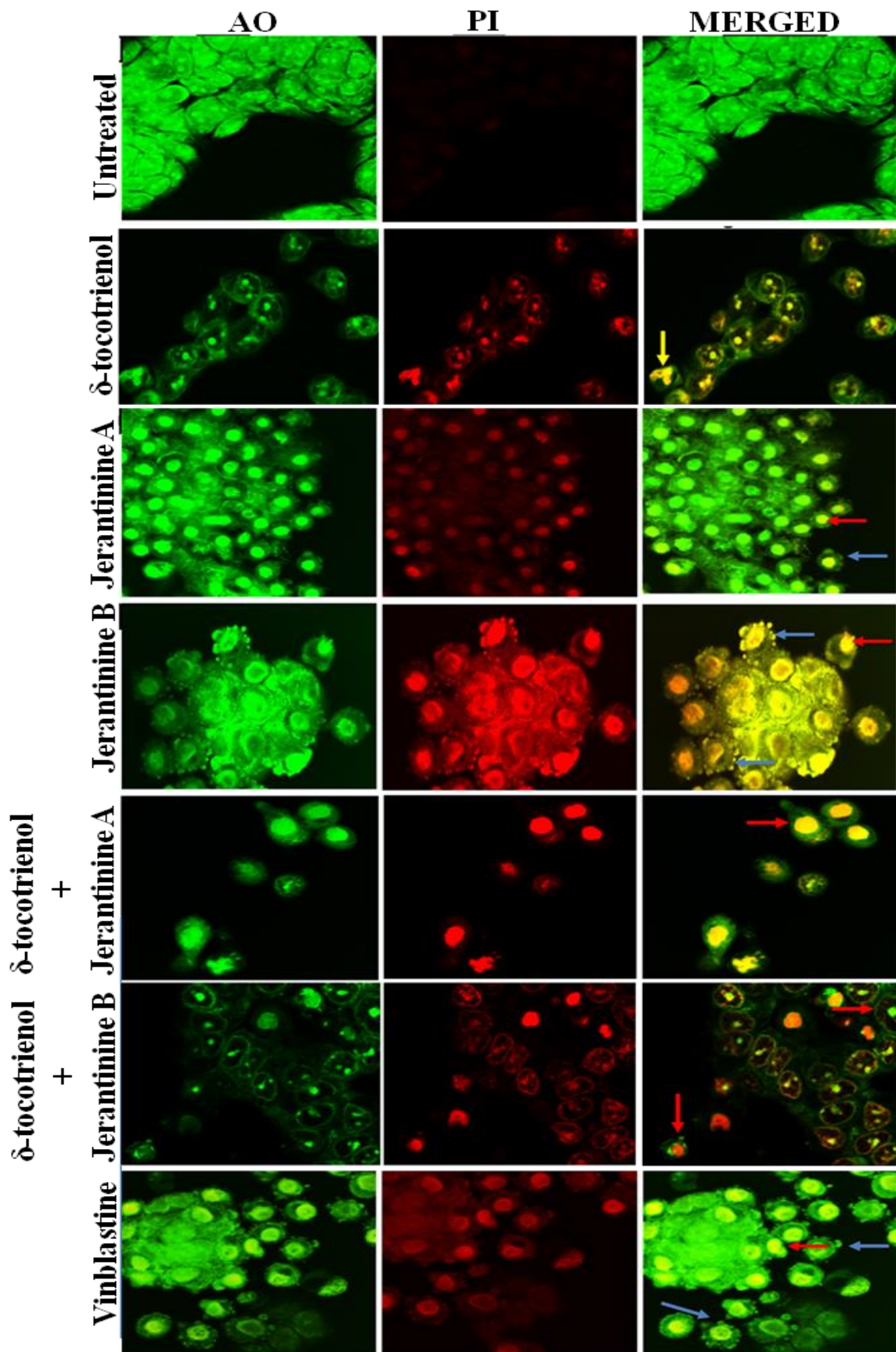
Fig 4.6: Representative AO/PI images of HT-29 cells treated for 24 h with individual and combined low-dose delta tocotrienol with IC_{20} doses of jerantinines (A and B). HT-29 cells treated with vinblastine and plain media containing DMSO served as positive and negative controls, respectively. Morphological features of apoptosis including nuclear chromatin condensation (red arrows), nuclear chromatin fragmentation (yellow arrows) and membrane blebbing (blue arrows) were evident. Images were captured at 40X magnification and merged using the NIS-Elements software under Nikon Ti eclipse confocal microscope observation.

4.3.4. DNA damage patterns of U87MG, A549 and HT-29 cells treated with individual and combined treatments

As shown in Fig. 4.7, a considerable double stranded DNA break (DSBs) was induced in U87MG cells treated with single IC_{50} dose of jerantinine A, jerantinine B, delta and gamma tocotrienols and control drug, vinblastine (Fig. 4.7a). Interestingly combined low-doses of jerantinine B with delta tocotrienol gave similar effects to single IC_{50} dose delta tocotrienol (Fig. 4.7a, c). Similarly, combined treatment of low-dose gamma tocotrienol with jerantinines A and B induced a slightly higher DSBs compared to single IC_{50} dose treatment with gamma tocotrienol. In contrast, minimal or no SSBs was evident on U87MG cells receiving individual and combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) (Fig. 4.7b, d). Similarly, control drug vinblastine did not induce SSBs in U87MG cells (Fig 4.7b, d).
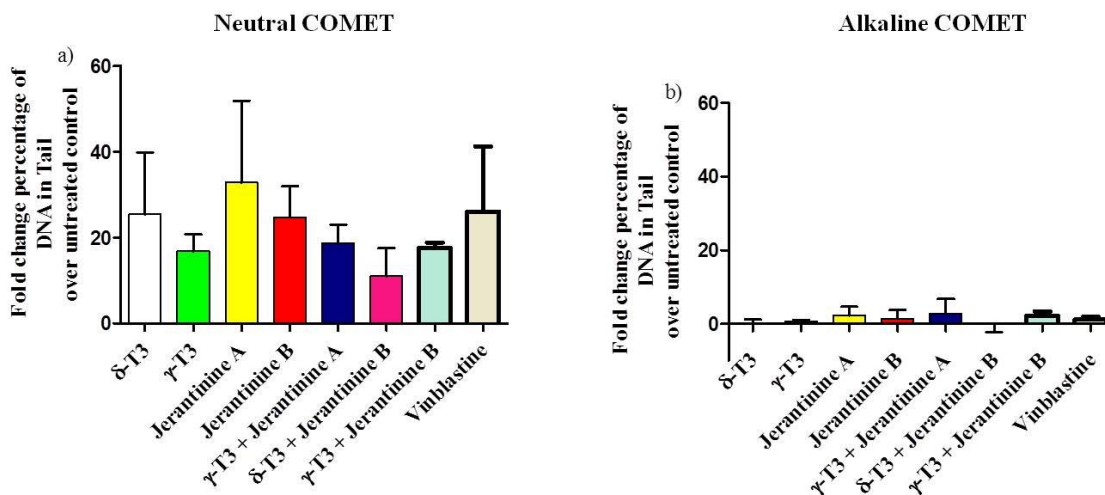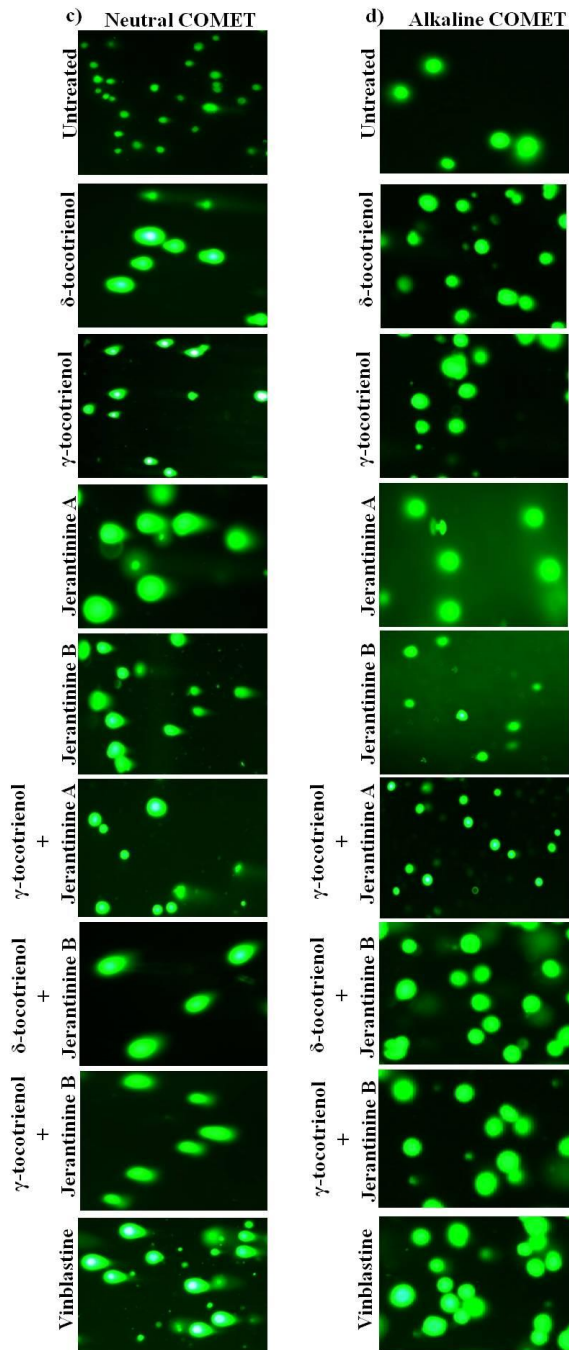
Fig 4.7: Induction of DNA damage by individual IC_{50} doses and combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} dose of jerantinines (A and B) on U87MG cells after 72 h. U87MG cells treated with plain media containing DMSO and IC_{50} dose of vinblastine served as negative and positive controls, respectively. Fold change in DNA damage over untreated controls under neutral (a) and alkaline conditions (b) represent DSBs and SSBs, respectively measured using OpenComet software from a population of 200 cells. Representative images of treated U87MG cells under (c) neutral and (d) alkaline conditions show migrating comets which indicate DNA damage. P*<0.05.

For A549 cells, individual treatments of IC_{50} doses of jerantinines A and B, and control drug vinblastine induced significant DSBs in A549 cells as confirmed by neutral COMET assay with over 50% tail DNA (Fig. 4.8a, c). A similar trend was also observed in A549 cells treated with delta tocotrienol and combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine A (Fig. 4.8a). In contrast, similar treatments could not induce SSBs as confirmed by alkaline electrophoresis, although a slight but non-significant increase in tail DNA percentage was observed in A549 cells treated with jerantinines A and B and delta tocotrienol compared to untreated control cells (Fig. 4.8b, d). Likewise, DSBs was also observed in HT-29 cells treated with jerantinine A, delta tocotrienol and combined low-dose treatment of delta tocotrienol with jerantinine A (Fig. 4.9a, c). As shown in Fig. 4.9a, jerantinine B and its combined low-dose treatment with delta tocotrienol also induced DSBs. In contrast, alkaline COMET revealed that jerantinines A and B and combined low-dose treatments but not delta tocotrienol and control drug vinblastine induced slight but non-significant increase of SSBs in treated HT-29 cells compared to untreated controls (Fig 4.9b, d).
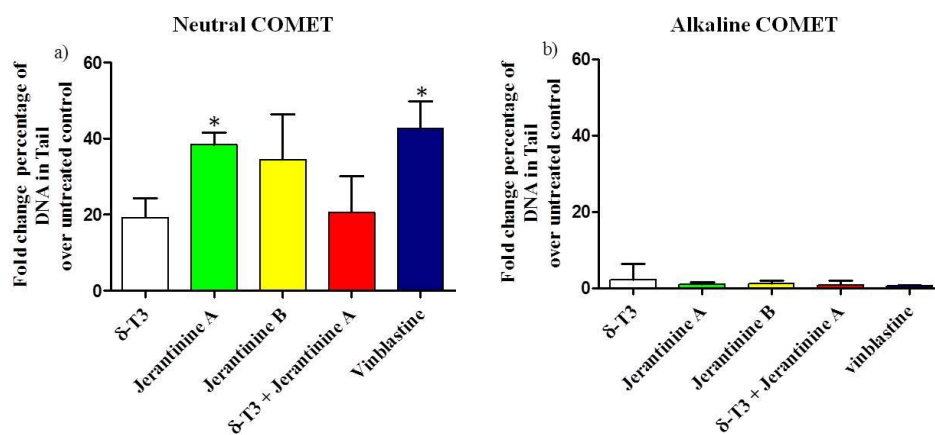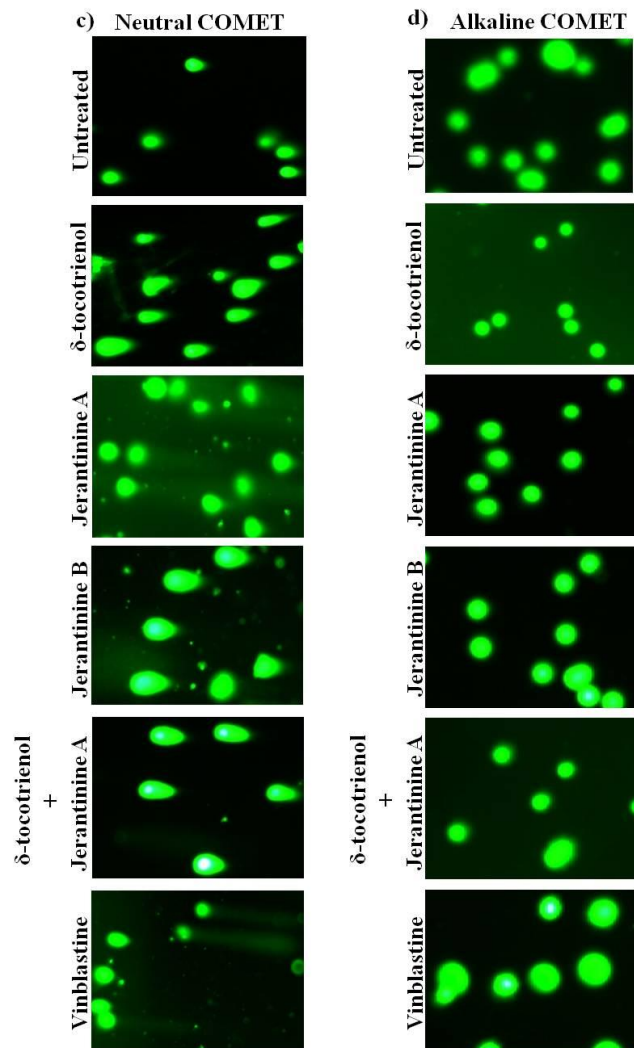
Fig 4.8: Induction of DNA damage by individual IC_{50} doses and combined low-dose treatments of δ-tocotrienol with IC_{20} dose of jerantinine A on A549 cells after 72 h. A549 cells treated with plain media containing DMSO and IC_{50} dose of vinblastine served as negative and positive controls, respectively. Fold change in DNA damage over untreated controls under neutral (a) and alkaline conditions (b) represent DSBs and SSBs, respectively measured using OpenComet software from a population of 200 cells. Representative images of treated U87MG cells under (c) neutral and (d) alkaline conditions show migrating comets which indicate DNA damage. $P^* < 0.05$.

Neutral COMET

a)

Fold change percentage of DNA in Tail over untreated control

δ-T3
Jerantinine A
Jerantinine B
δ-T3 + Jerantinine A
δ-T3 + Jerantinine B
Vinblastine

Alkaline COMET

b)

Fold change percentage of DNA in Tail over untreated control

δ-T3
Jerantinine A
Jerantinine B
δ-T3 + Jerantinine A
δ-T3 + Jerantinine B
Vinblastine

123
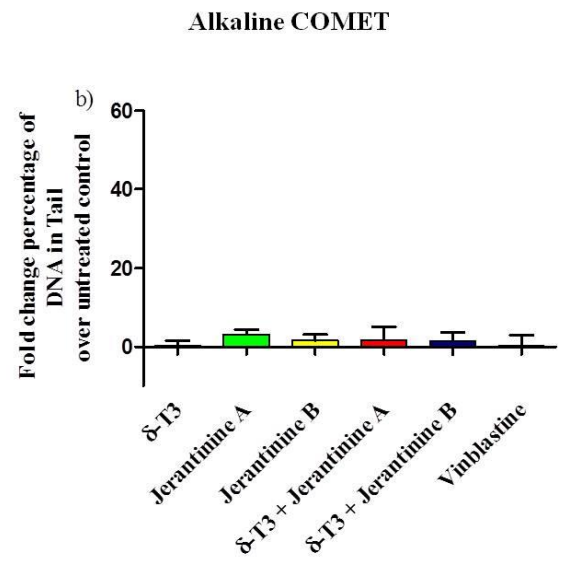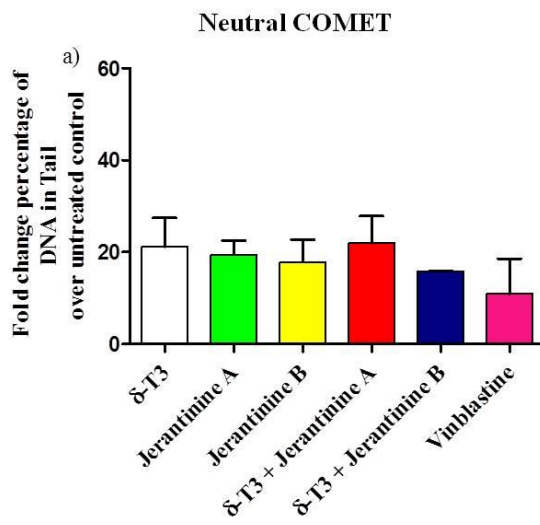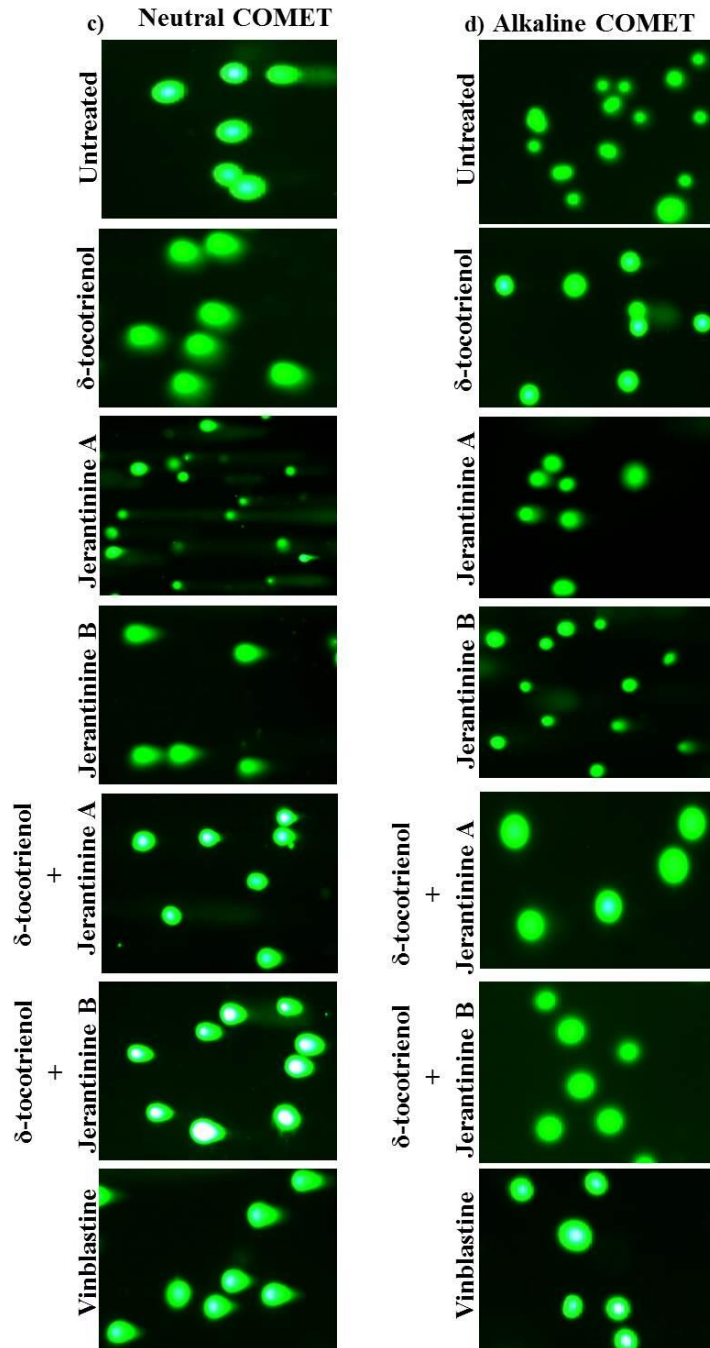
Fig 4.9: Induction of DNA damage by individual IC_{50} doses and combined low-dose treatments of δ-tocotrienol with IC_{20} dose of jerantinines (A and B) on HT-29 cells after 72 h. HT-29 cells treated with plain media containing DMSO and IC_{50} dose of vinblastine served as negative and positive controls, respectively. Fold change in DNA damage over untreated controls under neutral (a) and alkaline conditions (b) represent DSBs and SSBs, respectively measured using OpenComet software from a population of 200 cells. Representative images of treated U87MG cells under (c) neutral and (d) alkaline conditions show migrating comets which indicate DNA damage. P*<0.05.

4.4 Discussion

Apoptotic cell death has distinctively defined morphological features under the electron microscope which include an onset of cell and nucleus shrinkage, membrane blebbing, nuclear chromatin condensation and fragmentation into masses and its subsequent margination against nuclear membranes (Saraste and Pulkki, 2000). This results in disassembly of cells into smaller apoptotic bodies which either undergo secondary necrosis or in most cases occurrence of phagocytosis by neighbouring cells to avoid releasing its cellular content into other tissues and induce inflammation. In addition, DNA fragmentation and caspase activation are also used as biochemical hallmark for apoptosis.

Individual and combined low-dose treatments of tocotrienols (delta and gamma) with IC$_{20}$ doses of jerantinines (A and B) induced synergistic growth inhibition as reported in Chapter Three. To date there are no detailed studies on the effect of jerantinines (A and B) on morphology of cancer cells. This necessitated the present study to further investigate and determine the type of cell death induced by individual and combined low-dose treatments. Apoptosis cells undergoing cell shrinkage have low levels of Na$^+$ and K$^+$ that causes loss of contact with neighbouring cells and detachment from the extracellular matrix (ECM). This causes the cells to acquire rounded shaped morphology. This is followed by formation of blebs as a result of separation of the plasma membrane from cytoskeletons. Previous studies have demonstrated that in addition to previously perceived inhibition of cell to cell contact factors by caspases, cell membrane blebbing also requires phosphorylation of myosin light chain into an active form as well as

rearrangement of the actin cytoskeleton. Furthermore, condensation and subsequent formation of apoptotic bodies have been shown to correlate with dissolution of polymerized actin and degradation/disassembling of microtubules (Howard and Hyman, 2003; Stricker et al., 2010). In relation to the present study, treatment with jerantinines A and B and combined treatments with gamma or delta tocotrienol resulted in cell shrinkage, consistent membrane blebbing and formation of apoptotic bodies against all screened cell lines. This is consistent with the recent findings that showed disruption of microtubules by jerantinines A and B was accompanied by membrane blebbing (Qazzaz et al., 2016; Raja et al., 2014). Based on the similarities in apoptotic morphology with the present study, it is possible that the combined low-dose treatments may also evoke disruption of microtubule networks.

Unlike jerantinines A and B, there was no evidence of membrane blebbing although, other apoptotic features including nuclear chromatin condensation and fragmentation and cell shrinkage were evident in A549, U87MG and HT-29 cells treated with delta or gamma tocotrienol. This further corroborates previous studies illustrating the morphological features of apoptosis induced by tocotrienols. For instance, alpha, delta and gamma tocotrienols induced apoptosis in human lung and brain cancer cells by evoking changes in cell morphology including nuclear chromatin condensation and nuclear margination (Lim et al., 2014a). Similarly, nuclear chromatin condensation, cell blebbing and apoptotic bodies formation were also evident in breast cancer cells treated with gamma tocotrienol (Takahashi and Loo, 2004; Yu et al., 1999). Recent studies have also demonstrated that tocotrienols induce other forms of cell death. For instance, gamma

tocotrienol induced apoptosis and autophagy in prostate cancer cells (Jiang et al., 2012a). Similarly, gamma tocotrienol induced paraptosis cell death evoking changes in cellular morphology including swelling of mitochondria, endoplasmic reticulum and formation of intracellular vacuoles in SW620 human colon cancer cells (Zhang et al., 2013). In fact, tocotrienols have been shown to induce both apoptosis and other forms of cell death which may be dependent on dosage and microcellular environment. For instance, a high dose of delta tocotrienol (10μg/ml) isolated from *Kielmeryera coriacea* induced apoptotic features including chromatin condensation and fragmentation and features such as formation of pyknotic nuclei that is consistent with necrosis (De Mesquita et al., 2011). Similarly, combined treatment of gamma tocotrienol with 6-gingerol induced morphological features of cell death that included cell shrinkage and pyknosis (Yusof et al., 2015). However, in comparison to this study, features such as pyknotic nuclei that can be detected by H&E staining technique were not observed for individual and combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) as only features consistent with apoptosis were evident. These studies nonetheless further illustrate the dynamic potential of tocotrienols which may be dependent on the type of cell and concentration of tocotrienol administered. Histologically, apoptotic cells are single or cluster of cells that appear as circular or oval mass with dark cytoplasm and dense purple nuclear chromatin fragments upon staining with haematoxylin and eosin stains (Elmore, 2007). This is consistent with the features observed herein thereby implicating apoptosis as a mode of cell death induced by individual and combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B).

On the other hand, internucleosomal DNA fragmentation is used as a biochemical hallmark of apoptosis. Whilst the DNA laddering technique provides evidence for DNA damage illustrating the characteristic fragmentation, the DNA COMET assay allows for more detailed determination of SSBs or DSBs. Indeed, the present study showed that IC_{50} dose of jerantinine A, jerantinine B and vinblastine induced predominantly DSBs on A549, HT-29 and U87MG cells. Similarly, A549, HT-29 and U87MG cells treated with IC_{50} dose of delta or gamma tocotrienol showed evidence of DSBs breaks. Similar multitude of dominant DSBs were observed in combined low-dose treatments (Fig. 4.7, 4.8, 4.9) indicating a strong synergy as well as improved potency of tocotrienols. In addition, there was minimal or no evidence of induction SSBs on U87MG, A549 and HT-29 cells receiving individual and combined low-dose treatments (Fig 4.7b, 4.8b, 4.9b). Previous studies had shown that tocotrienol and vinblastine activate DSBs in cancer cells (Lim et al., 2014a, 2014b) which is consistent with the present study. Although in the present study slight but non-significant SSBs were also evident for A549 cells treated with delta tocotrienol. This could be attributed to the difference in automated scoring method adopted in this study compared to manual method in the previous study. In fact, SSBs and base damage account for 98% of overall DNA damage. However, the effect is insignificant in relation to cytotoxicity (Ismail et al., 2005). In relation to the present study, undoubtedly the apoptotic effects of tocotrienols, combined low-dose treatments with jerantinines (A and B) and vinblastine are as a result of DSBs. This is further supported by the observed high levels of DSBs compared to minimal or no evidence SSBs. To date, there are no reported studies on DNA damage patterns induced by

jerantinines and as such the results from the present study might be the first. Previous studies have shown that chemotherapeutic agents can induce both SSBs and DSBs. For instance, etoposide induces SSBs and DSBs by interacting with topoisomerase IIα (Fortoul et al., 2006). This results in p53 dependent and independent late S and G_2 cell growth arrests (Clifford et al., 2003; Lee and Xiao, 2012). Although a study demonstrated that jerantinine A induced G2/M growth arrest, the potential of jerantinine derivatives inhibiting topoisomerase II is unlikely considering that recent studies have confirmed inhibition and disruption of microtubule networks as mode of action for jerantinine derivatives (Frei et al., 2013; Raja et al., 2014). This is supported by the fact that etoposide that inhibits topoisomerase II has no effect on microtubules although its parent derivative podophyllotoxin disrupt microtubules to induce mitotic arrest (Lee and Xiao, 2012). Therefore, the apoptotic effects of jerantinines (A and B), tocotrienols and the combined low-dose treatments in this study are as a result of DSBs and the SSBs are insignificant effects at the onset of apoptosis that precedes the DSBs.

DSBs and DNA lesion prevent replication and transcription of DNA thus representing the lethal form of DNA damage that triggers a cascade of sensors consequently resulting in inhibition of cell cycle progression, DNA repair or induction of apoptosis. Phosphatidyl inositol 3-kinase related kinases ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) are key cellular sensors of DNA lesions that are activated directly or indirectly upon induction of DNA DSBs by cisplatin, irradiation (IR) or methylation agents (Norbury and Zhivotovsky, 2004). Although both pathways are different but are related at the downstream. ATR is primarily activated by SSBs that block and cause the

collapse of the DNA replication fork. Eventually though, these DNA lesions blocking replication fork may be converted to DSBs (Roos and Kaina, 2013). As demonstrated by the present study, jerantinine A, jerantinine B and the combined low-dose treatments with delta or gamma tocotrienol induced a dominant DSBs with minimal or no evidence of SSBs as such it is possible that both ATM and ATR are activated. Furthermore, the minimal SSBs observed in some treatments herein may be the resultant delayed conversion to DSBs.

In addition, p53 plays a vital role in DNA damage induced apoptosis via activation of ATM or ATR. ATM and ATR phosphorylate Ser15 of p53 causing direct activation. p53 is also indirectly phosphorylated via phosphorylation of upstream ChK2 (checkpoint kinase-2) and ChK1 (checkpoint kinase-1) by ATM and ATR upon induction of DSB and collapsed replication fork respectively (Guo and Kumagai, 2000; Shieh et al., 2000). Regardless of the type of DNA damage and kinases activated by both individual and combined treatments of jerantinines (A and B) with delta or gamma tocotrienol, it is highly possible that DNA damage induced apoptosis may be mediated via up-regulation of p53. Indeed, studies have shown the up-regulation of p53 by gamma tocotrienol and jerantinine A (Kannappan et al., 2010; Raja et al., 2014) further supporting the aforementioned proposition.

4.5 Conclusion

Treatments for U87MG, A549 and HT-29 cells with individual and combined low-dose tocotrienols (delta and gamma) with IC_{20} dose of jerantinines (A and B) caused changes in cellular morphological features including nuclear chromatin condensation and fragmentation, cell shrinkage and apoptotic bodies implicate apoptosis phenomenon without doubt. The apoptotic potential was further confirmed as jerantinines (A and B) and the combined low-dose treatments induced dominant DSBs on A549, HT-29 and U87MG. The similarities in DNA damage patterns and morphological features characterised by massive membrane blebbing induced by jerantinines (A and B) and control drug vinblastine indicate the possibility of these compounds having similar mechanism of action. Undoubtedly, the manifestations of minimal or no evidence of SSBs suggest that the apoptotic effects are as a result of DSBs which may be mediated via dependent or independent of p53.

5.0 CHAPTER FIVE

Induction of cell cycle arrest and disruption of microtubule mediated via caspase dependent and independent apoptosis by individual and combined treatments of tocotrienols (delta and gamma) and jerantinines (A and B)

5.1 Introduction

Results from Chapter Four revealed that single and combined treatments induced changes in cellular morphology that included membrane blebbing, cell shrinkage, nuclear chromatin fragmentation and condensation and formation of apoptotic bodies. In addition, there was an induction of DSBs pattern of DNA damage on U87MG, A549 and HT-29 cells that indicating an occurrence of apoptotic cell death. As in most cases, apoptosis is characterized by growth arrest at different points of the cell cycle. Activation of caspase enzymes is one of the key biochemical hallmarks for apoptosis although there is also evidence of caspase independent apoptotic cell death. In addition, the manifestation of morphological features such as membrane blebbing and DSBs type of DNA damage as shown in Chapter Four are similar to those of microtubule inhibiting control drug, vinblastine. Considering that disruption of microtubules has been suggested as a possible mechanism of apoptosis induction for jerantinine derivatives, it is imperative to determine the type of cell cycle arrest involved as it relates to antimicrotubule effects of jerantinines. Besides, the combined low-dose treatments would represent a shift in cell cycle arrest considering that both tocotrienols and jerantinines may induce different growth arrest patterns. Microtubule targeting agents mediate apoptosis via the mitochondria pathway although the death receptor pathway is also

activated. Hence, the potential roles of caspase enzymes as well as cell death dependent and independent of caspase enzymes were investigated.

Flow cytometry was used to evaluate the various checkpoints of cell cycle arrest. This is based on the univariate analysis of cellular DNA content stained with propidium iodide (PI) that allows for determination of cell distribution across the various phases (G1, S and G2/M) of the cell cycle presented as DNA histogram of a singlet cells (Nunez, 2001).

On the other hand, immunofluorescence microscopic technique was employed for morphological investigations on microtubule structures caused by the effects of individual and combined low-dose treatments. The technique is based on the use of antibody raised against tubulins. Tubulins (alpha and beta) are the monomers that make up the microtubules. Polymerization dynamics can be detected via morphological observation. Colorimetric techniques were conducted for quantitative evaluation of caspase enzymes activities. The technique is based on the spectrophotometric determination p-nitroanilide (pNA) after cleavage from the respective substrates of caspase 8 (IETD-pNA), caspase 9 (LEHD-pNA) and caspase 3 (DEVD-pNA). The cysteinyl aspartic acid-protease (caspase) enzymes play the key roles in mediating both death receptor and mitochondria pathway of apoptosis. Initiator caspase 8 and 9 trigger death receptor and mitochondria pathway, respectively that are executed via activation of executioner caspase 3. The mitochondria pathway is initiated upon loss of mitochondrial membrane potential and subsequently executed through activation of APAF-1 and caspase 9 by cytochrome c thereby forming an apoptosome (Fulda et al., 2010).

Therefore, caspase involvement in apoptotic pathway should be elucidated. Besides, cell viability studies were conducted in the presence of caspase specific inhibitors to determine the role of caspase in the cell death phenomenon induced by individual and combined low-dose treatments. The caspase 8, 9 and 3 specific inhibitors are synthetic peptides that irreversibly inhibit FLICE (FADD-like ICE), caspase 9 and caspase 3 related activities to block apoptosis.

The specific objectives of the present chapter are:

i)   To determine the checkpoints of cell cycle arrest on cancer cells treated with single and combined treatments by using flow cytometry.

ii) To determine the potential antimicrotubule effects of individual and combined low-dose treatments.

iii) To determine the role of caspase enzymes in mediation of apoptosis and cell death phenomenon induced by single and combined treatments.

5.1. Materials and Methods

5.1.1 Chemical reagent/kits and cell cultureware

The following reagents and kits listed in Table 5.1 were used to conduct the experiments.

Table 5.1: List of chemical reagents, kits and glassware used and the respective manufacturers.

s/no	Chemical reagents/kits	Manufacturer
1	Monoclonal antitubulin antibody and goat anti mouse IgG (FITC)	Santa Cruz Biotechnology, Santa Cruz, CA
2	Prolong gold antifade	Molecular Probes, Eugene, OR
3	4',6-diamidino-2-phenylindole (DAPI), triton X-100, sodium citrate, ribonuclease A solution, propidium iodide and bovine serum albumin (BSA)	Nacalai Tesque, Japan
4	Colorimetric caspase 8: (GTX 85543), caspase 9: (GTX 85538) and caspase 3: (GTX 85558) Kits	GeneTex, USA
5	Caspase specific inhibitors for caspase 8: Z-IE-(OME)-TD- (OME)-FMK (GTX47946), caspase 9: Z-LE-(OME)-HD-(OME)-FMK (GTX 47945) and caspase 3: ZD-(OME)-E-(OME)-VD-(OME)-FMK (GTX 47950)	GeneTex, USA
6	4-well chamber slides	SPL life sciences, Korea

5.1.1 Cell cycle analysis by flow cytometry

U87MG, A549 and HT-29 cells were seeded in 6-well plates and incubated for 24 h to facilitate attachment. Thereafter, the medium was replaced with treatment medium for single and combined low-dose treatments on the respective cell lines as indicated in Table 4.2. Following 24 h and 48 h incubation periods, a total of 1×10^6 cells were collected for cell cycle analysis according to the previously described protocol (Raja et

al., 2014). The treated and untreated cells were detached and washed twice with pre-chilled PBS with centrifugation at 2200rpm for 5min at 4°C. The cells were subsequently re-suspended in 500 µl flourochrome solution (0.1% Triton X-100, 0.1% sodium citrate, and 0.1mg ribonuclease and 50 µg propidium iodide) and stored overnight in the dark at 4°C. The cell cycle data was captured using the BD accuri c6 flow cytometer (BD biosciences, USA) and data from three repeats were analyzed using FCS express 5 (Denovo software, USA). The cell cycle checkpoint with the highest percentage increase in cell population compared to untreated controls was considered to be the point of cell cycle arrest.

5.1.2 Immunofluorescence staining for microtubules

U87MG, A549 and HT-29 cells were seeded at a density of 5 x 10^{3} cells in 4-well chamber slides and incubated for 24 h at cell condition to facilitate attachment. Thereafter, the medium was replaced with treatment medium for single and combined treatments on the respective cell lines as indicated in Table 4.2. Whereas, cells containing plain medium with equivalent amount of DMSO or IC$_{50}$ dose of vinblastine were used as negative and positive controls, respectively. After 72 h incubation, the cells were fixed and permeabilised with -20^{0}C methanol and 0.5% triton X-100 for 10 min. The cells were blocked with 10% BSA for 20 min and incubated with monoclonal antitubulin antibody at the dilution (1:200) in 1.5% BSA blocking buffer for 60min at 37°C. The slides were washed with three changes of PBS for 5min followed by incubation with goat anti mouse IgG (FITC) diluted at 1:200 in 1.5% BSA blocking buffer for 45 min at 37°C. Finally, the slides were stained with 10 µg/ml DAPI for 15 min and mounted with prolong gold

antifade. The slides were visualized and captured at 40X magnification under Nikon eclipse Ti confocal microscope (Nikon, Japan).

5.1.3 Caspase assay

U87MG, A549 and HT-29 cells were seeded at a density of 1 x 10^6 in 6-well plates (SPL Life Sciences, Korea) and incubated for 24 h to facilitate attachment. Subsequently, the medium was replaced with treatment medium for individual IC_{50} doses and combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) on the respective cell lines as shown in Table 4.2. Whereas, cells treated with plain medium containing equivalent amount of DMSO served as untreated control. Following incubation at different time points in between 1 h – 48 h, cells were detached and centrifuged at 2200 rpm for 5 min to pellet cells. The cell pellets were re-suspended in pre-chilled PBS and centrifuged at 2200 rpm for 5 min to remove all traces of trypsin and media. The pellets were suspended in 50 µl of respective cell lysis buffer, incubated on ice for 10 min and centrifuged at 10,000 rpm for 1 min to collect the supernatant which was stored at -80°C until analysis. Gene Tex caspase 8, 9 and 3 kits were used for evaluating the activity of caspase enzymes. Fifty microliter of the supernatant was mixed with 50 µl 2X reaction buffer containing 10mM dithiothreitol (DTT). Thereafter, 5µl of conjugated chromophore p-nitroanilide (pNA) substrate (4mM) for respective caspases 8, 9 and 3 was added and the mixture was incubated for 4-6 h. The absorbance for treated and untreated samples was read at 405nm using the Epoch multiplate spectrophotometer (Biotek, USA) and the fold increase in caspase activities was determined by comparing

the results to untreated controls. Results were presented as mean ± standard deviation (SD) from triplicate experiments.

5.1.4 Caspase inhibition assay

U87MG, A549 and HT-29 cells were seeded at a density of 5 x 10^3 in 96-well plates (SPL life Sciences, Korea). Following an overnight incubation, the cells were treated with individual IC_{50} doses or combined low-dose tocotrienols with IC_{20} dose of jerantinines (A and B) as shown in Table 4.2 with or without 1μM individual caspase 8, caspase 9: Z-LE-(OME)-HD-(OME)-FMK) and caspase 3: ZD-(OME)-E-(OME)-VD-(OME)-FMK specific inhibitors for 72 h. U87MG, A549 and HT-29 cells treated with plain media containing DMSO with or without individual caspase 8, 9 and 3 specific inhibitors were served as control. Following 72 h incubation, the percentage of cell viability was determined using the neutral red uptake assay as previously described (Repetto et al., 2008). Results were presented as mean ± SEM of triplicates conducted from three independent experiments.

5.1.5 Statistical analysis

Statistical analysis was conducted using one way ANOVA (Dunnets t-test) to compare between treated and untreated groups for cell cycle analysis, caspase assay and caspase inhibition assay. P values <0.001 (***), P <0.01 (**) and P <0.5 (*) were considered statistically significant.

5.2 Results

5.2.1. Determination of cell cycle arrest induced by single and combined treatments

Following 24 h and 48 h treatments, the effects of both individual and combined low-dose treatments on cell cycle were analyzed based on three independent experiments. In comparison to untreated control, the individual IC_{50} dose of delta tocotrienol induced an increase of G_0/G_1 cell population with 81.4% and 87.5% arrests in U87MG cells after 24 h and 48 h, respectively (Fig. 5.1). Similar patterns of growth arrest of 67.7% and 79.9% at G_0/G_1 were evident in U87MG cells receiving IC_{50} dose of gamma tocotrienol after 24 h and 48 h, respectively. As shown in Table 5.2, this represented up to 26.3% and 18.7% increase of G_0/G_1 cell population (P <0.001) for U87MG cells receiving IC_{50} dose of delta and gamma tocotrienols, respectively. In contrast, significant G2/M growth arrest was evident in U87MG cells treated with IC_{50} of jerantinine A (66.9% and 83.5%) and jerantinine B (67.9% and 57.3%) after 24 h and 48 h which represented a significant (P <0.001) increase of up to 67.6% and 52%, respectively (Fig. 5.1). Unlike single IC_{50} doses of tocotrienols (delta and gamma) and jerantinines (A and B), the combined treatment of low-dose gamma tocotrienol with IC_{20} dose of jerantinine A demonstrated a change in pattern of cell cycle arrest from 7.5% (P <0.001) increase in G2/M arrest to 23.6 % (P <0.001) increase in G0/G1 populations of U87MG cells after 24h and 48h, respectively. In contrast, the combined treatment of low-dose gamma tocotrienol with IC_{20} dose of jerantinine B induced slight increase of 3.6% in S population and significant increase of 13.4% (P <0.001) in G0/G1 population of U87MG cells after 24 h and 48 h, respectively. Similar trend was also evident for combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine B, although, there was significant (P <0.001)

increase in S (11.1%) populations after 24 h (Table 5.1). On the other hand, single IC_{50} dose of delta tocotrienol induced slight increase (6.2%) in G2/M and a significant increase of G0/G1 (17.8%) populations of A549 cells after 24 h and 48 h, respectively (Table 5.3). In contrast, as shown in Fig 5.2, jerantinines (A and B) and the combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine A induced significant increase (P <0.001) in G2/M populations of A549 cells after 24 h and 48 h (Table 5.3). As shown in Fig 5.3, single IC_{50} dose of delta tocotrienol induced slight increase in G0/G1 population (24 h) with a more significant increase (P <0.05) evident after 48h. In contrast IC_{50} doses of jerantinines (A and B) induced significant increase of up to 70% in G2/M populations of HT-29 cells after 24 h and 48 h treatments. On the other hand, an increase in G0/G1 populations was evident on HT-29 cells treated with combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine B for 24 h and 48 h. Whereas, an increase in G0/G1 and S populations were evident in HT-29 cells receiving combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine A for 24 h and 48 h, respectively (Table 5.4).

a) **Untreated**

% G0G1=61.2
% G2/M=15.9
% S = 22.9

δ-tocotrienol **γ-tocotrienol** **Jerantinine A** **Jerantinine B** **δ-tocotrienol + Jerantinine B** **γ-tocotrienol + Jerantinine A** **γ-tocotrienol + Jerantinine B**

24h

% G0/G1=81.4
% G2/M=5.8
% S = 12.9

% G0/G1=67.7
% G2/M=10.5
% S = 21.9

% G0/G1=15.2
% G2/M=66.9
% S = 17.9

% G0/G1=19.2
% G2/M=67.9
% S = 12.9

% G0/G1=56.7
% G2/M=12.8
% S = 30.5

% G0/G1=51.7
% G2/M=23.4
% S = 24.9

% G0/G1=58.8
% G2/M=14.7
% S = 26.5

48h

% G0/G1=87.5
% G2/M=4.8
% S = 7.7

% G0/G1=79.9
% G2/M=7.08
% S = 12.9

% G0/G1=16.5
% G2/M=83.5
% S = 0.00

% G0/G1=17.8
% G2/M=57.3
% S = 24.9

% G0/G1=72.3
% G2/M=15.5
% S = 12.2

% G0/G1=84.8
% G2/M=2.2
% S = 12.9

% G0/G1=74.6
% G2/M=11.6
% S = 13.8

Fig 5.1: a) Representative flow cytometric profiles of U87MG cells treated with individual and combined low-dose treatments for 24 h and 48 h. Histogram profiles of flow cytometric data for 24 h (b) and 48 h (c) based on three independent experiments. Significant difference for higher cell populations in treated compared to untreated groups was calculated at $p < 0.5$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Table 5.2: Induction of cell cycle arrest on U87MG cells by single and combined treatments.

Samples	Changes over untreated control (%)						Designated cell cycle arrest			
	G0/G1		G2/M		S		Stage	p-value	Stage	P-value
	24h	48h	24h	48h	24h	48h	24h		48h	
δ-T3	+20.2	+26.3	-10.1	-11.1	-10.0	-15.2	G0/G1	***	G0/G1	***
γ-T3	+6.5	+18.7	-5.4	-8.8	-1.0	-10.0	G0/G1	***	G0/G1	***
JA	-46.0	-44.7	+51.0	+67.6	-5.0	-22.9	G2/M	***	G2/M	***
JB	-42.0	-43.4	+52.0	+41.4	-10.0	+2.0	G2/M	***	G2/M	***
δ-T3+ JB	-4.5	+11.1	-3.1	-0.4	+7.6	-10.7	S	***	G0/G1	**
γ-T3 + JA	-9.5	+23.6	+7.5	-13.7	+2.0	-10.0	G2/M	***	G0/G1	***
γ-T3 + JB	-2.4	+13.4	-1.2	-4.3	+3.6	-2.1	S	-	G0/G1	***

The points of cell cycle arrest tested at 24h and 48h are shaded, statistically significant percentage of populations higher in treated comparing to untreated groups are set at $P < 0.001$ (***), $P < 0.01$(**), $P < 0.05$(*).

Untreated

% G0/G1=68.7
% G2/M=11.3
% S = 20.0

δ-tocotrienol
+

δ-tocotrienol   **Jerantinine A**   **Jerantinine B**   **Jerantinine A**

24h

% G0/G1=69.5
% G2/M=17.5
% S = 13.1

% G0/G1=19.9
% G2/M=65.9
% S = 14.2

% G0/G1=21.7
% G2/M=66.7
% S = 11.4

%G0/ G1=35.1
% G2/M=52.7
% S = 12.2

48h

% G0/G1=86.5
% G2/M=9.1
% S = 4.5

% G0/G1=31.3
% G2/M=60.7
% S = 8.0

% G0/G1=8.3
% G2/M=87.2
% S = 4.6

% G0/G1=40.7
% G2/M=52.5
% S = 6.8

a)

145

b)

Fig 5.2: a) Representative flow cytometric profiles of A549 cells treated with individual and combined low-dose treatments for 24 h and 48h. Histogram profiles of flow cytometric data for 24 h (b) and 48 h (c) based on three independent experiments. Significant difference for higher cell populations in treated compared to untreated groups was calculated at $p < 0.5$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Table 5.3: Induction of cell cycle arrest on A549 cells by single and combined treatments.

Samples	Changes over untreated control (%)						Designated cell cycle arrest			
	G0/G1		G2/M		S		Stage	p-value	Stage	P-value
	24h	48h	24h	48h	24h	48h	24h		48h	
δ-T3	+0.8	+17.8	+6.2	-2.2	-6.9	-15.5	G2/M	-	G0/G1	***
JA	-48.8	-37.4	+54.9	+49.4	-5.8	-12.0	G2/M	***	G2/M	***
JB	-47	-60.7	+55.4	+75.9	-8.6	-15.4	G2/M	***	G2/M	***
δ-T3+ JA	-33.6	-28.0	+41.4	+41.2	-7.8	-13.2	G2/M	***	G2/M	***

The points of cell cycle arrest tested at 24h and 48h are shaded, statistically significant percentage of populations higher in treated comparing to untreated groups are set at P < 0.001 (***), P < 0.01(**), P < 0.05(*).

a) **Untreated**

% G0/G1=67.4
% G2/M=21.4
% S = 11.2

δ-tocotrienol

δ-tocotrienol + Jerantinine A

δ-tocotrienol + Jerantinine B

δ-tocotrienol   **Jerantinine A**   **Jerantinine B**

24h

% G0/G1=70.7
% G2/M=17.4
% S = 11.9

% G0/G1=27.2
% G2/M=66.1
% S = 6.7

% G0/G1=19.1
% G2/M=76.8
% S = 4.2

% G0/G1=69.1
% G2/M=18.1
% S = 12.8

% G0/G1=68.4
% G2/M=21.2
% S = 10.4

48h

% G0/G1=72.7
% G2/M=20.4
% S = 6.9

% G0/G1=6.5
% G2/M=91.4
% S = 2.1

% G0/G1=11.7
% G2/M=88.0
% S = 0.3

% G0/G1=66.5
% G2/M=16.9
% S = 16.7

% G0/G1=71.4
% G2/M=17.5
% S = 11.2

149

b)

Percentage of cell

80

60

40

20

0

24h

G0/G1

S

G2/M

*** *** ***

Untreated

δ-tocotrienol

Jerantinine A

Jerantinine B

δ-tocotrienol + Jerantinine A

Untreated

δ-tocotrienol

Jerantinine A

Jerantinine B

δ-tocotrienol + Jerantinine A

Untreated

δ-tocotrienol

Jerantinine A

Jerantinine B
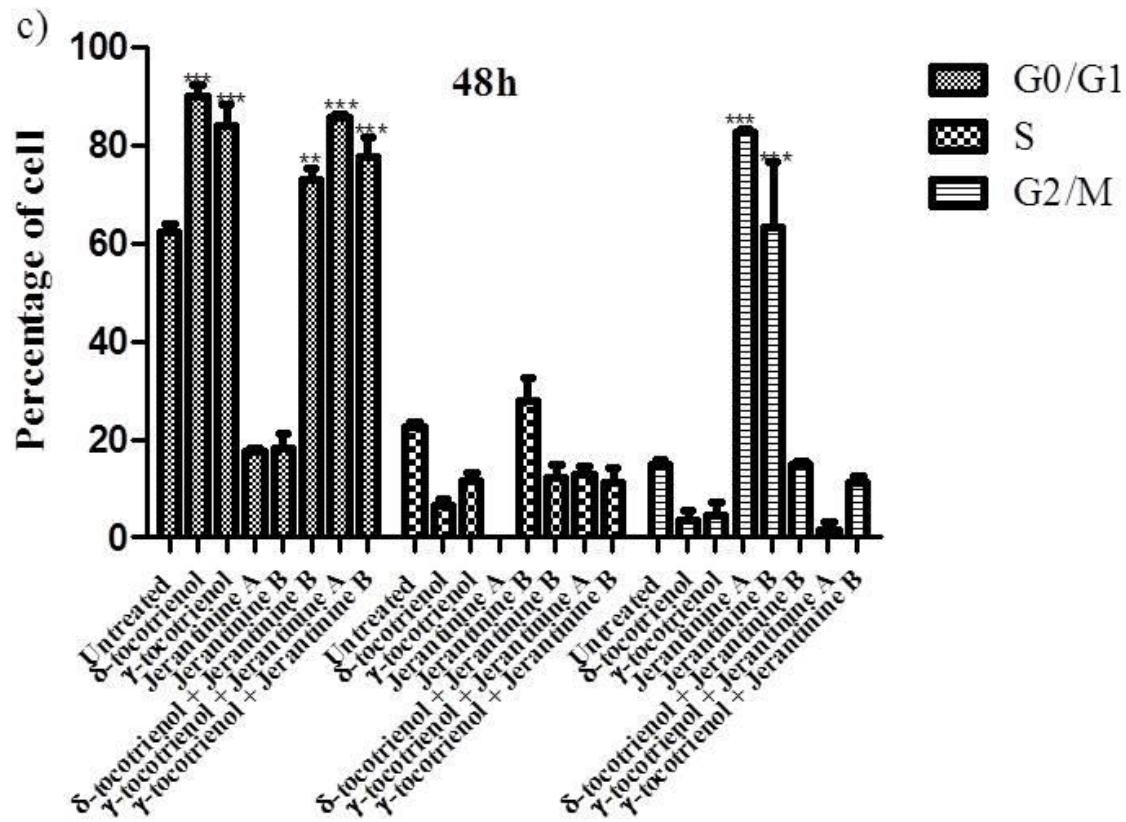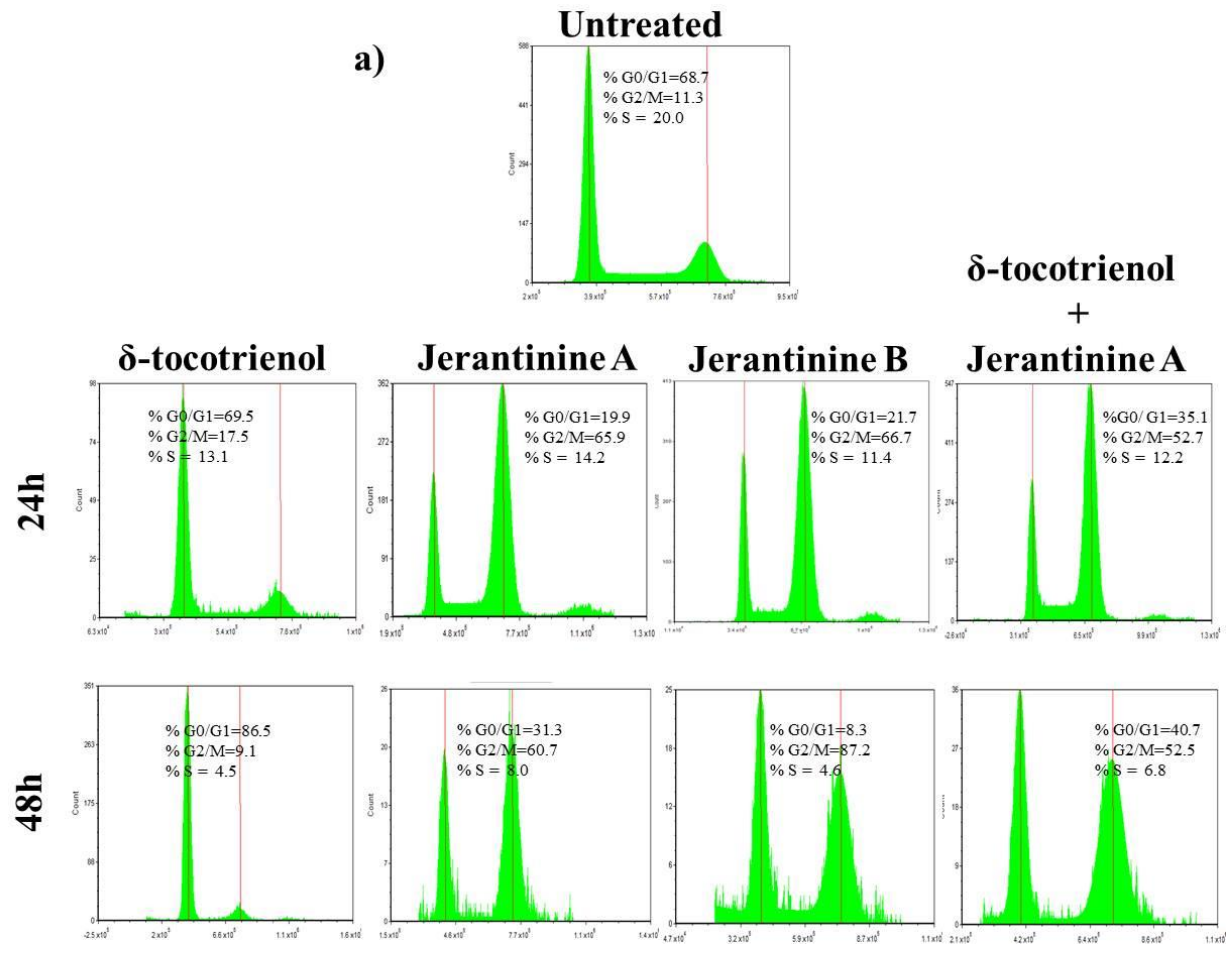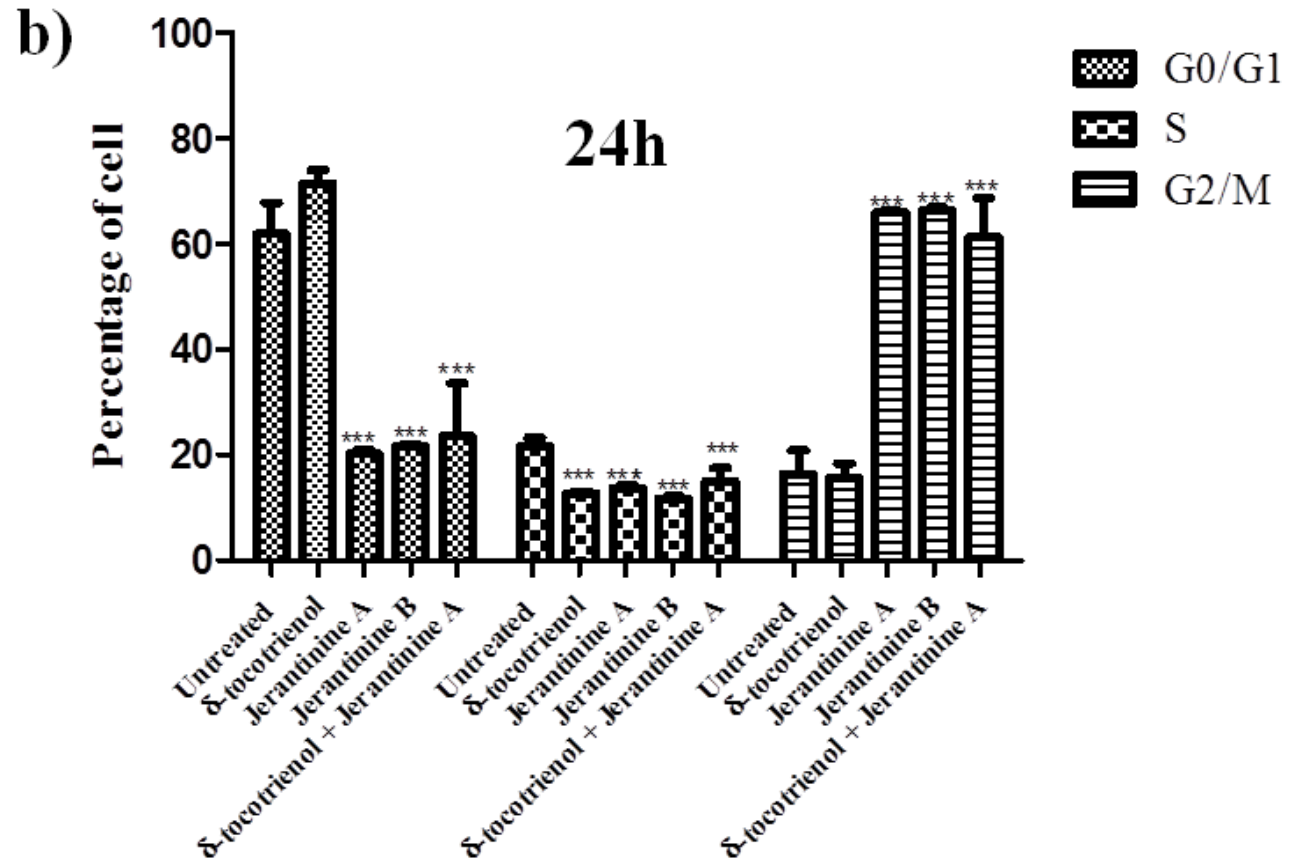
δ-tocotrienol + Jerantinine A

Fig 5.3: a) Representative flow cytometric profiles of HT-29 cells treated with individual and combined low-dose treatments for 24 h and 48 h. Histogram profiles of flow cytometric data for 24 h (b) and 48 h (c) based on three independent experiments. Significant difference for higher cell populations in treated compared to untreated groups was calculated at $p < 0.5$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Table 5.4: Induction of cell cycle arrest on HT-29 cells by single and combined treatments.

Samples	Changes over untreated control (%)						Designated cell cycle arrest			
	G0/G1		G2/M		S		Stage	p-value	Stage	P-value
	24h	48h	24h	48h	24h	48h	24h		48h	
δ-T3	+3.0	+5.3	-3.0	-1.0	+0.7	-4.3	G0/G1	-	G0/G1	*
JA	-60.9	-40.2	+44.7	+70	-9.1	-4.5	G2/M	***	G2/M	***
JB	-48.3	-55.7	+55.4	+66.6	-7.0	-10.9	G2/M	***	G2/M	***
δ-T3+ JA	+1.7	-0.9	-3.3	-4.5	+1.6	+5.5	G0/G1	-	S	-
δ-T3+ JB	+1.0	+4.0	-0.02	-3.9	-0.8	0.0	G0/G1	-	G0/G1	-

The points of cell cycle arrest tested at 24 h and 48 h are shaded, statistically significant percentage of populations higher in treated comparing to untreated groups are set at $P < 0.001$ (***), $P < 0.01$(**), $P < 0.05$(*).
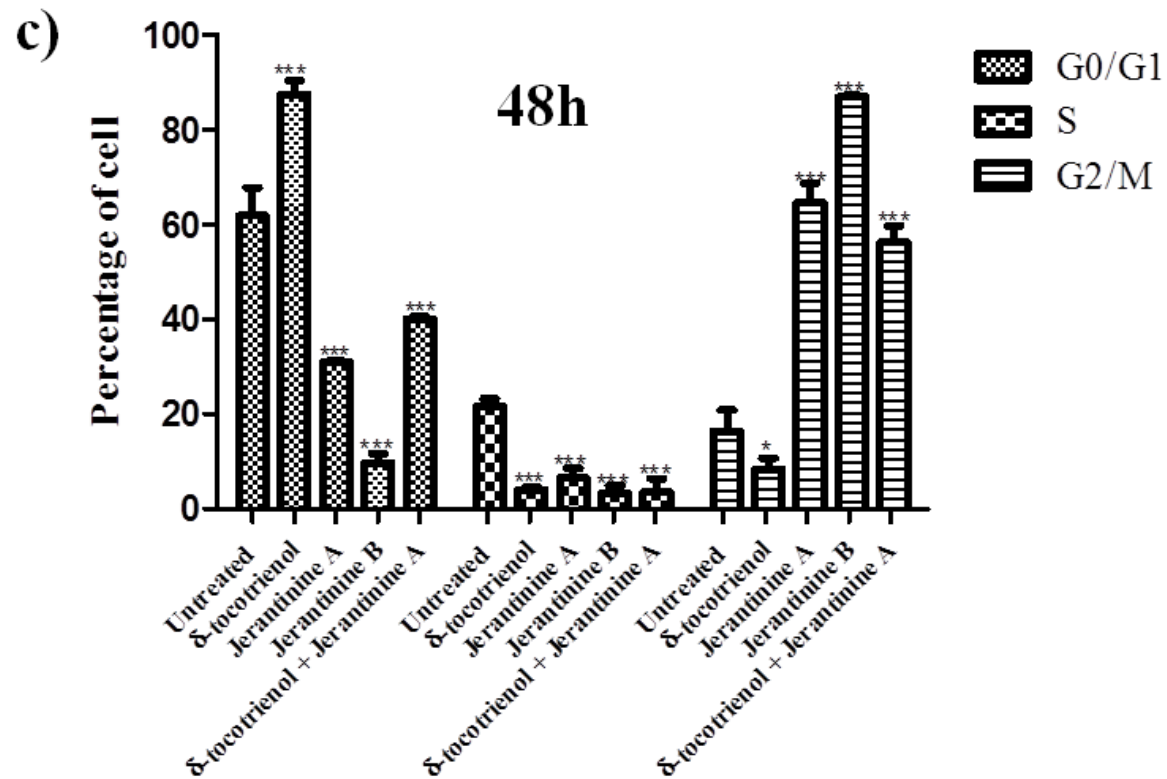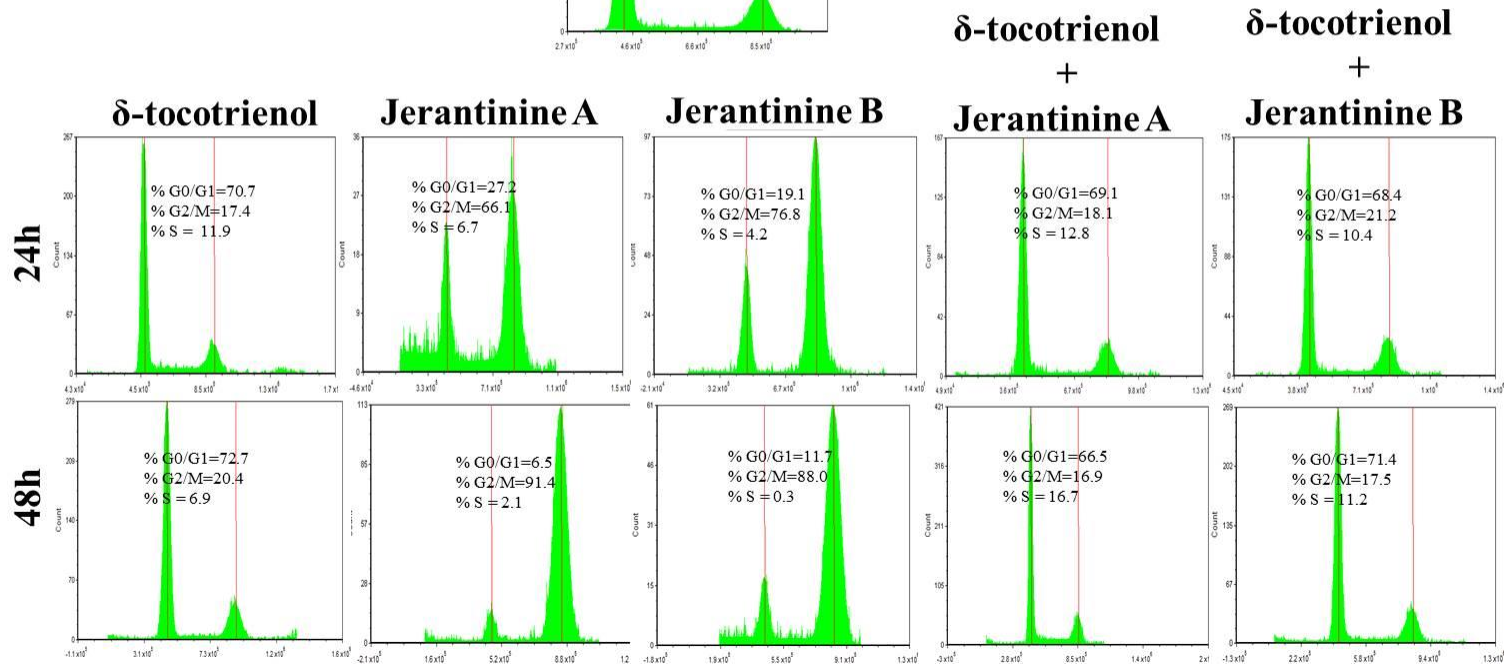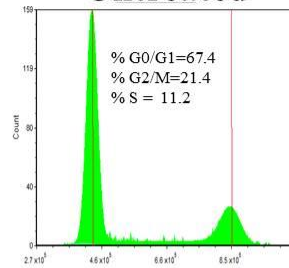
5.2.2. Observation of microtubules following single and combined treatments

Following 72 h incubation, immunoflourescense staining showed disruption of alpha tubulin by IC_{50} doses of jerantinines (A and B) on U87MG cells (Fig. 5.4). Similar effects were also observed in U87MG cells treated with IC_{50} dose of control drug, vinblastine (Fig. 5.4). In contrast, treatment of U87MG cells with IC_{50} dose of delta or gamma tocotrienol did not cause disruption of microtubules as confirmed by the intact alpha tubulin structures with extended cytoplasm in Fig. 5.4. Furthermore, A549 and HT-29 cancer cells receiving treatment for 72 h with IC_{50} doses of jerantinines (A and B) and vinblastine caused considerable disruption of microtubules as confirmed by alpha tubulin structures in Fig. 5.5 and 5.6, respectively. In a similar trend to that observed in U87MG cells, IC_{50} doses of delta tocotrienol did not induce disruption of microtubules in A549 (Fig. 5.5) and HT-29 cells (Fig. 5.6) thereby exhibiting similar pattern of intact alpha tubulin compared to untreated controls.

Interestingly, unlike single IC_{50} doses of delta or gamma tocotrienol, combined treatment of low- dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) caused considerable disruption of microtubules in U87MG cells (Fig. 5.4). Similar trend was also evident in A549 and HT-29 cells receiving combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B). This strongly indicates an improve potency of delta and gamma tocotrienols that results from its synergy with jerantinine A and B derivatives.

	α-tubulin	Dapi	Merged
Untreated			
δ-tocotrienol			
γ-tocotrienol			
Jerantinine A			
Jerantinine B			
γ-tocotrienol + Jerantinine A			
δ-tocotrienol + Jerantinine B			
γ-tocotrienol + Jerantinine B			
Vinblastine			
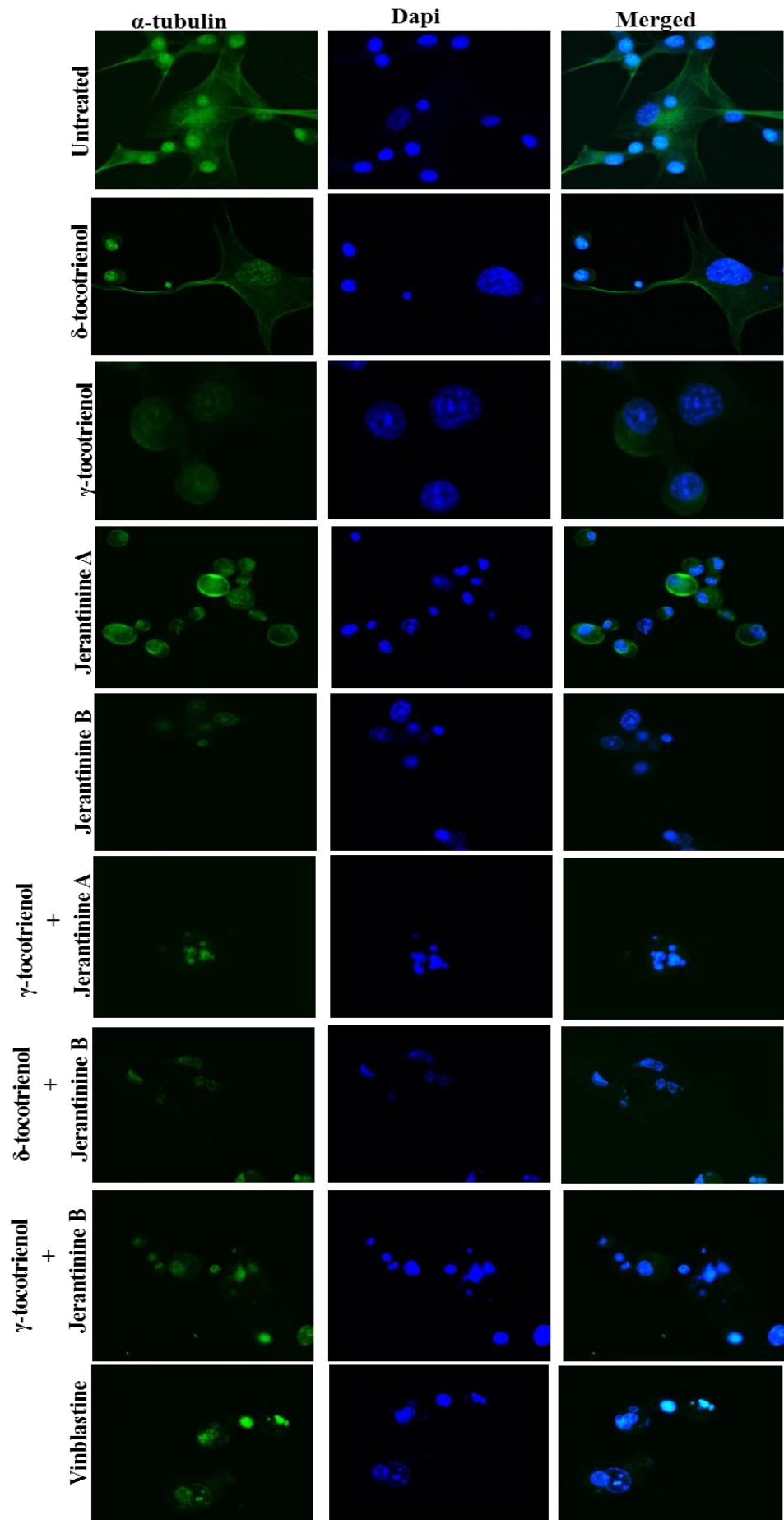
Fig 5.4: Immunofluorescence stained microtubules of U87MG cells reacted with monoclonal tubulin antibody. U87MG cells were treated with plain media containing DMSO (untreated control), IC_{50} dose of vinblastine (positive control) and single IC_{50} dose or combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) for 72 h. Vinblastine, jerantinines (A and B) and combined treatment of low-dose tocotrienols with IC_{20} doses of jerantinines (A and B) but not tocotrienols alone caused disruption of microtubules. Images were captured at 40X magnification under Nikon Ti eclipse confocal microscope (Nikon Japan).

	α-tubulin	Dapi	Merged
Untreated			
δ-tocotrienol			
Jerantinine A			
Jerantinine B			
δ-tocotrienol + Jerantinine A			
Vinblastine			

Fig 5.5 Immunofluorescence stained microtubules of A549 cells reacted with monoclonal tubulin antibody. A549 cells were treated with plain media containing DMSO (untreated control), IC_{50} dose of vinblastine (positive control) and single IC_{50} dose or combined treatment of low-doses delta tocotrienol with IC_{20} doses of jerantinines (A and B) for 72 h. Vinblastine, jerantinines (A and B) and combined treatment of low-dose delta tocotrienol with jerantinines (A and B) but not delta tocotrienol alone caused disruption of microtubules. Images were captured at 40X magnification under Nikon Ti eclipse confocal microscope (Nikon, Japan).

	α-tubulin	Dapi	Merged

Untreated

δ-tocotrienol

Jerantinine A

Jerantinine B

δ-tocotrienol + Jerantinine A

δ-tocotrienol + Jerantinine B
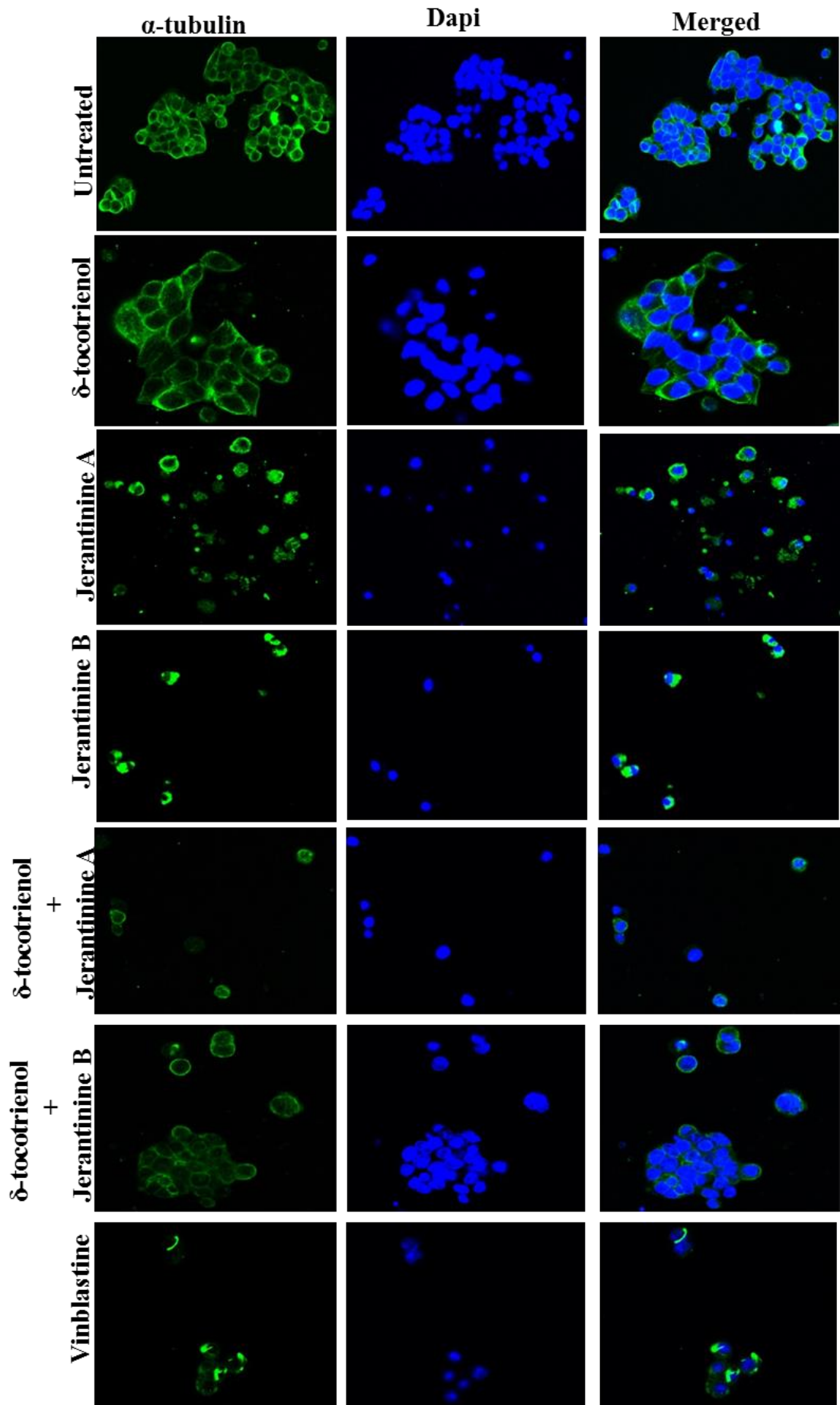
Vinblastine

Fig 5.6 Immunofluorescence stained microtubules of HT-29 cells reacted with monoclonal tubulin antibody. HT-29 cells were treated with plain media containing DMSO (untreated control), IC_{50} dose of vinblastine (positive control) and single IC_{50} dose or combined treatment of low-dose delta tocotrienol with IC_{20} doses of jerantinines (A and B) for 72 h. Vinblastine, jerantinines (A and B) and combined treatment of low-dose delta tocotrienol with IC_{20} doses of jerantinines (A and B) but not delta tocotrienol alone caused disruption of microtubules. Images were captured at 40X magnification under Nikon Ti eclipse confocal microscope (Nikon, Japan).

5.2.3. Evaluation of the effects of individual and combined treatments on caspase enzyme activity and caspase independent cell death

Following a kinetic study, the respective time points for peak activity of caspase 8, 9 and 3 upon treatment of cells were determined (see appendix 2). Similarly, 1 μM of caspase specific inhibitors suppressed the basal level of caspase enzymes (see appendix 3a,b). Following 1 h individual treatment with IC_{50} dose of delta and gamma tocotrienols an increase in caspase 8 activities was evident on A549 (36.6%, P <0.05), U87MG (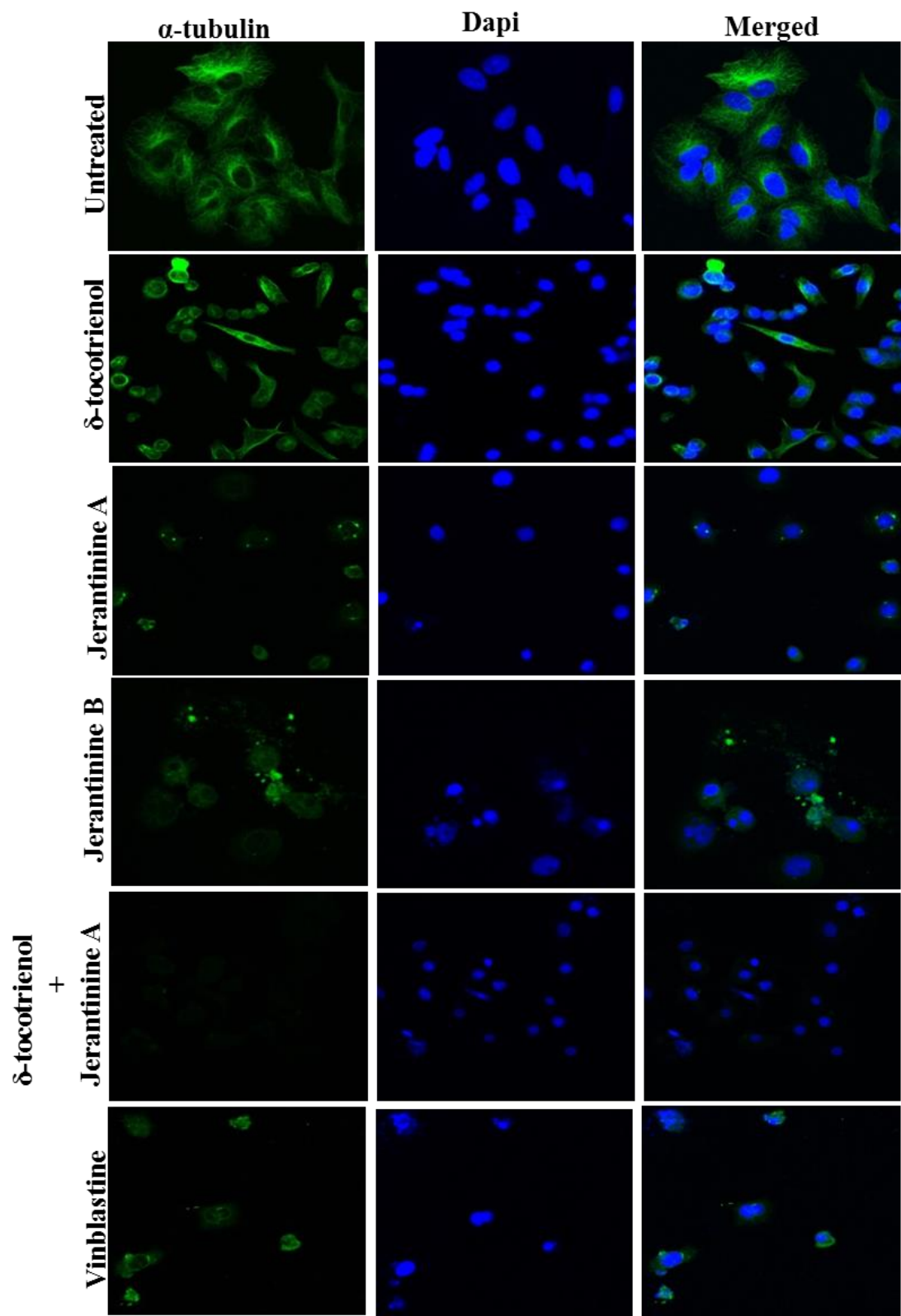21.4% and 11.3%) and HT-29 (18.7%) cells (Fig. 5.7 a, d, g). Similar increased caspase 8 activities compared to untreated controls were also observed after 9 h treatment with IC_{50} dose of jerantinine A (23.3%, 28.7% and 9.3%), jerantinine B (16.7%, 31.9% and 14.5%) and the respective combined low-dose treatments with delta or gamma tocotrienol on U87MG, A549 and HT-29 cells, respectively (Fig. 5.7 b, e, h). In contrast, an upregulation of caspase 9 levels was only evident on A549 cells after 24 h treatment with IC_{50} dose of delta tocotrienol (13.7%) but not on both HT-29 and U87MG cells treated with delta or gamma tocotrienol. Whereas, with the exception of HT-29 cells treated with jerantinine B, an increased caspase 9 activity was evident on U87MG (7.8%), A549 (12.1%) and HT-29 (5.2%) cells treated with single IC_{50} dose of jerantinine A and U87MG (1.6%) and A549 (12.0%) treated with single IC_{50} dose of jerantinine B (Fig. 5.7 b, e, h). Combined low-dose treatments of delta tocotrienol with IC_{20} dose of jerantinine B (14.5%), delta tocotrienol with IC_{20} dose of jerantinine A (24.3%) and delta tocotrienol with IC_{20} dose of jerantinine A (5.5%) induced the highest increase of caspase 9 levels on U87MG, A549 and HT-29 cells, respectively. Although such increased activity was at much lower level compared to caspase 8, nonetheless, this also demonstrates potential involvement of both initiator caspases in apoptosis. On the other hand, U87MG, A549 and HT-29

cancer cells receiving IC_{50} doses of delta or gamma tocotrienol, jerantinine A, jerantinine B and the combined low-dose treatments for 48 h induced an appreciable increase in caspase 3 activity (Fig. 5.7 g, h, i). In fact, significant increase compared to untreated controls was evident on U87MG (31.8%, $P < 0.05$) and HT-29 (20.4%, $P < 0.01$) receiving combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine B. Similar significant increase of caspase 3 enzyme activity was also evident only on HT-29 cells receiving IC_{50} doses of delta tocotrienol (26.1%, $P < 0.001$), jerantinine A (19.9%, $P < 0.01$) and jerantinine B (19.8%, $P < 0.01$).
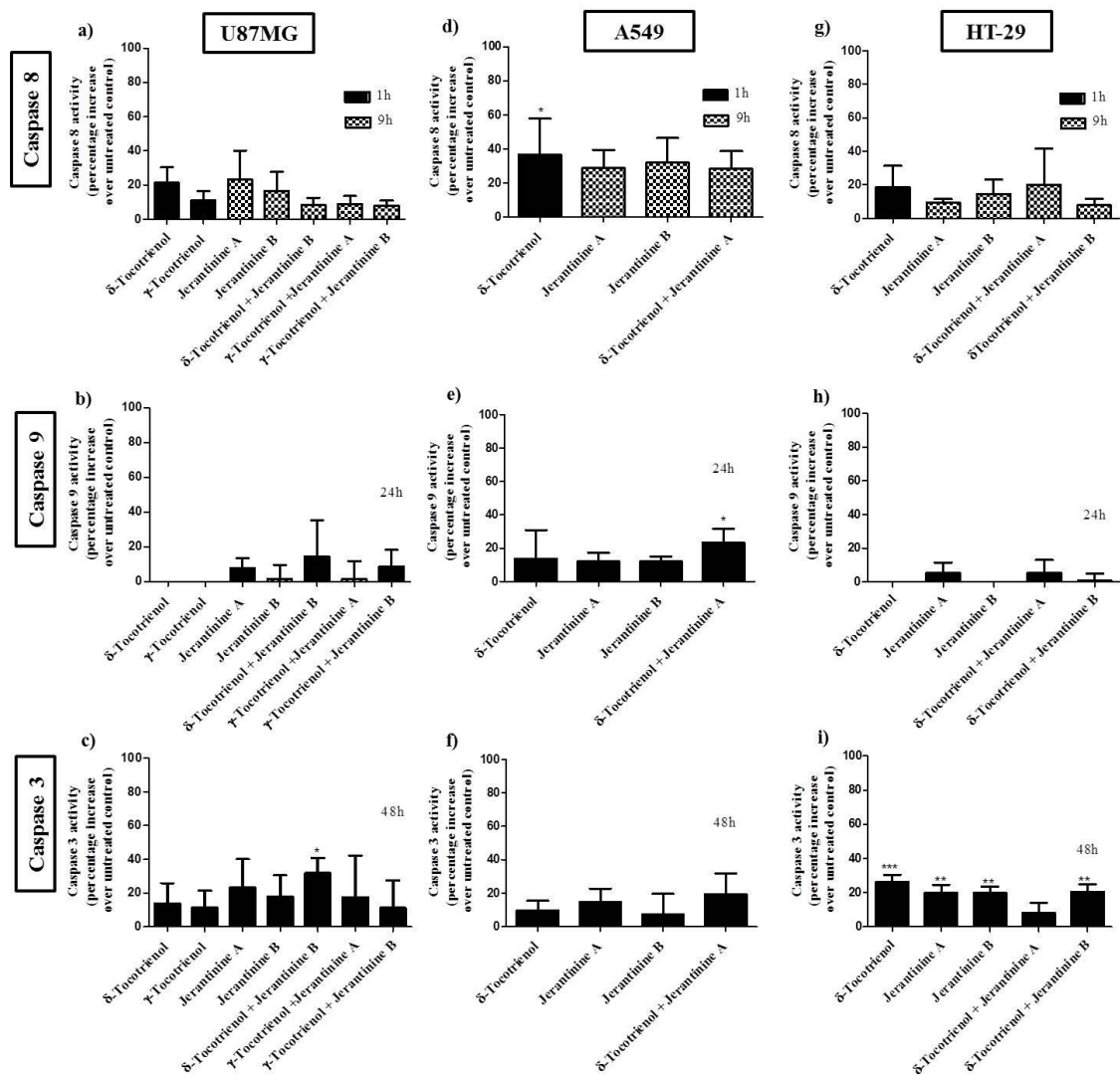
Fig 5.7: Induction of caspase 8, 9 and 3 enzymatic activities by individual IC_{50} dose and combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) on U87MG (a, b, c), A549 (d, e, f) and HT-29 cells (g, h, i). A total of 1×10^{6} cells were treated with respective treatments followed by protein extraction and subsequent determination of caspase activity. Results are presented as percentage increase of respective enzymatic activity over untreated control cells. P <0.001 (***), P <0.01 (**), P <0.05 (*) indicate significant increase of caspase enzyme activity for treated groups compared to untreated controls.
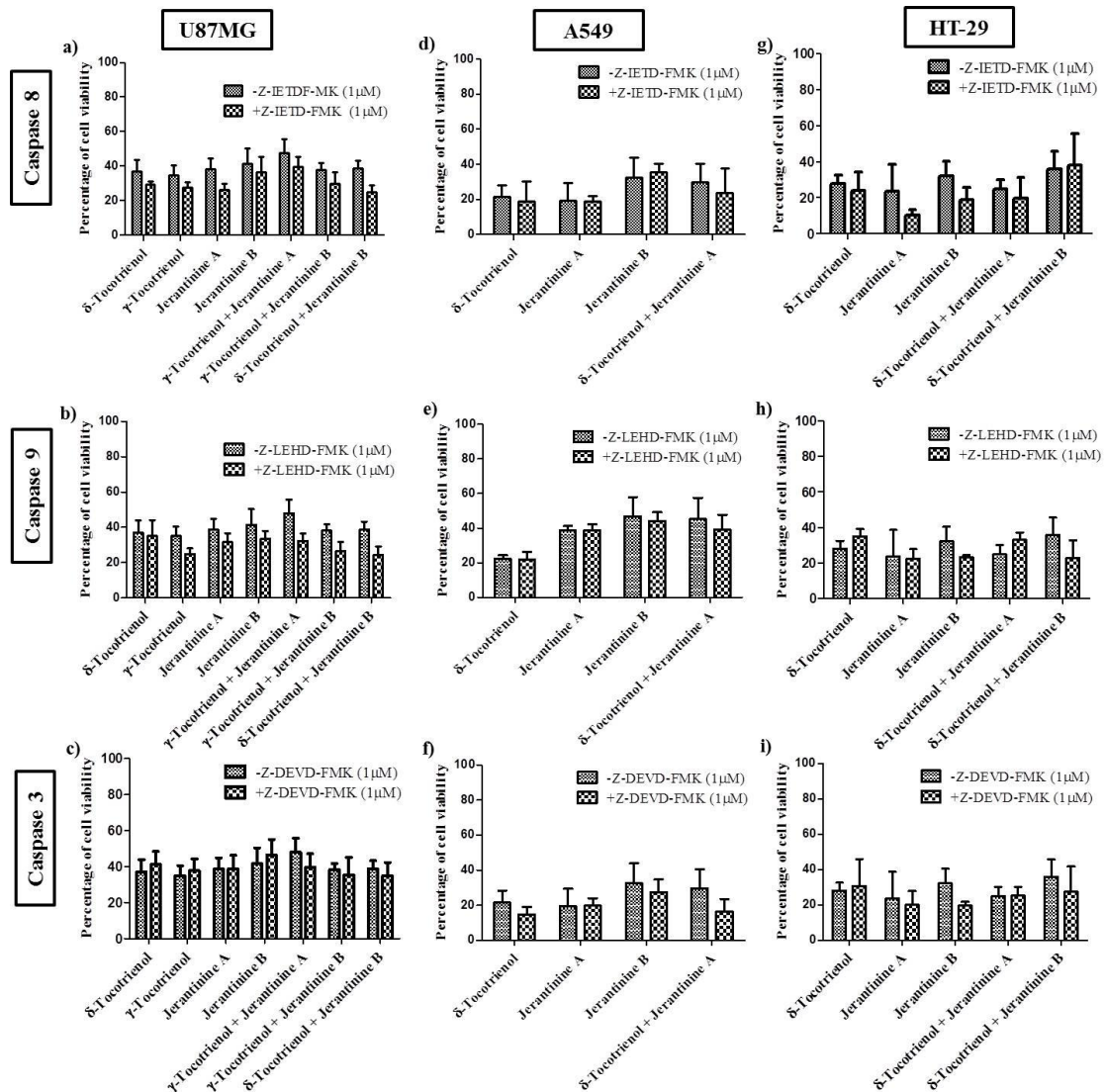
Fig 5.8: Cell viability profiles of U87MG (a, b, c), A549 (d, e, f) and HT-29 (g, h, i) cells treated with or without caspase specific inhibitors. A total of 5 x 10^{3}cells were treated with respective treatments of individual IC$_{50}$ dose or combined low-dose tocotrienols (delta and gamma) with IC$_{20}$ doses of jerantinines (A and B) with or without 1 μM of caspase 8 (Z-IETD-FMK), caspase 9 (Z-LEHD-FMK) and caspase 3 (Z-DEVD-FMK) inhibitors for 72h and the cell viability was determined by neutral red uptake assay. Results are presented as mean ± SEM percentage of cell viability comparing to untreated controls conducted in triplicates from three independent experiments. Both individual IC$_{50}$ doses and combined low-dose treatments of tocotrienols (delta and gamma) with IC$_{20}$ doses of jerantinine (A and B) induce cell death independent of caspase activation in U87MG, A549 and HT-29 cells. Statistical significance between groups with or without inhibitors was calculated at P <0.001 (***), P <0.01 (**), P <0.05 (*).

The percentage viability of treated cells with or without 1 µM of individual caspase 8, 9, 3 specific inhibitors for 72 h was determined using the neutral uptake assay. Caspase specific inhibitors at concentration of up to 10 µM induce cell death as instructed by kit manufacturer whereas, concentration of 1 µM was found to inhibit caspase activity without causing profound damage to murine mammary cancer cells (Shah et al., 2009). In light of this evidence, 1 µM of caspase inhibitors was used in study. In fact, a separate study demonstrated that it did not cause any profound damage to all cells with 80% and more cell viability (see appendix 3a). As shown in Fig. 5.8 (a, b, c), treatments of A549, U87MG and HT-29 cells with individual IC_{50} doses of tocotrienols (delta and gamma), jerantinines (A and B) and the combined low-dose treatments of tocotrienols with IC_{20} doses of jerantinines (A and B) in the presence of 1 µM of caspase 8 inhibitor could not block cell death after 72 h incubation. Similar trend was also evident in all treated cell lines which were given with 1 µM of caspase 9 specific inhibitor (Fig 5.8 d, e, and f) and caspase 3 specific inhibitor (Fig. 5.8 g, h and i). In fact, the cell viability below 50% was evident on U87MG, A549 and HT-29 cells receiving individual and combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) with or without caspase specific inhibitors. Nevertheless, statistical analysis revealed there was no significant difference between cell viability of treated groups without inhibitors and treated groups containing 1 µM of caspase 8, 9 and 3 specific inhibitors.

5.3. Discussion

Cell proliferation is essential for growth and development of organisms. However, uncontrolled cell growth due to defect in cell cycle checkpoints causes cancer. Considering its significant role in cancer, drugs targeting cell cycle progression have consonantly been developed. In fact, some of these drugs are administered to patients in combination for the purpose of maximizing potent effects on cancer cells as well as minimizing undesirable effect to normal cells (Diaz-Moralli et al., 2013). The results from Chapter Four revealed that both individual and combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) induced morphological changes and DSBs indicative of apoptosis. DSBs are triggers of cell cycle arrest that eventually stops the uncontrolled proliferation of cancer cells thereby committing cells to apoptosis (Collins et al., 1997). It is therefore imperative to investigate the effects of both individual and combined low-dose treatments on cell growth. Evidently, IC_{50} doses of delta and gamma tocotrienols consistently triggered G0/G1 cell cycle arrest after 24 h and 48 h treatments on U87MG, A549 and HT-29 cells. This is consistent with previous studies that reported the induction of G0/G1 growth arrest by tocotrienols (Fernandes et al., 2010; Hodul et al., 2013; Lim et al., 2014a, 2014b). Although a slight increase in G2/M population was observed in A549 cells receiving IC_{50} dose of delta tocotrienol after 24 h, nonetheless this eventually resulted in significant (P <0.001) G0/G1 cell cycle arrest after 48 h treatment. In contrast, a more profound G2/M arrest (P <0.001) was consistently evident on U87MG, A549 and HT-29 cells treated for 24 h and 48 h with IC_{50} doses of jerantinines A and B conforming to the recently published studies (Qazzaz et al., 2016; Raja et al., 2014). Antimicrotubule agents such as taxanes, paclitaxel etc have been shown to induce G2/M arrest (Bhalla, 2003; Perez, 2009), thus suggesting that

jerantinines A and B herein may target microtubule as a mechanism of apoptosis induction. Interestingly, only combined treatment of low-dose delta tocotrienol with IC_{20} dose of jerantinine A induced G2/M arrest (P<0.001) on A549 cells after 24 h and 48 h. Whereas, there was a time dependent shift in pattern of cell cycle arrest for the remainder of the combined low-dose treatments on U87MG and HT-29 cells. This includes a shift in increasing S population (24 h) to G0/G1 population (48 h) for combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} dose of jerantinine B on U87MG cells. Similarly, there was a shift from G2/M to G0/G1 and G0/G1 to S after 24 h and 48 h for combined treatment of low-dose gamma tocotrienol with IC_{20} dose of jerantinine A on U87MG cells and combined treatments of low-dose delta tocotrienol with IC_{20} dose of jerantinine A on HT-29 cells, respectively. This is not surprising considering that these are sub-effective doses which may take longer time to induce a potent cell cycle arrest. Furthermore, this may be attributed to the different pattern of cell cycle arrests induced by individual doses of tocotrienols (delta and gamma) and jerantinines (A and B) which are predominantly arrested at G0/G1 and G2/M, respectively. On the other hand, based on the pattern of cell cycle arrests, the results herein suggest that combined low-dose treatments may not induce microtubule inhibition. Although, this may not be exclusive as considering that there was evidence of G2/M arrest on U87MG cells receiving combined treatment of low-dose gamma tocotrienol with IC_{20} dose of jerantinine A after 24 h.

Microtubules compose primarily of beta (β) and alpha (α) tubulin dimers which are the integral part of cytoskeleton. The vast roles of microtubules in cells include maintaining cell structure, cellular mobility, mitosis and cell signaling (Hawkins et

al., 2010). The important role of microtubules in cellular division has made them desirable targets for chemotherapeutic agents especially in rapidly growing cells. Subsequent research over the years has led to discovery and clinical use of microtubule inhibitors that are primarily classified into microtubule stabilizing and destabilizing agents. A few prominent examples of the stabilizing agents include the taxanes, paclitaxel whereas vinca alkaloids are exemplified of the destabilizing agents (Perez, 2009). Despite the limiting problems that include toxicity and multi drug resistance, microtubule interacting drugs have recorded a certain degree of success in cancer chemotherapy. This necessitates the development of more improved and robust alternatives.

Previous preliminary study on synthetic derivative of jerantinine E revealed a disruption of microtubule network and inhibition of tubulin polymerization in A549 cells as a possible mechanism of apoptotic induction (Frei et al., 2013). Further mechanistic studies revealed that jerantinines A and B evoked G2/M growth arrest consistent with that of tubulin interacting drugs. Further evaluation revealed that disruption of tubulin dynamics would result in multipolar spindle and irregular chromosome segregation (Qazzaz et al., 2016; Raja et al., 2014). On the other hand, although tocotrienols evoked G0/G1 cell growth arrest, Lim et al.,(2014a) has suggested that inhibition of microtubules may be the possible mode of action based on the observed similarities with control drug, vinblastine on the pattern of DNA damage induced in A549 and U87MG cells. Considering that the data from this study is consistent with the evidence from the aforementioned studies, it was interesting to investigate the tubulin inhibitory effects of individual and combined low-dose treatment of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B).

As such, in this study, immunofluorescence technique was employed to achieve this objective.

Indeed, results from immunofluorescence assay herein demonstrated disruption of microtubules by IC_{50} doses of jerantinines (A and B). In contrast, U87MG, A549 and HT-29 cells receiving IC_{50} dose of delta or gamma tocotrienol displayed intact alpha tubulin structures similar to that of untreated controls. Interestingly, owing to the original antimicrotubule effects of jerantinines A and B, the addition of IC_{20} dose of jerantinine A or B to either delta or gamma tocotrienol has caused considerable disruption of microtubules. This was also accompanied by cell shrinkage and decreased intensity of stained alpha tubulin compared to untreated controls which can be attributed to the loss of microtubule networks. On the other hand, although tocotrienols (delta and gamma) induced dissimilar effects, the results herein do not conclusively rule out the potential effects of tocotrienols towards microtubules. Nevertheless, this would represent an improved potency and efficacy of tocotrienols resulting from its synergy with jerantinines A and B thus adding a multidimensional approach that would include inhibition of microtubules in addition to the pre-established mechanism of apoptotic induction by tocotrienols. This is surprising for combined low-dose treatments on U87MG and HT-29 cells that induced predominantly G0/G1 arrest. This observation was not consistent with G2/M arrest that is often attributed to most antimicrotubule agents. Therefore, it is possible that the low doses of tocotrienols (delta and gamma) are potent enough to induce profound G0/G1 arrest without significant number of U87MG and HT-29 cells to proceed to the G2/M phase. This potentially explains the antimicrotubule effects of the combined low-dose treatments despite the induction of G0/G1 growth arrest which indicates co-

integrative synergistic interaction between combined treatments. On the other hand, the antimicrotubule effect of combined low-dose treatment of delta tocotrienol with IC_{20} dose of jerantinine A on A549 cells was accompanied by G2/M arrest. This suggests that in contrast to combined treatments on U87MG and HT-29 cells, the low-dose tocotrienol was not potent enough to induce a profound G0/G1 arrest on A549 cells.

Furthermore, these results are contrary to the aforementioned proposition by Lim et al.,(2014b) that tocotrienols may also inhibit microtubules. This is not a surprise considering that tocotrienols inhibit cyclin D1 thereby inducing G0/G1 growth arrest in lung carcinoma (A549 and H1650) cells and glioblastoma (U87MG) cancer cells (Ji et al., 2012; Lim et al., 2014a, 2014b). Cyclin D1 is a regulator protein expressed in the G1/S phase transition (Ji et al., 2012). However, this is not exclusive to only cyclin D1 as a few studies have also demonstrated that delta tocotrienol and its peroxy dimer from *Kielmeyera coriacea* induced G2/M growth arrest in HL-60 (leukaemia) cancer cells (de Mesquita et al., 2011). Similarly, delta tocotrienol down regulated retinoblastoma, cyclin D1, cyclin B1, cyclin dependent kinase 4 and 1 (CDK4 and CDK1) in MDA-MB-231 breast cancer cells. Although G2/M regulatory proteins were down regulated, inhibition of proliferation was marked by loss of cyclin D1 and G0/G1 growth arrest (Elangovan et al., 2008). On the other hand, jerantinine A down regulated cyclin B1, the protein regulating the G2/M transition phase of the cell cycle (Raja et al., 2014). These aforementioned studies have shown that tocotrienols could potentially evoke G2/M arrest (depending on cellular microenvironment) consistent with microtubule interacting drugs, however to date, there are no reports suggesting tocotrienols as antimicrotubule agents. Undeniably, the low doses of delta and gamma

tocotrienols herein have potentiated the antimicrotubule effects of IC_{20} doses of jerantinines A and B. However, such potentiating effects of low-dose tocotrienols towards the antimictotubule effects of IC_{20} doses of jerantinines A and B are not reflected by the pattern of G0/G1 growth arrest. Alternatively, the effect could be potentiated via up regulation of p53.

The presence of lower level of p53 protein present in healthy cells increases with phosphorylation upon induction of different types of cellular damage such as DNA damage and inhibition of microtubules. According to a previous study, jerantinine A caused an increased p53 level (Raja et al., 2014), thus, it is speculated that the antimicrotubule effect of jerantinine A, jerantinine B and the respective combined low-dose treatments with delta or gamma tocotrienol are mediated via up-regulation of p53. In contrast, although studies have illustrated the upregulation of p53 by gamma tocotrienol (Kannappan et al., 2010b), there are no reports on disruption of microtubule as the possible mechanism of action. The pattern of p53 phosphorylation in cells with disrupted microtubules is distinctively different from cells undergoing only cellular DNA damage. In fact, the pattern of p53 phosphorylation in cells treated with microtubule inhibiting compounds is varied by the types of cells and microtubule inhibitors (Perez, 2009). For instance, there was an increased phosphorylation of p53 on serine-15 only in epithelial tumor cells but not fibroblasts cells treated with microtubule inhibitors (Stewart et al., 2001). Hence, p53 upregulation by tocotrienols may be in response to cellular DNA damage. Nonetheless, this study demonstrated the antimocrotubule effects of jerantinine A and jerantinine B that may involve upregulation of p53 which is further amplified when combined in lower dose with delta or gamma tocotrienol.

The disruption of microtubules as well as DNA damage and manifestation of morphological hallmarks of apoptosis indicate an induction of apoptotic cell death by both individual and combined low-dose treatments. Considering that microtubule targeting agents execute apoptosis predominantly via the mitochondria and also death receptors, it is therefore necessary to investigate the potential role of caspase enzymes. Apoptotic cell death is mediated through extrinsic and intrinsic pathways which may be dependent on activity of initiator and executioner caspases. In addition to evidence of caspase independent apoptosis, researches have illustrated the cross linkage of both pathways through the activities of executioner caspase. The extrinsic pathway is mediated by the tumor necrosis factor (TNF) receptor super family such as CD95 and TNF-related apoptosis inducing ligand (TRAIL). Upon induction by chemotherapeutic agents, activated receptors recruit adaptor molecules such as Fas-associated death domain (FADD) which activates caspase 8 and subsequently caspase 3 (Fulda and Debatin, 2006a). Alternatively, caspase 8 can also trigger the mitochondria pathway via truncation and subsequent activation of BH3 interacting domain agonist (BID) (Elmore, 2007). On the other hand, the mitochondria pathway is triggered upon disruption and permeabilization of the mitochondrial membrane by the proapoptotic B-cell lymphoma protein (Bcl) family proteins thereby releasing proteins including cytochrome C, second mitochondrial activator of caspase/direct inhibitor of apoptosis binding protein with low PI (Smac/Diablo), apoptosis inducing factor (AIF), High temperature requirement (HtrA2/Omi) and endonuclease G in the cytosol. These proteins activate caspase 9 to induce apoptosis or alternatively activate caspase independent cell death (Fulda et al., 2010).

Herein, increased caspase 8 and 3 activities were evident in U87MG, A549 and HT-29 cells after 1h treatment with IC_{50} dose of delta or gamma tocotrienol (A549, U87MG, and HT-29). Indeed, previous studies on tocotrienols revealed the role of caspase enzymes for the induction of apoptosis. These studies showed that induction of apoptosis by alpha, beta, delta and gamma tocotrienols in human brain and lung cancer cells was mediated through upregulation of caspase 8 activities (Lim et al., 2014a, 2014b). Similarly, gamma tocotrienol and tocotrienol rich fraction (TRF) increased intracellular activities of caspase 8 and 3 but not caspase 9 in murine mammary cancer cells (Shah et al., 2003). This is in agreement with the present study for caspase 8 and 3 activities. Dissimilarity on increased caspase 9 activity was evident in A549 cells treated with delta tocotrienol. Such contrasting evidence on caspase activation could be attributed to the response of different isomers to different cellular microenvironment. For example, gamma tocotrienol induced upregulation of caspase 8, 9 and 3 activities in human hepatoma Hep3B cells (Sakai et al., 2006). On the other hand, jerantinines A and B induced slight increase in caspase 9 activities in three cell lines (U87MG, A549, HT-29) and two cell lines (U87MG and A549), respectively. Owing to the ability of jerantinines (A and B) to induce caspase 9 activity, the respective combined low-dose treatment of tocotrienols with IC_{20} doses of jerantinines (A and B) caused slight upregulation of caspase 9 (Fig. 4.1). This demonstrates an improved multidimensional mechanism of apoptosis induction for combined low-dose treatments as compared to individual IC_{50} doses of delta and gamma tocotrienols.

Although caspase 8 has been linked to death receptor pathway, the mechanisms of action of tocotrienols elucidated from previous studies demonstrated the ambiguity for

both pathways that may be dependent on cellular microenvironment. For instance, gamma tocotrienol induced apoptosis via caspase 8 activation independent of the death receptor signaling pathway in +SA mammary tumor cells which are known to be resistant to death receptor induced apoptosis (Shah and Sylvester, 2004). Similarly, beta tocotrienol was reported to induce caspase 8 dependent apoptosis via mitochondria and death receptor pathways in lung and brain cancers (Lim et al., 2014b). In contrast, gamma tocotrienol induced TRAIL receptor death receptor mediated apoptosis in human colon cancers. This was dependent on ROS, extracellular signal regulated kinase 1 (ERK-1), p53 and Bax as deletion of the genes or sequestering of ROS abolished the upregulation of death receptors (Kannappan et al., 2010b). In light of the aforementioned evidence, it is possible that activation of caspase 8 and subsequently caspase 3 herein by delta and gamma tocotrienols is not exclusively mediated through the death receptor or mitochondria pathway. On the other hand, combined treatments of jerantinine A or B with delta or gamma tocotrienol on A549, U87MG or HT-29 cells demonstrated a similar trend of dominant caspase 8 and 3 activities. This is an addition to the increased caspase 9 activities although at a much lower level compared to the former caspase enzymes. Undoubtedly, this demonstrates an improved activity compared to single high-dose tocotrienols that would involve both death receptor and mitochondria pathways. However, unlike tocotrienol, reports on the potential role of caspase enzymes in jerantinine derivatives induced apoptosis are limited. In fact, thus far, there are no studies on jerantinine B. The only recent mechanistic study showed that jerantinine A induced an increased intracellular activity of caspase 3 (Raja et al., 2014) by which the present study showed similar finding. The activation of caspase 8, 9 and 3 by jerantinines A and B as demonstrated in this study provides new information which

strongly indicates potential of both death receptor and mitochondria mediated pathways. The activation caspase 8 enzyme after 1h and 9h by tocotrienols (delta and gamma) and jerantinines (A and B), respectively was indicative of the fact that caspase mediated apoptosis occurred at an earlier stage in the former compared to the latter. Interestingly, unlike single IC_{50} doses of tocotrienols, the combined low-dose treatments with IC_{20} doses of jerantinines (A and B), activation of caspase 8 after 9h treatment represented a change in time required for the manifestation of apoptosis. This is not surprising and could be attributed to the fact that the lower dose of tocotrienols would take longer time to induce apoptosis compared to IC_{50} doses.

Induction of both caspase dependent and independent cell death has been reported as a potential alternative to circumventing drug resistance and limitations of tocotrienol potency (Constantinou et al., 2008). Available literatures on the potential induction of caspase independent cell death by tocotrienols have been contradictory. For instance, treatment with 1μM of caspase 8 and 3 inhibitors blocked the induction of apoptosis by gamma-tocotrienol and tocotrienol rich fraction (TRF) in murine mammary cancer cells (Shah et al., 2009). Similarly, Takahashi and Loo, (2004) reported that gamma tocotrienol induced apoptosis in MDA-MB-231 breast cancer cells via disruption of mitochondria membrane, followed by cytochrome C release and subsequent manifestation of chromatin condensation and fragmentation. However, further mechanistic studies did not detect poly (ADP-ribose)-polymerase (PARP) cleavage or changes in expression Bax and BCL-2 proteins indicating that apoptosis was independent of caspase enzyme and also cytochrome C release was not crucial to the induction of apoptosis (Takahashi and Loo, 2004). In addition, gamma tocotrienol induced paraptosis like cell death in SW620 human colon cancer cells via suppression

of Wnt signalling pathway (Zhang et al., 2013). In some studies, the induction of both apoptotic and non-apoptotic cell death by tocotrienols were also observed. For instance, gamma tocotrienol induced apoptosis, necrosis and autophagy in human PC-3 and LNCaP prostate cancer cells via intracellular upregulation of dihydrosphingosine and dihydroceramide (Jiang et al., 2012b). Similarly, both natural and synthetic homolog's of vitamin E induced caspase dependent and independent DNA damage and dominant caspase independent programmed cell death in prostate cancer cells (Constantinou et al., 2012, 2009). Evidently, data from the present study showed a trend of induction of cell growth inhibition independent of caspase 8, 9 or 3 activation by both individual and combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) on A549, HT-29 and U87MG cells as there was no significant difference between cells treated with or without caspase specific inhibitors. This further corroborates the aforementioned literatures. These studies undoubtedly suggest the potential role of cellular environment in determining the induction and possibly control of apoptotic pathways. This suggestion is further supported by studies that gamma tocopherol induced caspase 9 activation in LNCaP prostate cancer cells (Jiang et al., 2004) but not colon cancer cells (HT-29, HCT-15, HCT-116 and, SW480) (Campbell et al., 2006). Alternatively, caspase independent cell death could be regulated by lysosomes as a study has demonstrated that cells deficient of lysosomal cathepsin D were resistant to alpha tocopheryl succinate (Constantinou et al., 2008; Neuzil et al., 2002).

Previous studies reported that microtubule targeting drugs such as paclitaxel and epothilone B induced caspase independent cell death in NSCLC cells via upregulation of cathepsin B (Bröker et al., 2004). Similarly, paclitaxel triggered AIF induced

caspase independent cell death in human ovrian carcinoma cells (Ahn et al., 2004). On the other hand, there are no available literatures on possible induction of caspase independent cell death by jerantinine A or B. The present study has demonstrated an induction of cell growth inhibition independent of caspase 8, 9 and 3 activation by jerantinine A or B and its combined low-dose treatment with delta and gamma tocotrienol on A549, U87MG and HT-29 cells. In addition, it further demonstrates that this mechanism of action is not cell specific thus providing new information on the mechanistic action of jerantinine derivatives.

Mechanism of caspase independent apoptosis recruits an intricate action of genes that are dependent or independent of mitochondria. Pro-apoptotic proteins including AIF and endonuclease G released by mitochondria translocate to the nucleus causing nuclear chromatin condensation and DNA fragmentation as a latter event of apoptosis (Joza et al., 2001; Li et al., 2001). In addition, AIF and Endonuclease G have dual roles including the induction of caspase independent cell death (Elmore, 2007). As illustrated by previous studies, lysosomal cathepsin D also triggers caspase independent release of AIF in caspase $3^{-/-}$ and Apaf $1^{-/-}$ cells that are characterized by cell shrinkage and chromatin condensation. This was mediated through cathepsin D activation of Bax through conformational change. Subsequently Bax is relocated to outer mitochondria membrane in a Bid independent manner where it induces mitochondrial release of AIF for apoptosis induction (Bidère et al., 2003; Susin et al., 2000). Furthermore, addition of broad caspase inhibitor z-VAD-FMK could neither block translocation of AIF from the mitochondria nor its subsequent apoptotic effect (Bröke et al., 2005). Although there are studies indicating induction of caspase independent cell death by tocotrienols, elucidation on the possible mechanism of

action is still lacking. It is noteworthy to say that the aforementioned action could be mediated via cathepsin D upregulation of Bax and subsequent release of AIF as demonstrated in T-lymphocytes (Bidère et al., 2003).

5.4 Conclusion

This study demonstrated the apoptotic potential of tocotrienols (delta and gamma) and jerantinines (A and B) on U87MG, A549 and HT-29 cells. Analysis of cell cycle data confirmed induction of G0/G1 arrests by individual IC_{50} doses of delta and gamma tocotrienols and combined low-dose treatments on U87MG and HT-29 cells. Whereas, jerantinines A and B induced G2/M cell cycle arrest on U87MG, A549 and HT-29 cells. This demonstrated that the pattern of cell cycle arrest (G2/M) induced by jerantinines A and B was related to the respective antimicrotubules effects in contrast to that of combined low-dose treatments even though induction of antimicrotubule effects by IC_{20} dose of jerantinines A and B was evident. The study demonstrated an improved potent effect of combined low-dose treatments via multi targeted approach that involves disruption of microtubules compared to individual doses of tocotrienols (delta and gamma) that could be mediated potentially via death receptor and mitochondria pathways. Further studies on death receptors and markers of mitochondria pathways would be conducted to further elucidate the mechanism of action.

6.0 CHAPTER SIX

Alteration of apoptosis related genes expression in U87MG cells by individual and combined treatments of tocotrienols (delta and gamma) and jerantinines (A and B)

6.1 Introduction

Previous findings from Chapters Three and Four demonstrated an improved potency for combined treatments via synergistic interaction compared to individual tocotrienol treatments thus justifying the need to further elucidate the mechanism of action. Investigations from Chapter Five revealed that mechanism mediating the individual and combined low-dose treatments of tocotrienols (delta and gamma) and jerantinines (A and B) involved caspase dependent and independent apoptotic cell death. The activation of caspase enzymes suggested the possible involvement of mitochondria pathway and/or death receptor pathways. Furthermore, disruption of microtubules was evidenced in cells treated with jerantinines (A and B) and the combined low-dose treatments with tocotrienols but not individual treatment of tocotrienols (delta and gamma) suggests an additional mechanism that involves possible recruitment of the p53 tumor suppressor gene which is attributed by DNA damage or inhibition of microtubules. The major pathways (extrinsic and intrinsic) of apoptosis are mediated through complex mechanisms that involve death receptors, caspase enzymes and pro- and anti- apoptosis related proteins (Norberg et al., 2010). Therefore, it is imperative to investigate the potential role of these proteins towards the execution of apoptosis by individual and combined low-dose treatments.

Apoptosis antibody array is a rapid and accurate technique that allows detection and characterization of expression profiles of several apoptosis related proteins (Mai et al.,

2014) and therefore was employed herein to determine the potential of pro-apoptotic and anti-apoptotic proteins. The Human Apoptosis Antibody Array G-series (Ray Biotech, USA) used in this study utilizes the sandwich enzyme linked immunosorbent assay (ELISA) principle. This is based on the ability of sample proteins to bind to antibodies imprinted on histology slide after which, signals are developed with biotinylated detection antibodies and streptavidin-conjugated flour. Further Western blotting studies and ELISA were conducted to validate and confirm proteins (Bid, p53 and cytochrome C) of interest. BID plays a key role in apoptosis induction by transferring apoptotic stimuli form death receptors into mitochondria. This activates the mitochondria pathway via disruption of mitochondrial membrane potential and subsequent release of apoptosis related proteins including cytochrome C. On the other hand, p53 activation is triggered in response to DNA damage, microtubule disruption and also plays key roles in cell cycle arrest and mediating mitochondria pathway for apoptosis (Vazquez et al., 2008). The Western blotting technique is based on the principle of antigen-antibody reaction that is detected though chromogenic substrates or chemiluminiscence. Whereas, Human cytochrome C ELISA kit (R&D Systems, USA) was used to investigate the cytochrome C release as a marker for the confirmation of mitochondria pathway for mediation of apoptosis. This technique is based on the solid phase sandwich ELISA that allows for quantification of cellular cytochrome C levels.

U87MG brain cancer cells thus far are the least studied in terms of cytotoxic activity of tocotrienols in comparison to A549 and HT-29 cells. In fact, there are no reported studies on the cytotoxic effect of jerantinine derivatives on U87MG cells. In addition, unlike A549 and HT-29 cells, three different combinations of tocotrienols (delta and

gamma) with jerantinines (A and B) herein induced synergistic effects with only mild toxicity towards MRC5 cells. Furthermore, reports have shown that tocotrienols can penetrate the blood brain barrier. Therefore, U87MG cells were selected for further mechanistic studies considering the aforementioned reasons in addition to the fact that brain cancer has poor prognosis and high incidence rate in children.

The specific objectives of the study are:

a) To determine the roles of both pro- and anti-apoptotic proteins found in U87MG cells receiving individual and combined treatments

b) To determine the role of cytochrome C as a marker for mitochondria mediated apoptotic pathway

c) To propose the potential apoptotic mechanisms of action for individual and combined low-dose treatments

6.2 Materials and Methods

6.2.1 Chemical reagent/kits and cell cultureware

The following reagents and kits listed in Table 6.1 were used to conducts the experiments.

Table 6.1: List of chemical reagents, kits and glassware and the respective manufacturers.

s/no	Chemical reagents/kits	Manufacturer
1	Human Apoptosis Antibody Array G-series (AAH- APO-G1-8)	Ray Biotech, USA
2	BCA protein assay Kit	Fisher Scientific, USA
3	Quantikine Human cytochrome C ELISA kit	R&D Systems, USA
4	Bovine serum albumin (BSA)	Sigma, USA
5	p53 (DO-1: SC-126) mouse monoclonal antibody, BID (B-3: SC-373-939) mouse monoclonal antibody	Santa Cruz Biotechnology INC, USA
6	Pierce rabbit anti mouse (PB 186444) secondary horse radish peroxidise (HRP) linked antibody, Spectra multicolour protein ladder	Thermo Scientific, USA
7	Tris-base	Promega, USA
8	Glycine, 30% acrylamide, Ammonium perisulphate, Tris-CL	Biorad USA
9	Methanol	Merck, Germany
10	3,3',5,5'-Tetramethylbenzidine (TMB) peroxidase substrate	KPL, USA
11	Tetramethylethylenediamine (Nacalai Tesque, Japan), Sodium dodecyl sulphate	Sigma, USA
12	0.45 micron nitrocellulose membrane	Thermo Scientific, USA
13	90mm cell culture petri dish	SPL Life sciences, Korea
14	Tween 20	Nacalai Tesque, Japan

6.2.2 Apoptosis array

U87MG cells were seeded at a density of 1 x 10^6 and treated with individual or combined treatments of tocotrienols (delta and gamma) and jerantinines (A and B) as previously described in Table 4.2. Cells treated with plain media containing DMSO to serve as untreated control. Following 72 h treatment, the cells were detached using trypsin and centrifuged at 2200 rpm for 5 min. The cell pellets were suspended in pre-chilled PBS and centrifuged for 5min at 2200 rpm to remove any trace of trypsin. Thereafter, the cell pellets were re-suspended in 1x lysis buffer supplemented with 1x protease cocktail inhibitor supplied by Raybiotech, USA and incubated on ice for 10 min. The cell suspension was centrifuged at 10,000 rpm for 1 min and the supernatant protein lysate was collected. The protein concentration was determined using the Graphpad Prism 6 from a standard curve prepared using the BCA protein assay kit (see appendix 4). The protein lysate was stored at -80°C until analysis.

For apoptosis array, the glass chip chamber was assembled and allowed to air dry for 60 min followed by 30 min incubation with 100 µl of 1x blocking buffer. Thereafter, 600 µg of protein lysate was loaded in each well followed by overnight incubation at 4°C. The sample was decanted and the chambers were then washed with gentle shaking for5 times, each for 2 min and then 2 times for 2 min each with 150 µl of 1x wash buffer I and II, respectively. Seventy microliter of biotin conjugated antibodies was added to each well and incubated for 2 h at room temperature. Seventy microliter of 1500 fold diluted Hilyte plus conjugated streptavidin was added to each subarray followed by 2 h incubation in the dark. Each subarray was washed twice for 2 min with 1x wash buffer I at room temperature. The slide was disassembled, immersed into 50 ml tube filled with wash buffer I (twice) and II (once) and rinsed with gentle

shaking for 10 min. Finally, the slide was completely air dried and signals were scanned using compatible laser scanner at Raybiotec Inc, USA.

6.2.3 Western blotting analysis

U87MG cells were seeded in 90 mm dish and incubated in cell culture conditions. Upon confluency, the cells were treated with individual IC_{50} doses or combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) as shown in Table 4.2 for 24 h. After treatments, cells were detached and a total of 4×10^6 U87MG cells were suspended in pre-chilled PBS and centrifuged at 2200 rpm for 5 min (twice) to pellet cells and remove traces of media and trypsin. The pellets were then suspended in 200 µL of 1x laemmli sample buffer followed by gentle vortexing and sonication. Following the lysis step, the cell samples were incubated on ice for 20 min. The mixture was centrifuged 10000 rpm for 1min and the supernatant was collected and stored at -80°C until analysis. For Sodium dodecyl-sulphate poly acrylamide gel electrophoresis, 12% gradient gel (for recipe, see Appendix 6) was prepared. The samples were heated at 99°C for 5 min and 20 µL was then loaded into each well. The gel was run at 150V for 60 min in 1x Tris/glycine/SDS running buffer (for recipe see, Appendix 6). Thereafter, the protein bands were transferred in wet condition to 0.45 µM nitrocellulose membrane using the X-Cell II blot module (Invitrogen, USA). The transfer was run for 80 min at 25V using 1x Towbin transfer buffer (for recipe, see Appendix 3). After completion of transfer, the membrane was blocked in 3% BSA in 1x Tris-buffered saline with tween 20 (TBST) (for recipe, see Appendix 6) for 60 min followed by separate overnight incubation with diluted primary antibodies (anti- glyceraldehyde -3-phosphate dehydrogenase (GAPDH) at 1:3000), (anti-BID at 1:1000), (anti-p53 at 1:1000). The

antibody was discarded and the membrane was washed with three changes of TBST for 5 min each. The membrane was then incubated for 2 h with rabbit anti-mouse HRP linked secondary antibody diluted to ratio 1:5000 in TBST. Finally, the membrane was washed with four changes of TBST for 5 min each followed by TMB substrate wash until bands were visible. The membrane was washed with distilled water, air dried and images were subsequently captured using GS-800 calibrated densitometer (Bio-Rad, USA).

6.2.4 Enzyme-linked immuno sorbent assay (ELISA) for cytochrome C detection

U87MG cells were seeded into different treatment groups in 90 mm cell culture dish and incubated for 24 h to facilitate attachment. Thereafter, the cells were treated with single IC_{50} doses or combined low-dose treatment of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) as stated in Table 4.2. U87MG cells that received plain media containing DMSO served as an untreated control. A total of 1.5 x 10^6 cells were pelleted after 24 h treatment and washed three times with cold PBS. The cells, were suspended in 1ml of cell lysis buffer 2 provided by manufacturer (R&D Systems, USA) and incubated at room temperature for 1 h with gentle shaking. The cells were centrifuged at 1000 x g for 15 min and a 10-fold dilution of the supernatant in 200 μL of calibrator diluent was prepared and stored in -80°C until use. Prior to analysis, all reagents and working standards were prepared according to manufacturer's instruction. Calibrator diluents (100 μl) (R&D Systems, USA) diluted at 1: 10 in distilled water was added into each well. For generation of calibration curves to quantify cytochrome C, 100 μL of human cytochrome C standards prepared at a serial dilution (0, 0.625, 1.25, 2.5, 5, 10 and 20 ng/ml) were added to each well of the 96-well plate pre-coated with monoclonal antibody raised against cytochrome C.

For treated U87MG cells, a standard curve was prepared using Pierce BCA protein assay kit (Thermo Fisher Scientific, USA) and the protein content of samples was quantified by interpolation using the Graphpad Prism 6. Then, 100 µL of treated and untreated control samples were added to pre-coated wells containing 100 µL of calibrator diluent followed by 2 h incubation at room temperature. Each well was then aspirated and washed four times with 400 µL of wash buffer and the plate was inverted and blotted against a paper towel to completely remove any liquid. Two hundred microliter of human cytochrome C conjugate was added to each well and incubated for 2 h at room temperature. The wells were aspirated and washed four times with wash buffer and 200 µL substrate solution was added in to each well and incubated for 30 min at room temperature protected from light. Finally, 50 µL of stop solution was added and the optical density was determined using Epoch microplate spectrophotometer (Biotek, USA) at wavelength of 450 nm.

6.2.5: Statistical analysis

One way analysis of variance using the Dunnet's t-test was used to compare the differences in expression levels between the treated and untreated controls for all three assays. $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*) were indicated as significant difference.

6.3 Results

6.3.1 Assessment of apoptosis related gene expression levels altered by individual and combined treatments on U87MG cells via apoptosis array

Following the scanning of signals, the data obtained was analysed using the Ray biotech analysis tool and results were presented as mean ± SD fold change over untreated U87MG cells from two replicates. Following 72 h treatment with IC_{50} dose of delta tocotrienol, there was a slight upregulation of pro-apoptotic proteins, Bax, Bid, Bim, p27 and death receptors, TRAIL-R (1 and 2). This is contrary to the evident downregulation of bad, p53 and in fact over 2-folds downregulation of FAS death receptor. This indicates that delta tocotrienol induced apoptosis was not mediated through FAS death receptor. As shown in Fig 6.1, there was a general downregulation of anti-apoptotic proteins including Bcl-2, XIAP, cIAP-2. Surprisingly, there was an upregulation of the anti-apoptotic protein, survivin and in fact over 2-folds upregulation of livin. On the other hand, as shown in Fig. 6.2, U87MG cells treated with IC_{50} dose of gamma tocotrienol showed an upregulation of pro-apoptotic including Bad, Bax, Bid and death receptors, TRAILR-1 and TRAILR-2. The upregulation was however below 2 folds comparing to untreated controls. In a similar trend to that of U87MG cells treated with delta tocotrienol, there was below 2 folds downregulation of some pro-apoptotic proteins including FAS, p53, Bim and cytochrome C. In contrast to delta tocotrienol U87MG cells, there was evidence of a slight upregulation of most anti-apoptotic including Bcl-2, Bcl-2 and cIAP-2. Similar up regulations were also evident on livin and survivin in contrast to over 2-folds downregulation of XIAP. The results demonstrated similar expression profiles of pro-apoptotic and anti-apoptotic proteins on U87MG cells treated with IC_{50} doses of delta and gamma tocotrienols after 72 h.

Fig 6.1: Protein expression profiles of U87MG cells receiving individual IC_{50} dose of delta tocotrienol for 72 h. Protein was extracted from 1×10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. P <0.001 (***), P <0.01 (**) and P <0.05 (*) indicate significant difference between treated and untreated groups.
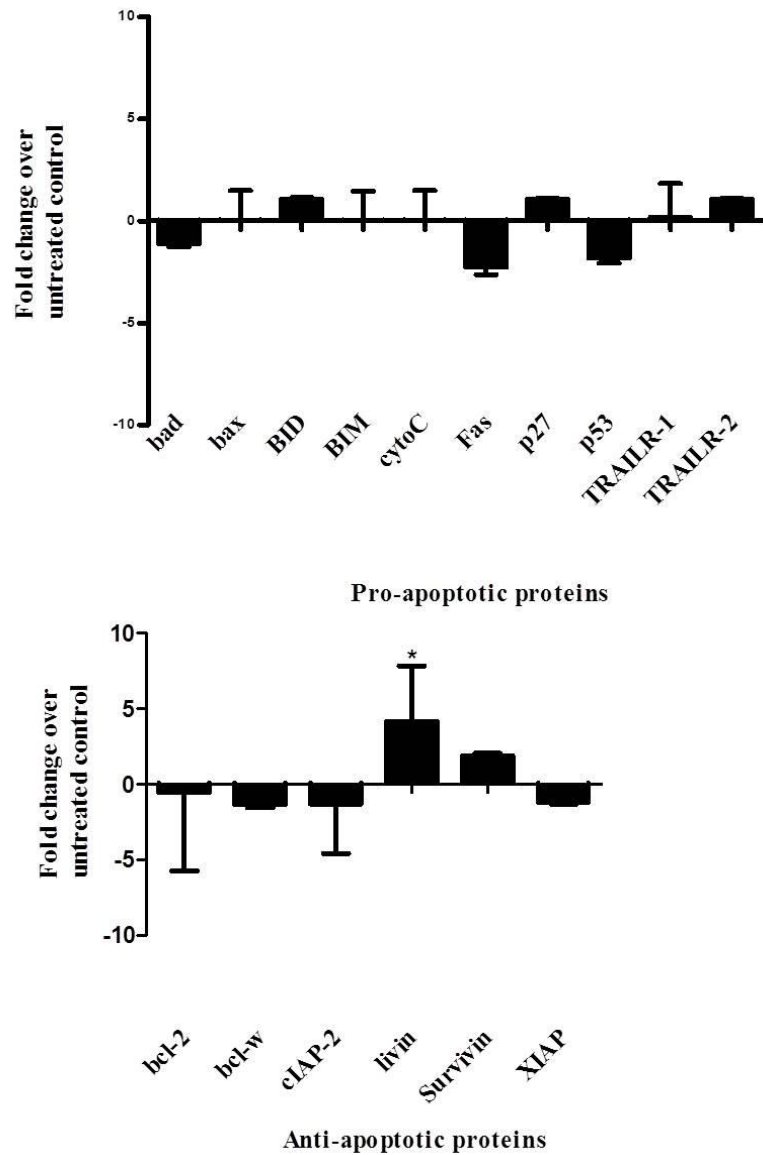
Fig 6.2: Protein expression profiles of U87MG cells receiving individual IC_{50} dose of gamma tocotrienol for 72 h. Protein was extracted from 1×10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. $P < 0.001$ (***), $P < 0.01$ (**) and $P < 0.05$ (*) indicate significant difference between treated and untreated groups.
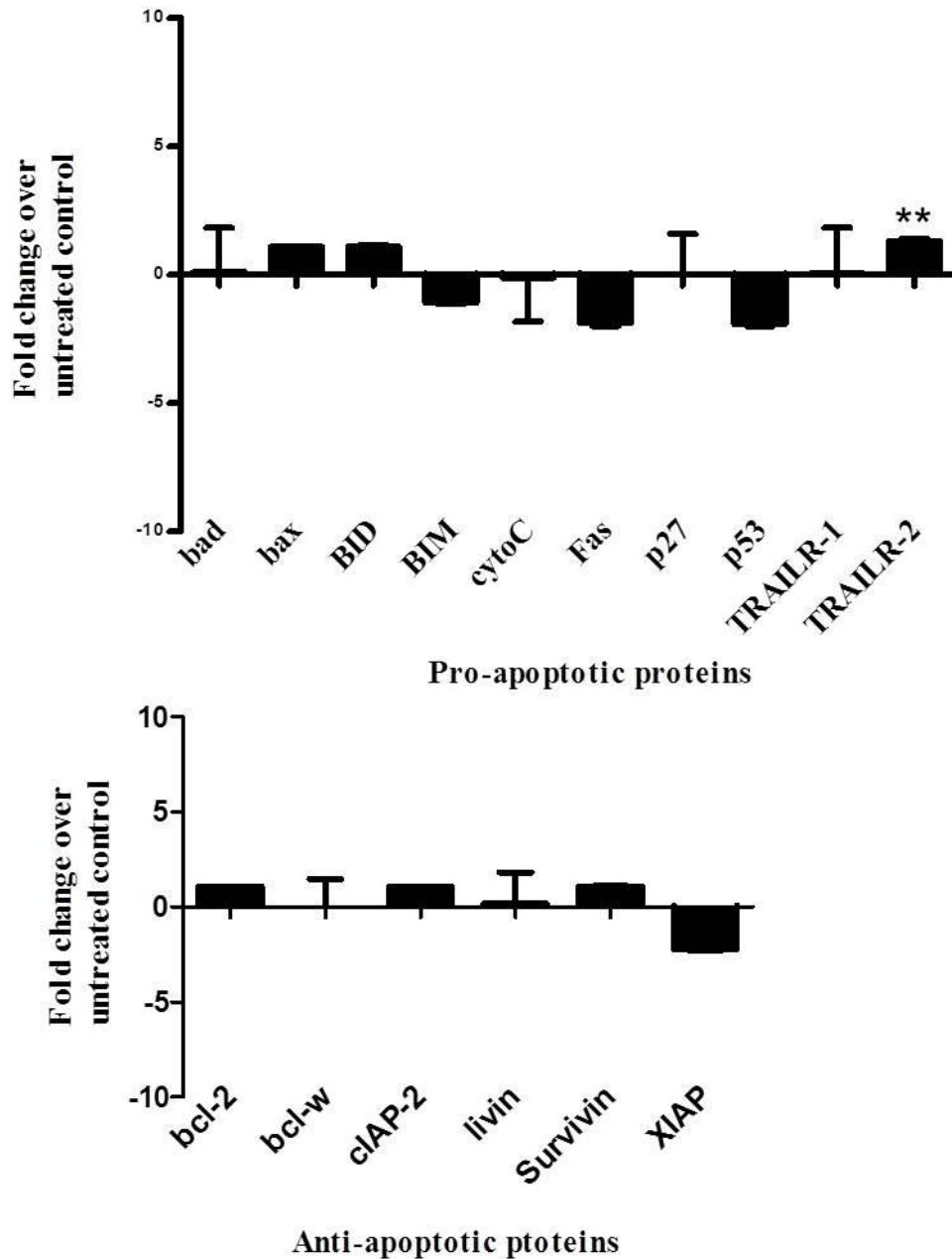
U87MG cells treated with IC_{50} dose of jerantinine A showed marked increase on the pro- apoptotic proteins including Bid, p27, Bad and cytochrome C. In fact, unlike U87MG cells treated with IC_{50} doses of tocotrienols (delta and gamma), there was over 1.5-fold and 2 folds upregulation of Bim and p53, respectively as shown in Fig. 6.3. Similarly, there was also over 1.4-fold upregulation level of FAS death receptor. But, unlike tocotrienols (delta and gamma), jerantinine A induced an upregulation of only TRAILR-1 death receptor. However, similar upregulation that was below 2 folds change of anti-apoptotic proteins including Bcl-2, Bcl-w, cIAP-2 were evident. In fact, there was over 2-fold upregulation level of survivin.

U87MG cells receiving IC_{50} dose of jerantinine B demonstrated an upregulation of over 1.1-fold change for pro-apoptotic proteins including Bad, Bax, Bid, Bim, and p53 amongst others. There was also an upregulation of death receptors, FAS and TRAILR-1 although TRAILR-2 was down regulated. This is similar to the expression profiles of death receptors of U87MG cells receiving IC_{50} dose of jerantinine A (Table 6.2). In comparison to jerantinine A, U87MG cells receiving IC_{50} dose of jerantinine B also demonstrated greater expression profiles of Bad and Bax proteins with up to 1.1-fold increase compared to the 0.12-fold evident in the former. Except for XIAP, there was a general upregulation of anti-apoptotic proteins including Bcl-2, Bcl-w, cIAP-2 and livin. In fact, there was over 2-folds upregulation level of survivin.

Fig 6.3: Protein expression profiles of U87MG cells receiving individual IC$_{50}$ dose of jerantinine A for 72 h. Protein was extracted from 1 x 10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. P <0.001 (***), P <0.01 (**) and P <0.05 (*) indicate significant difference between treated and untreated groups.
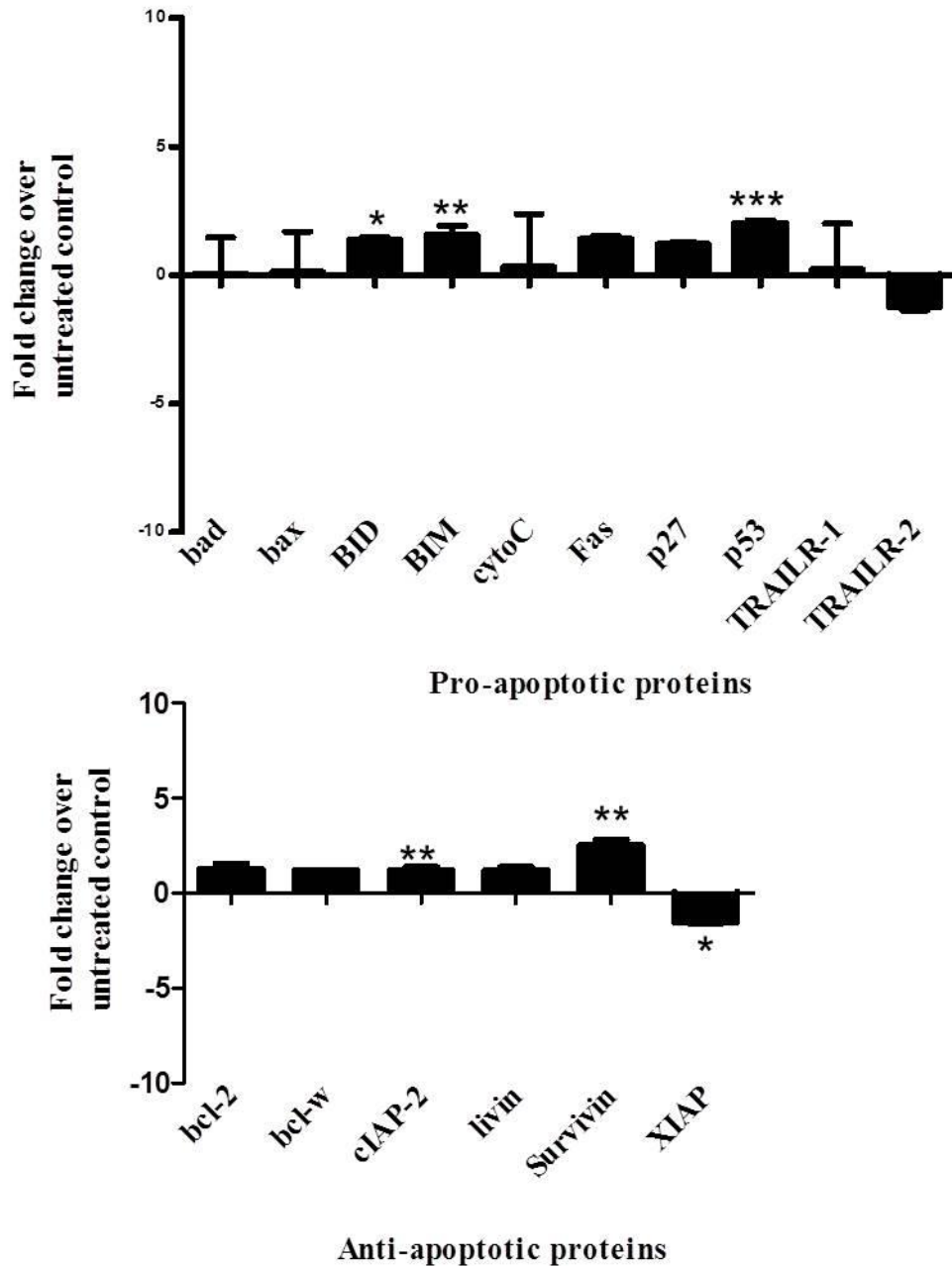
Fig 6.4: Protein expression profiles of U87MG cells receiving individual IC_{50} dose of jerantinine B for 72 h. Protein was extracted from 1 x 10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. P <0.001 (***), P <0.01 (**) and P <0.05 (*) indicate significant difference between treated and untreated groups.
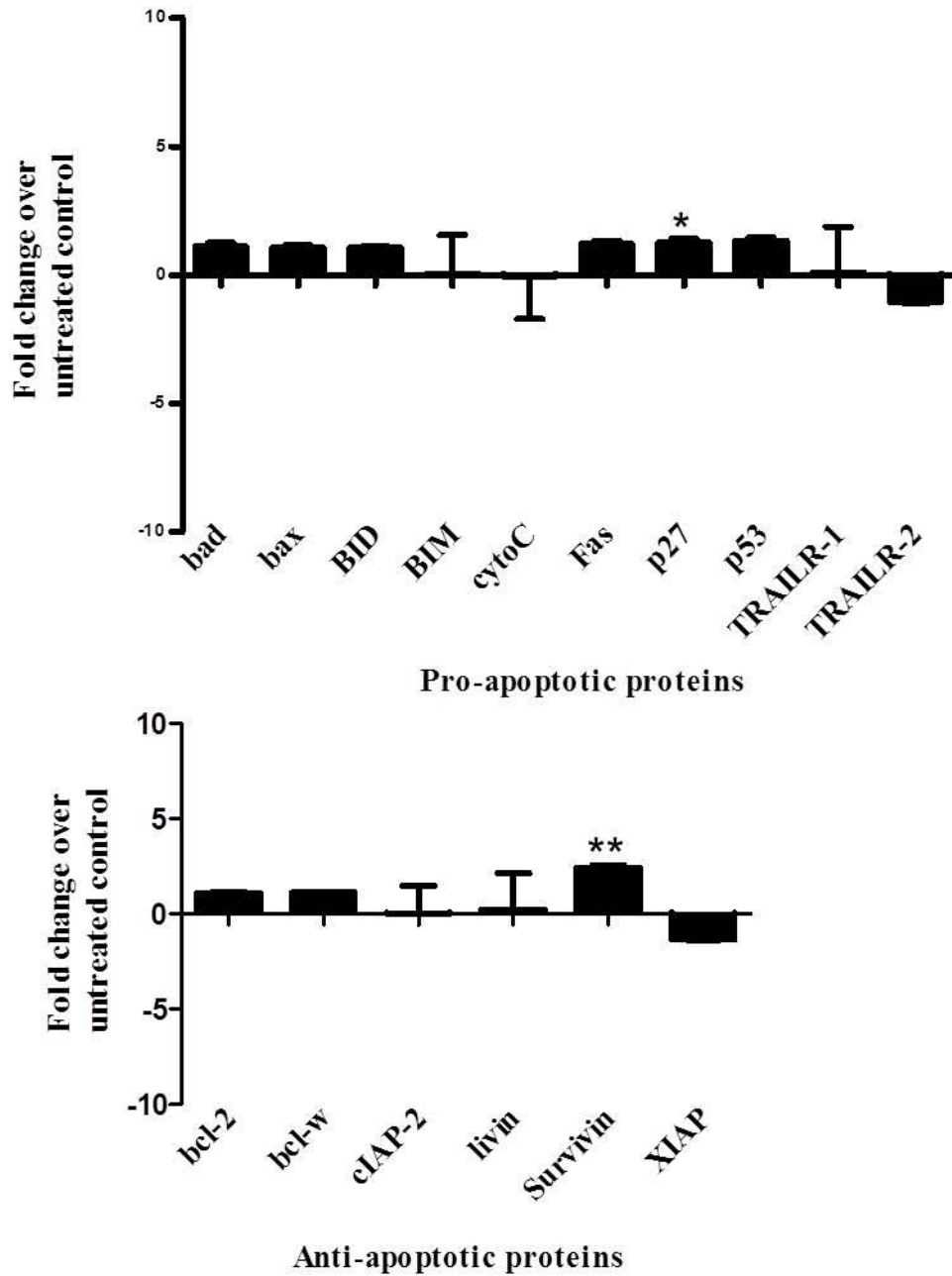
Combined treatment of low doses demonstrated an improved potency by causing upregulation of pro-apoptotic protein of both death receptor and mitochondria pathway. The combined treatment of low-dose delta tocotrienol with IC_{20} dose jerantinine B induced upregulation of BH3 proteins (Bax, Bid, Bim and Bad), FAS, p27, p53 and TRAIL-R (1 and 2) (Fig 6.5). This is similar to the expression profiles on U87MG cells receiving combined low–dose treatment of gamma tocotrienol with jerantinine B. This is contrary to the observation of downregulation of p53, FAS, Bad in U87MG cells treated with individual dose of delta tocotrienol. In addition, there was an overall increase in expression of pro-apoptotic proteins for up to 1.9-fold compared to 1.1-fold observed for U87MG cells treated with individual dose of delta tocotrienol. As shown in Table 6.3 there was an upregulation (< 2-fold) of anti-apoptotic proteins except for XIAP which had been down regulated (1.2-fold) on U87MG cells treated with combined low-dose delta tocotrienol with IC_{20} dose of jerantinine B. This is similar to the expression profile observed in U87MG cells treated with IC_{50} dose of jerantinine B although the expression of survivin was below 2-fold change. As shown in Fig. 6.6, combined low-dose treatment of gamma tocotrienol with IC_{20} dose of jerantinine A caused a general up regulation of pro-apoptotic proteins including cytochrome C, p27, Bad, Bax. In fact, there was over 3-fold upregulation of p53. Similar upregulation of death receptors, FAS and TRAIL-R1 was also evident. However, there was a downregulation of Bid (0.01-fold) and TRAIL-R2 (0.3-fold) (Fig. 6.6). On the other hand, there was a general upregulation of anti-apoptotic proteins including XIAP and with over 2 fold increase evident for survivin. In comparison to individual IC_{50} dose of gamma tocotrienol, the combined low-dose treatment caused improved expression of pro-apoptotic proteins especially p53 (3.7-fold), FAS (1.4-fold), cytochrome C (1.9-fold) and p27 (1.5-fold). Similarly,

the combined low-dose treatment also caused improved p53 expression (3.7-fold) as compared to the p53 (2-fold) level observed in U87MG cells treated with individual IC_{50} dose of jerantinine A (Fig 6.3). However, in contrast to individual doses of gamma tocotrienol and jerantinine A, the combined low-dose treatment did not cause the downregulation of XIAP.

Similarly, combined low-dose treatment of gamma tocotrienol with IC_{20} dose of jerantinine B caused upregulation of p53 although this was below 2-fold increase in comparison to combined treatments with jerantinine A (Fig. 6.7). Nonetheless, there was an improved upregulation of Bad, Bim, Bax, Bid, FAS, TRAIL R-1, and TRAIL R-2. In fact, this also demonstrated improved expression of proteins particularly p53 (1.6-fold), FAS (0.4-fold), p27 (1.3-fold) and BIM (1.2-fold) comparing to individual dose of gamma tocotrienol (Table 6.2). There was a similar upregulation profile of anti-apoptotic proteins except for XIAP in comparison to individual dose of gamma tocotrienol. Whereas, unlike the individual dose of jerantinine B, the upregulation of survivin induced by the combined treatment of low-dose gamma tocotrienol with jerantinine B was below 2-fold change.

Fig 6.5: Protein expression profiles of U87MG cells receiving combined low-dose treatment of delta tocotrienol with IC_{20} dose of jerantinine B for 72 h. Protein was extracted from 1 x 10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. P <0.001 (***), P <0.01 (**) and P <0.05 (*) indicate significant difference between treated and untreated groups.
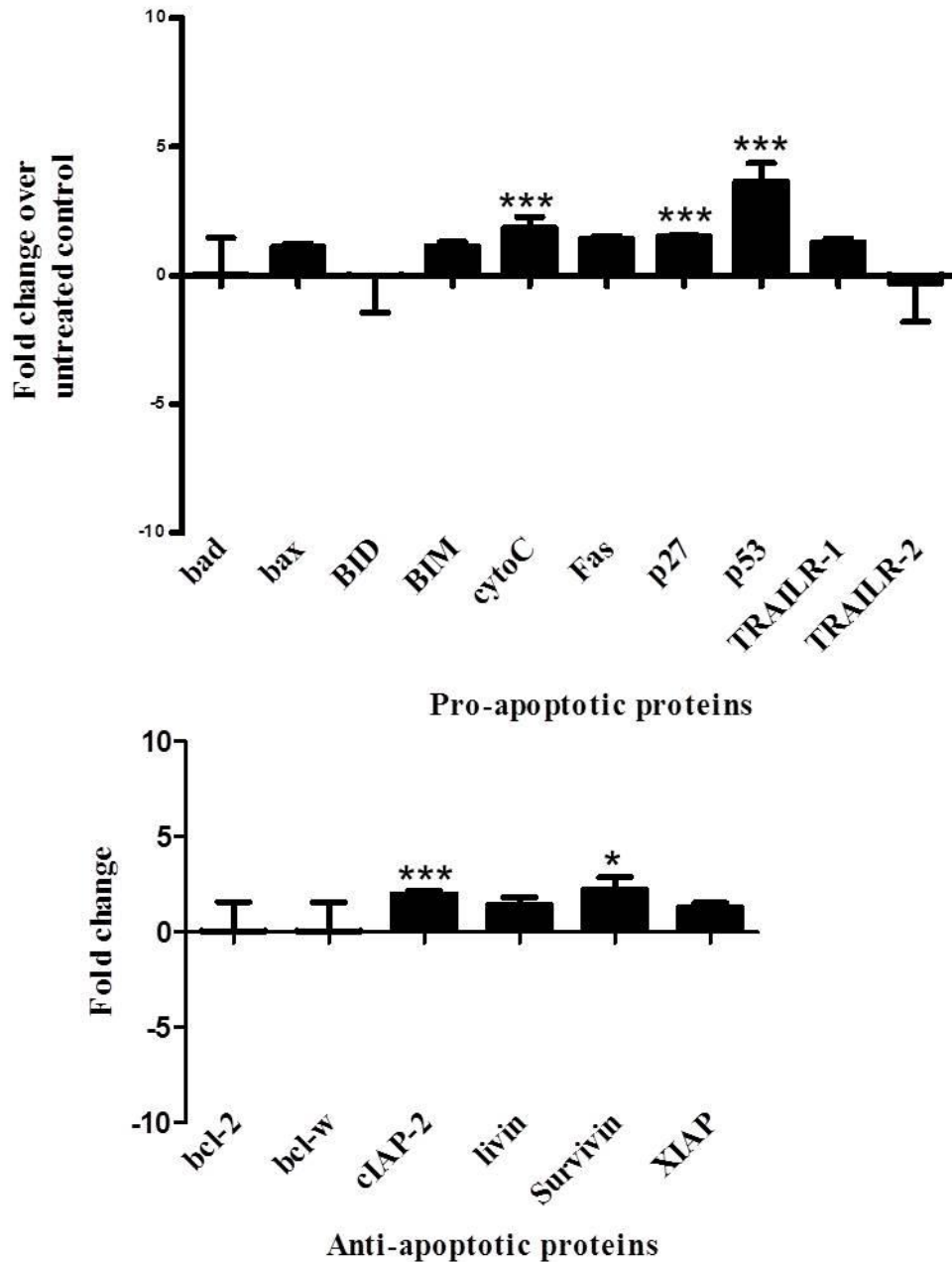
Fig 6.6: Protein expression profiles of U87MG cells receiving combined low-dose treatment of gamma tocotrienol with IC_{20} dose of jerantinine A for 72 h. Protein was extracted from 1 x 10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. P <0.001 (***), P <0.01 (**) and P <0.05 (*) indicate significant difference between treated and untreated groups.
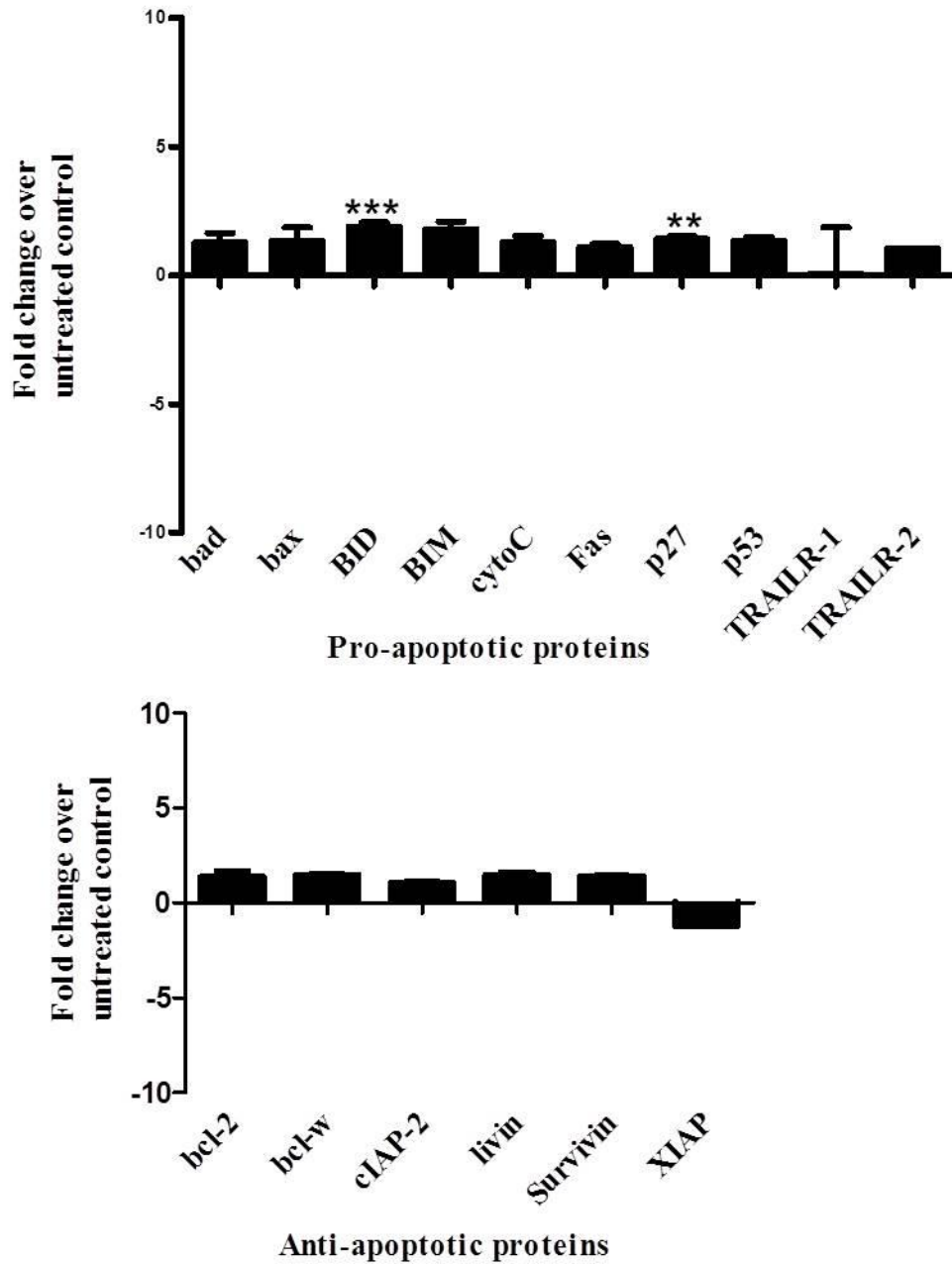
Fig 6.7: Protein expression profiles of U87MG cells receiving combined low-dose treatment of gamma tocotrienol with IC_{20} dose of jerantinine B for 72 h. Protein was extracted from 1 x 10^6 U87MG cells and the expression levels of pro- and anti-apoptotic proteins were determined using apoptosis array analysis. Results are reflected by the fold change of treated over the untreated control cells from two repeats. $P < 0.001$ (***), $P < 0.01$ (**) and $P < 0.05$ (*) indicate significant difference between treated and untreated groups.
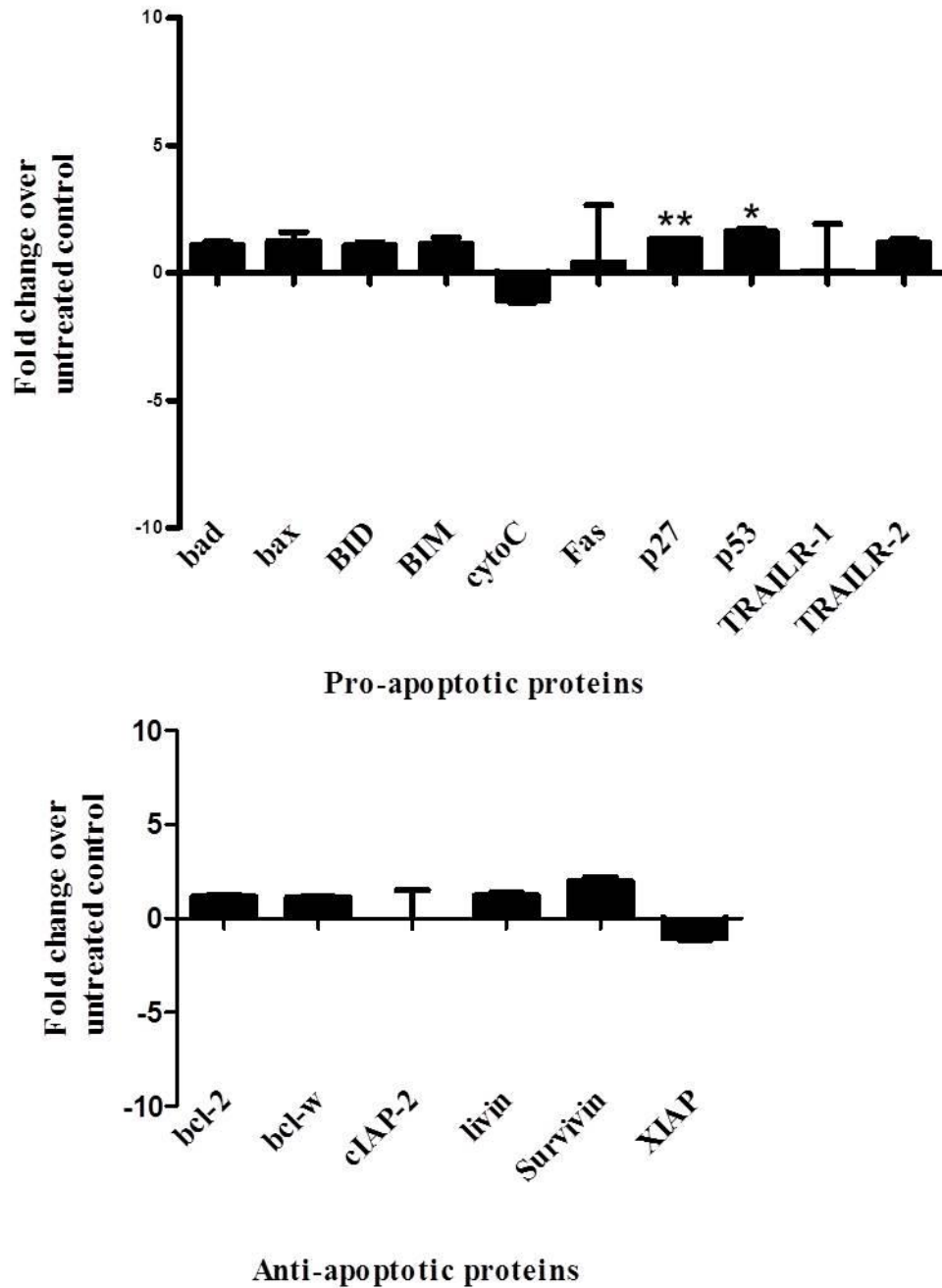
Table 6.2: Summary of fold change in expression profiles of pro-apoptotic proteins in U87MG cells

Treatments	Fold change over untreated control									
	Bad	Bax	Bid	Bim	Cyto C	Fas	p27	p53	TRAIL-R1	TRAIL-R2
δ-T3	-1.1	+0.02	+1.1	+0.01	+0.04	**-2.3**	+1.1	-1.8	+0.17	+1.1
γ-T3	+0.1	+1.1	+1.1	-1.1	-0.1	-1.9	0.00	-1.9	+0.03	+1.3
JA	+0.01	+0.1	+1.4	+1.6	+0.3	+1.4	+1.2	**+2.0**	+0.2	-1.3
JB	+1.1	+1.1	+1.0	+0.1	-0.06	+1.2	+1.3	+1.3	+0.1	-1.0
δ-T3+JB	+1.3	+1.4	+1.9	+1.8	+1.3	+1.1	+1.4	+1.3	+0.1	+1.0
γ-T3+JA	+0.01	+1.1	-0.01	+1.1	+1.9	+1.4	+1.5	**+3.7**	+1.3	-0.3
γ-T3+JB	+1.1	+1.2	+1.1	+1.2	-1.1	+0.4	+1.3	+1.6	+0.1	+1.2

(-) and (+) indicate down and upregulations of proteins compared to untreated controls. Proteins with 2-fold and above changes in expression are bolded and shaded.

Table 6.3: Summary of fold change in expression profiles of anti-apoptotic proteins in U87MG cells

Treatments	Fold change over untreated control					
	Bcl-2	Bcl-w	cIAP-2	Livin	Survivin	XIAP
δ-T3	-0.5	-1.3	-1.3	**+4.2**	+1.9	-1.2
γ-T3	+1.1	0.0	+1.0	+0.15	+1.1	**-2.2**
JA	+1.3	+1.2	+1.2	+1.2	**+2.5**	-1.5
JB	+1.1	+1.1	+0.02	+0.2	**+2.4**	-1.3
δ-T3+JB	+1.4	+1.5	+1.1	+1.5	+1.4	-1.2
γ-T3+JA	+0.01	+0.04	+1.9	+1.4	**+2.2**	+1.3
γ-T3+JB	+1.2	+1.1	+0.01	+1.2	+1.9	-1.1

(-) and (+) indicate down and upregulations of proteins compared to untreated controls. Proteins with 2-fold and above changes in expression are bolded and shaded.

6.3.2 Western detection of Bid and p53 levels altered by individual and combined treatment on U87MG cells

Western blotting analysis of U87MG cells following 24 h treatment with individual or combined low-dose treatments corroborated data obtained from apoptosis array on the activation of BID by individual IC_{50} doses and combined low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) (Fig 6.8, 6.9). Similarly, an upregulation of p53 was only evident in jerantinines (A and B) and combined low-dose treatments with tocotrienols (delta and gamma) but not individual IC_{50} doses of tocotrienols (Fig. 6.8, 6.9).
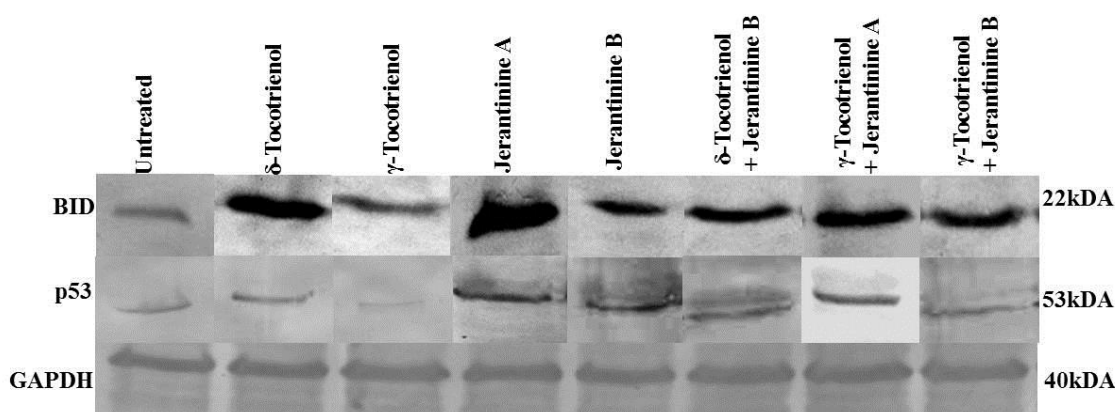
Fig 6.8: Western blotting profiles of Bid and p53 expression detected in protein lysate of U87MG cells treated with individual IC_{50} doses or combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinine (A and B) for 24 h. Bid and p53 proteins with molecular sizes of 22kDA and 53kDA, respectively are detected. GAPDH is served as a loading control for the assay.
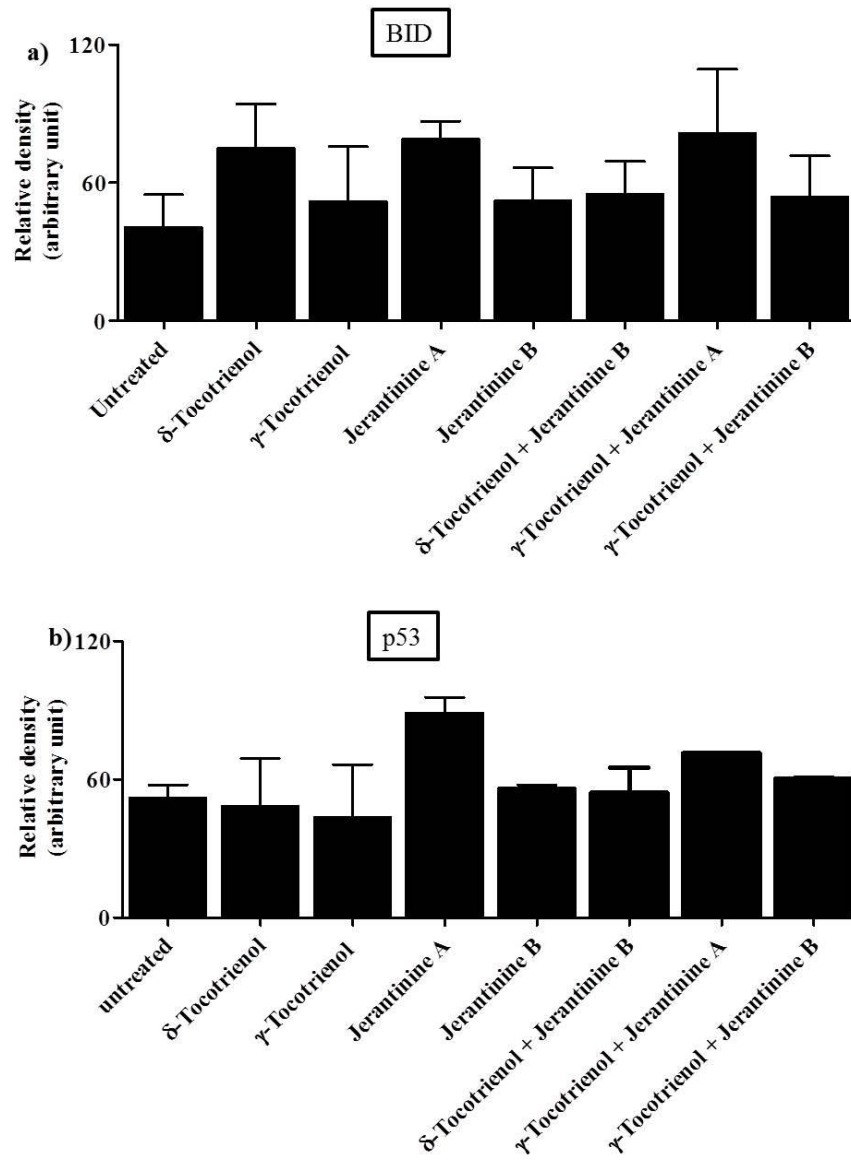
Fig. 6.9: Relative density profiles quantitated from the western protein bands (Fig. 6.8) of a) Bid and b) p53 obtained from U87MG cells receiving individual and combined treatments. Results are represented as mean ± SD from two repeats. Statistical analysis was conducted using one way ANOVA (Dunnet's t-test) and statistical significance between treated and untreated was set at P <0.05.

6.3.3 Evaluation of cytochrome C level altered by individual and combined treatments on U87MG cells via ELISA

Following 24 h treatments with individual IC_{50} doses of tocotrienols (delta and gamma), jerantinines (A and B) and the respective combined low-dose treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B), the cytochrome C levels were determined as a marker for apoptosis via ELISA. Results showed that individual IC_{50} doses of tocotrienols (delta and gamma) and jerantinines (A and B) augmented cytochrome C levels compared to untreated controls (Fig. 6.10). Similarly, combined low-dose treatments of gamma tocotrienol with IC_{20} doses of jerantinines (A and B) and delta tocotrienol with IC_{20} dose of jerantinine B also induced increased cytochrome C levels at the same multitude to that of individual doses as compared to untreated controls (Fig. 6.10)
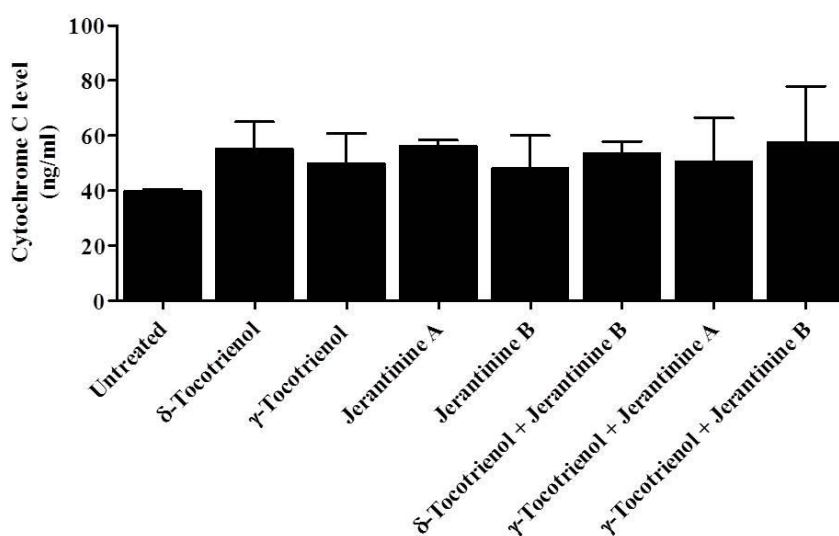
Fig. 6.10: Cytochrome C levels of U87MG cells receiving individual and combined treatments. A total of 1.5 x 10^6 cells were treated with individual IC_{50} doses and combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B). Cells receiving plain media with DMSO served as untreated control. The cytochrome C levels were quantified and results are presented as mean ± SD from three repeats. Statistical analysis was conducted using one way ANOVA (Dunnet's t-test) and statistical significance between treated and untreated was set at $P < 0.05$

6.3. Discussion

Apoptosis is a programmed cell death characterized by striking balance of activities between pro- and anti-apoptotic proteins which can be dependent and independent of caspase enzyme activity. The timely activation of apoptotic proteins that results in regulation of signaling pathways is mediated via best understood pathways of death receptor (extrinsic) and mitochondria (intrinsic). Tocotrienols have been shown to induce apoptosis in cancer cells via death receptor or mitochondria pathway depending on the nature of cellular microenvironment (Ling et al., 2012; Sun et al., 2009). In fact, recent studies demonstrated that tocotrienols caused upregulation of caspase 8, Bid and cytochrome C suggesting potential activation of both death receptor and mitochondria mediated pathways (Lim et al., 2014a, 2014b). Indeed, results from Chapter Five in the present study have demonstrated the activation of predominantly caspase 8 and 3 enzymes by delta and gamma tocotrienols. Furthermore, it demonstrated the potential of cell death independent of activation of caspase enzymes. As such, it was therefore necessary to elucidate the mechanism of action for delta and gamma tocotrienols herein to confirm the potential involvement of death receptor or mitochondria pathway.

Apoptosis induced via the death receptor pathway involves interaction between death domain and respective ligands for death receptors of the tumor necrosis factor (TNF) family resulting in recruitment of adaptor proteins and subsequent transmitting of death signals from cell surface to intracellular signaling pathways. The adaptor proteins (TRADD and FADD) activate caspase 8 to mediate apoptosis via subsequent activation of caspase 3 or alternative release of mitochondria related proteins via truncation of Bid to tBid (Elmore, 2007). Herein, there were upregulations of

TRAILR-1 and TRAILR-2 thus indicating that activation of caspase 8 by delta tocotrienol was mediated via death receptor. On a similar note, gamma tocotrienol induced upregulation of TRAILR-2 indicating that caspase 8 is activated via death receptor and subsequent recruitment of adapter proteins such as TRADD. Tocotrienols induction of caspase 8 mediated apoptosis can be dependent or independent of death receptors depending on the type of isomer as well as cell lines studied. For instance, previous study had shown that gamma tocotrienol promoted TRAIL induced apoptosis in prostate cancer cells (Kannappan et al., 2010a) in contrast to gamma tocotrienol induced upregulation of caspase 8 independent of death receptors in neoplastic mammary epithelial cells (Shah and Sylvester, 2004). The balance between pro- and anti-apoptotic Bcl2 family proteins determines the fate of cells undergoing apoptosis. As shown in Fig 6.1 and 6.2, there were general downregulation of anti-apoptotic proteins in U87MG cells receiving delta tocotrienol, whereas, slight but non-significant upregulation of anti-apoptotic proteins were evident in cells receiving gamma tocotrienol. Nonetheless, the balances between the pro- and anti-apoptotic proteins committed the U87MG cells to death. Results from the apoptosis array showed down regulation of cytochrome C in cells treated with gamma tocotrienol after 72 h, whereas, there was slight increase in cells treated with delta tocotrienol. However, this did not rule out the involvement of cytochrome C towards apoptosis execution by both delta and gamma tocotrienols as ELISA study showed cytochrome C levels peak after 24h (Fig 6.10). Similar trend was also evident in study for Bid activity as demonstrated by Western blotting data shown in Fig. 6.8. Undoubtedly, the induction of caspase 8 enzyme activity by both delta and gamma tocotrienols triggered Bid subsequently activating the mitochondria pathway and

cytochrome C release. These results confirmed that both delta and gamma tocotrienols triggered the death receptor and mitochondria pathways in U87MG cells.

On the other hand, there was a marked increase in expression levels of death receptor protein FAS in U87MG cells after 72 h treatment with IC_{50} dose of jerantinine A or jerantinine B. Although in contrast to delta tocotrienol there was a slight upregulation of TRAILR-1 only for both jerantinines A and B. The activation of caspase enzymes by jerantinines A and B as shown in Chapter Five suggested the potential role of both death receptor and mitochondria pathways. Undoubtedly the activation of FAS and TRAILR-1 death receptors herein by both jerantinines A and B which subsequently resulted in activation of BID and cytochrome C release confirmed the activation of both pathways in mediation of apoptosis (Ozören and El-Deiry, 2003). Although there are a few mechanistic studies on the mechanism of action of jerantinines A and B (Qazzaz et al., 2016; Raja et al., 2014), to date, there are no reports on the potential role of death receptors. Nonetheless, the reported activation of caspase 3 by jerantinine A on lung and colon cancer cells (Raja et al., 2014) is in agreement with data generated from this study. In fact, activation of caspase 3 can be triggered by both death receptor and mitochondria pathways (Saraste and Pulkki, 2000).

The combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines A and B have demonstrated an improved potency as compared to single high-dose tocotrienols as reported in previous chapters. Similarly, as shown in Fig. 6.1, 6.2, 6.5, 6.6 and 6.7 herein, unlike single IC_{50} dose of gamma or delta tocotrienol, the combined treatment of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) triggered the death receptor pathway via

upregulation of TRAIL and FAS death receptors. The subsequent activation of BID and eventual release of cytochrome C as evidenced in ELISA and Western blotting studies confirmed the role of mitochondria pathway in the execution of apoptosis. More importantly, this demonstrated an improved multi-targeted mechanism via recruitment of both TRADD and FADD death domain as compared to the potential recruitment of only TRADD by individual IC_{50} dose of delta and gamma tocotrienols. The individual IC_{50} doses of jerantinine A and B and combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinine A and B in this study induced upregulation of p53 and p27 in U87MG cells after 72 h treatment suggesting potential roles of these proteins towards G2/M and G0/G1 cell cycle arrests as evidenced in Chapter Five. In addition to potential induction of cell cycle arrest, p53 protein can engage the mitochondria pathway indirectly by transcriptional activation of proapaptotic proteins such as Bid, Noxa or Puma (Fulda and Debatin, 2006; Oda et al., 2000; Sax et al., 2002; Yu et al., 2001). Alternatively, p53 can directly trigger the mitochondria pathway via activation of Bax or Bak or through deactivation of anti-apoptotic Bcl-2 proteins (Chipuk, 2004; Fulda and Debatin, 2006; Mihara et al., 2003; Moll et al., 2005). Undoubtedly, the upregulation of p53 as well as Bcl-2 pro-apoptotic proteins including Bax and Bid by individual IC_{50} doses of jerantinines (A and B) and the combined low-dose treatment with tocotrienols (delta and gamma) on U87MG cells confirmed the recruitment of mitochondria pathway in mediation of apoptosis.

Survivin is a bifunctional that regulates cell division and also suppresses apoptosis. Survivin inhibits apoptosis by blocking SMAC and subsequently binding to XIAP (Johnson and Howerth, 2004; Mita et al., 2008) . In this study, there was a general

upregulation of survivin with over 2-fold increase as evidenced in U87MG cells receiving IC_{50} doses of jerantinines (A and B) and combined treatment of low-dose gamma tocotrienol with IC_{20} dose of jerantinine A. Interestingly though, there was an upregulation of cytochrome C as shown in Fig 6.6 suggesting that survivin did not suppress the mitochondria pathway. XIAP was generally downregulated and thus not available for binding to survivin and probably accounting for the increased levels observed and its inability to suppress the mitochondria pathway. SMAC/DIABLO in addition to AIF, endonuclease G and cathepsin have been reported to be positive modulators of caspase independent cell death (Bröker et al., 2005, 2004; Candé et al., 2002; Lockshin and Zakeri, 2004; Lorenzo and Susin, 2004). Considering that both individual and combined treatments induced caspase independent cell death (Chapter Five) suggest potential activation of SMAC/DIABLO which competes with survivin for binding of XIAP (Pavlyukov et al., 2011). In addition, previous studies have also shown that constitutive activation of STAT3 may lead to upregulation of survivin and thus inhibitors of oncogenic signal such as STAT3, phosphatidylinositol 3-kinase (PI3-K), Akt , EFGR and MEK1 can suppress survivin (Johnson and Howerth, 2004). Previous studies have indeed shown that tocotrienols can suppress these oncogenic signals as well (Ling et al., 2012; Samant and Sylvester, 2006; Weng et al., 2009). In fact, another study showed that delta tocotrienol suppression of antitumor activity of survivin in non–small cell lung cancer cells was associated with downregulation of Notch-1 and VEGF (Ji et al., 2011). Similarly, cytoprotection offered by survivin has been shown to be ineffective in FAS induced apoptosis (Johnson and Howerth, 2004). Hence, although survivin is upregulated, the potential inhibition of the mitochondria pathway is probably mitigated by suppression of oncogenic signal by delta and gamma tocotrienols as well as Fas induced apoptosis by jerantinines A and B.

6.4 Conclusion

Data gathered from apoptosis array, ELISA and Western blotting analysis herein showed that delta and gamma tocotrienols triggered both death receptor and mitochondria pathways mediated via TRAIL activation thereby triggering caspase enzyme activity, BID activation and cytochrome C release in U87MG cancer cells. On the other hand, jerantinines A and B induced apoptosis in U87MG cells via death receptors (FAS and TRAIL) and mitochondria pathways as confirmed by activation of Bid and cytochrome C release. Similarly, combined low-dose treatments of jerantinines (A and B) with tocotrienols (delta and gamma) mediated apoptosis via FAS, TRAIL death receptor and mitochondria pathway associated with BID activation and cytochrome C release. In addition, unlike single delta and gamma tocotrienols, individual doses of jerantinines A and B and the respective combine low-dose treatments activated p53 pathway with peak activation evidenced in combined treatment of jerantinine A with gamma tocotrienol. Overall, an improved multi-targeted mechanism of apoptosis induction on U87MG cells by combined low-dose treatments compared to individual dose of tocotrienols (delta and gamma) is potentiated via this study.

7.0 CHAPTER SEVEN

General Discussion and Conclusions

7.1 Background

Anticancer agents sourced from nature have continuously been explored for development of more efficient and potent cytotoxic drugs. These natural products in fact still constitute the vast majority of recent anticancer drugs. However, toxicity and drug resistance have limited potential clinical application of some of these drugs and most prominently, the vitamin E isomers (tocotrienols) are amongst these therapeutic candidates. Whilst tocotrienols have shown great pharmacological values especially *in vitro*, the potential clinical application has been limited due to high-dose led toxicity such as hemorrhage, stroke and others which are often the consequences of the need to deliver higher dosage for potent effects (Lonn et al., 2005). Furthermore, delivery of high dosage in particular orally overloads the transport protein resulting in metabolic degradation of tocotrienols thus causing low levels of therapeutic doses (Shirode and Sylvester, 2010). Recent literatures have suggested combinatorial treatment that would reduce the doses of tocotrienols as a possible alternative to circumvent such aforementioned limitations. Indeed, drug combinations can result in sequential, concurrent or concerted pharmacological interactions between two or more chemotherapeutic agents which may successively minimize toxicity, improve therapeutic potency of candidates and overcome drug resistance (Boik, 2001; Lim et al., 2013). In light of this, the present combination study as sequentially reported in Chapter Three to Chapter Six was undertaken with the aims of procuring the minimized potent dosage of the individual drug candidates and investigating towards the elucidation of mechanisms of action for potent treatment combinations.

Ficus species was chosen for combinatorial treatment with tocotrienols based on its history of traditional medicinal use as anticancer agents (Lansky et al., 2008; Singh et al., 2011) and recent pharmacological studies that suggested the application of *Ficus* species as adjunct cytotoxic agents (Nugroho et al., 2013, 2012). On the other hand, jerantinines A and B, the pure compounds isolated from *Tabernaemontana corymbosa*, have demonstrated an interesting anticancer activity, most notably on reversal of drug resistance. However, as demonstrated by limited preliminary and mechanistic studies, jerantinine derivatives induce similar toxic effects to non-cancerous cells (Qazzaz et al., 2016; Raja et al., 2014). Therefore, jerantinines A and B were selected in this study as combined treatment candidates with hopes to minimize such non-selective toxicity and exert more importantly different mechanisms of action in comparison to tocotrienols to potentiate improvement in potency via multi-targeted approach. The study started with initial antiproliferative screening to determine the growth inhibitory potency and the pharmacological interaction of combined treatments. Upon confirmation of synergism, further studies were conducted to investigate the morphological changes, DNA damage patterns and cell cycle arrest pattern. Based on the results of DNA damage and cell cycle arrest, investigations on possible antimicrotubule effects and potential role of caspase enzymes towards the mediation of apoptosis were conducted. Finally, molecular and cell-based assays were conducted to establish the potential roles of pro- and anti-apoptosis related proteins.

7.2 Combined low-dose treatments exert synergistic anticancer effects on cancer cells and minimize toxicity towards non-cancerous cells

As reported in Chapter Three, three different *Ficus* species (*Ficus hispida, Ficus fistulosa* and *Ficus schwarzii*) were collected from different locations in Malaysia for antiproliferative screening. *Ficus hispida* and *Ficus fistulosa* were selected for this study based on pharmacological and ethno-medicinal uses as potential anticancer treatments (Lansky et al., 2008; Peraza-Sánchez et al., 2002; Pratumvinit et al., 2009; Subramaniam et al., 2009; Tuyen et al., 1998; Zhang et al., 2002). Whereas, *Ficus schwarzii* was selected to provide new valuable information on its bioactivity considering that to date there is no published study on the bioactivity of this plant. Ethanol and alkaloid crude extracts were prepared for bark and leaf parts of each plant to allow for determination if antiproliferative potency of *Ficus* spcies was due to alkaloids that present in the crude extracts. The antiproliferative study revealed that crude alkaloid extracts from leaves and bark of *Ficus* species generally exerted more potent antiproliferative effects compared to ethanol extracts. Furthermore, based on NCI standard for crude extracts, the antiprofilerative potency was ranked as *F. fistulosa > F. schwarzii > F. hispida* in a decreasing order of potency. This is in agreement to a recent study that demonstrated the antiproliferative potency (IC_{50} = 2-7 µM) of *F. fistulosa* alkaloids, e.g. fistulopsines (A and B) on HCT 116 and MCF7 cells lines (Yap et al., 2016). Whereas, the lower potency of *F. hisipida* extracts observed in current study is contrary to the published potent effects of *F. hisipida* alkaloids including *O*-Methyltylophorindine and hispiloscine (Peraza-Sánchez et al., 2002; Yap et al., 2015). This is attributed to the fact that crude extracts contain other non-cytotoxic compounds that may have antagonistic effects. Furthermore, the type of

cell lines screened, location and time of plant collection may also account for the observed difference in potency.

On the other hand, jerantinines (A and B), the pure compounds isolated from *T. corymbosa* induced potent antiproliferative effects on U87MG, A549 and HT-29 cells with IC_{50} values fall below 4 μg/ml. However, similar potent effect was also evident on non-cancerous MRC5 cells thereby corroborating recent studies on the cytotoxic potency of jerantinines (Frei et al., 2013; Qazzaz et al., 2016; Raja et al., 2014). This is contrasted to the mild toxicity induced by tocotrienols (delta and gamma) towards non-cancerous MRC5 cells as shown in current study. However, the potency of tocotrienols (delta and gamma) tested on U87MG, A549, and HT-29 cancer cells, was lower as compared to jerantinines (A and B).

The combined treatment at lower dosage was adopted in this study to reduce the required potent doses thereby eliminating the need for single high tocotrienol dosage. This would circumvent the limitations of high dosage and minimize toxicity towards non-cancerous cells. In addition, the combined treatment would improve potency via multi-targeted mechanism compared to individual tocotrienol doses. The combined treatment of *F. fistulosa* with tocotrienols (delta and gamma) induced the most potent synergistic effect on cancer cells compared to the additive or antagonistic effects observed for combined treatment of *F. schwarzii* with tocotrienols (delta and gamma). This is in agreement with recent studies that demonstrated the application of *Ficus* species as adjunct treatments (Nugroho et al., 2013, 2012). However, the type of pharmacological interaction between adjunct treatments was not determined (Nugroho et al., 2013). In fact, the potential toxicity to non-cancerous cells was not determined

(Nugroho et al., 2012). In contrast, the present study demonstrated the synergistic potencies of *Ficus* species. In addition, the combined treatment approach adopted in this study showed that the use of low-dose *Ficus* species causes a considerable reduction of potent doses of adjunct treatments thereby minimizing toxicity towards non-cancerous cells. However, further mechanistic studies on *F. fistulosa* and *F. schwarzii* was discontinued despite the display of remarkable synergistic potency. This was justified by the fact that crude extracts contain numerous compounds that would make it difficult to properly elucidate for the underlying mechanisms of action and hence generating less interest and value compared to pure compounds of the study. Whereas, purified alkaloids jerantinines (A and B) have shown promising anticancer activity. However, the underlying mechanism of action is not fully understood. Therefore, the present study was focused on establishing the mechanism of action for apoptosis induction by jerantinines A and B and tocotrienols. Nevertheless, the potentials of these plant species as an adjunct anticancer treatment cannot be underestimated if pure bioactive compounds should have been isolated.

The combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) have promisingly shown reductions of required potent doses and toxicity levels of jerantinines (A and B) towards non-cancerous MRC5 cells. The combined treatments that induced synergistic effects (IC_{50} values fall below 4 µg/ml) and showed good selectivity window of at least 2-fold between cancer and non-cancerous cells were selected for further mechanistic studies.

7.3 Tocotrienols induce apoptosis via death receptor and mitochondria pathway

Tocotrienols have been reported to possess potent anticancer effects against a broad spectrum of cancer cell lines (Nesaretnam and Yew, 2007; Sen et al., 2007; Sylvester et al., 2010). However, the potency has been limited due to metabolic degradation and combined treatment that is capable of improving potency has been suggested as an alternative. Hence, in this study, in addition to the development of combined treatments, the individual doses of delta and gamma tocotrienols were also studied. This was to allow for comparison between individual doses of tocotrienols and combined low-dose treatments to determine if the combined treatments have resulted in an improved potency possibly via a multi-targeted pathway. The data herein confirmed that the potent antiproliferative effects of tocotrienols (delta and gamma) occurred on cancer cells especially U87MG and HT-29 cells selectively with only trivial toxicity exerted on non-cancerous MRC5 cells. This effect was characterized by manifestation of the morphological and biochemical features of apoptosis that included cell shrinkage, nuclear chromatin condensation and fragmentation, G0/G1 cell cycle arrest and double stranded DNA breaks. Further investigations revealed the recruitment of initiator caspase enzymes 8 and 9 and executioner caspase 3 as well as potential induction of apoptosis independent of caspase activation. This is in agreement with recent studies that confirmed activation of caspase 8 and 3 enzymes preceded the double stranded DNA breaks by tocotrienols on U87MG and A549 cells (Lim et al., 2014a, 2014b). In fact, these studies suggested the potential recruitment of both death receptor and mitochondria pathways by tocotrienols.

Tocotrienols (delta and gamma) herein induced the most potent antiproliferative effects on U87MG cells. In addition, to date, very few studies have been conducted on

the cytotoxic potency of tocotrienols on U87MG cancer cells. Therefore, taking into consideration on the aforementioned, U87MG cells was selected for further mechanistic studies using molecular and cell-based assays for the mechanism of action elucidation. These tocotrienol studies have generally confirmed the recruitment of TRAIL death receptors, Bid activation and mitochondria pathway through cytochrome C release. A proposed mechanism of action is shown in Fig 7.1 and suggests that TRAIL activation of caspase 8 triggered an activation of Bid. Eventually, the truncated tBid activated the mitochondria pathway via Bax activation and leading to subsequent release of cytochrome C. This is in agreement with the suggestion by Lim et al. (2014b) on the potential recruitment of death receptor and mitochondria pathways. The study also conforms to a previous report stating the TRAIL activation by gamma tocotrienol (Kannappan et al., 2010). However, this is contrary to a previous report that tocotrienol induced caspase 8 activation on +SA mammary cells was unrelated to death receptors (Shah and Sylvester, 2004). It is noteworthy to mention that +SA mammary cells have been proven resistant to death receptor induced apoptosis (Shah and Sylvester, 2004).
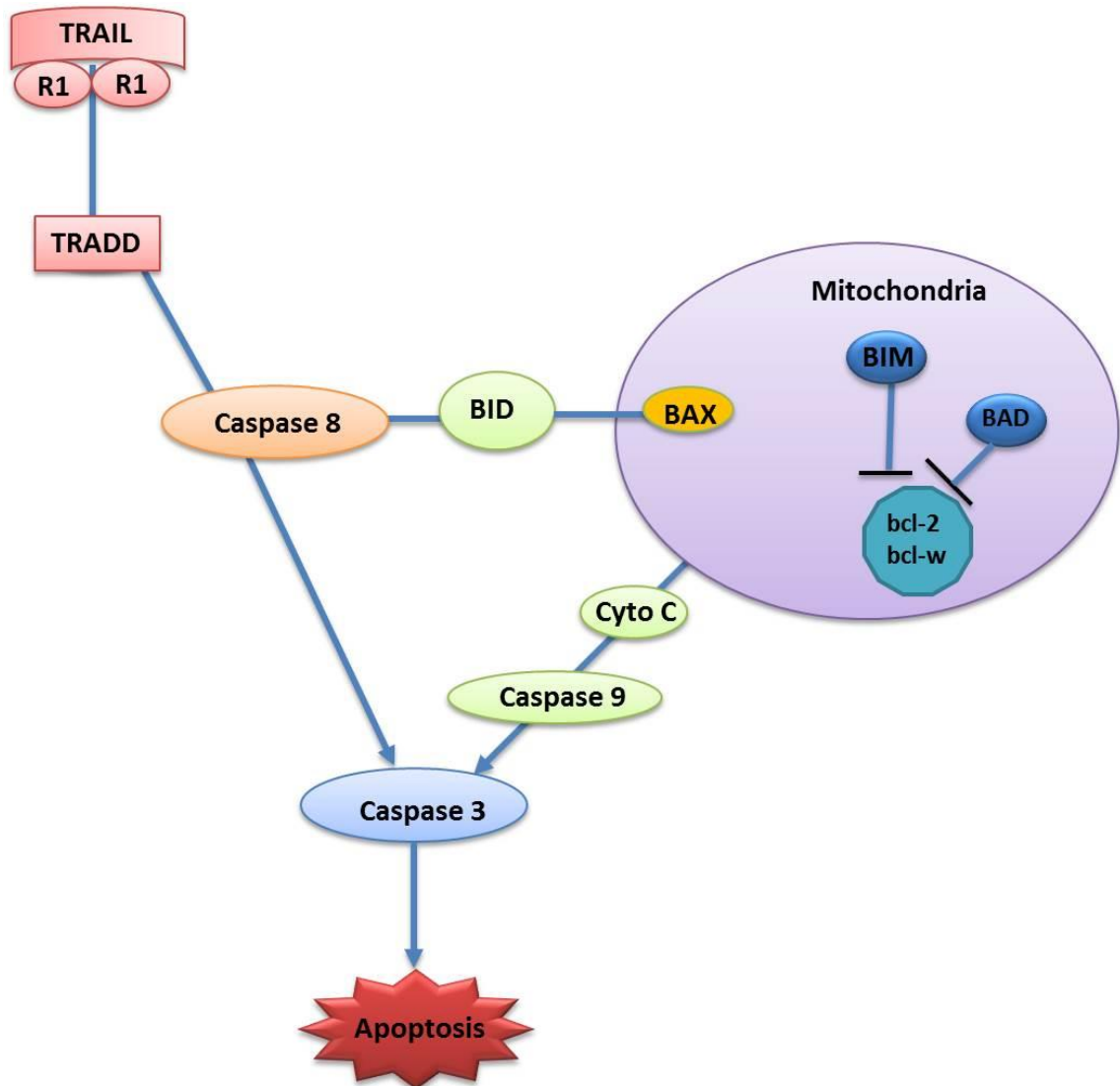
Fig 7.1: Schematic representation on the proposed mechanism of action for tocotrienols (delta and gamma). Apoptosis was induced via death receptor (TRAIL-R1 and R2) and mitochondria pathways. The activation of caspase 8 by TRAIL caused BID activation and subsequent release of cytochrome C from mitochondria to induce apoptosis.

7.4 Jerantinines target Fas and p53 linked apoptosis with anti-microtubule effects

As reported in Chapters Four and Five, both jerantinines (A and B) induced similar mode of apoptotic cell death. This was characterized by morphological features that included membrane blebbing and apoptotic body formation as well as induction of double stranded breaks as a biochemical hallmark of apoptosis. The similarities of morphological features and DNA damage patterns between jerantinines (A and B) and vinblastine (control) prompted the investigations on the potential antimicrotubule effects of jerantinines (A and B). Jerantinines (A and B) induced G2/M cell cycle arrest and caused disruption of microtubule networks. The disruption of microtubules was accompanied by cell shrinkage and lower alpha tubulin signals as compared to untreated control. The pattern of cell cycle arrest of Jerantinines (A and B) is consistent with that of microtubule targeting agents (Perez, 2009). Furthermore, this finding corroborated previous studies that confirmed jerantinines (A and B) are antimicrotubule agents indeed (Frei et al., 2013; Qazzaz et al., 2016; Raja et al., 2014). However, similar to microtubule targeting agents, jerantinines (A and B) showed non-selective toxicity to have induced similar toxic effects to both cancer and non-cancerous MRC5 cells. Investigation on the possible mechanism revealed that jerantinines (A and B) involved caspase 8 activation which was triggered by Fas death receptor. Fas death receptor induced apoptosis is mediated through Fas associated death domain (FADD). The activation of Bid, Bax and cytochrome C by jerantinines (A and B) also confirmed the involvement of the mitochondria pathway of apoptosis (Fig 7.2). This is in agreement with previous reports that death receptors can directly trigger apoptosis via caspase 8 and 3 or alternatively activate the mitochondria pathway through Bid activation (Elmore, 2007; Fulda and Debatin, 2006a; Ozören

and El-Deiry, 2003; Saraste and Pulkki, 2000). On the other hand, it is strongly suggested that jerantinines (A and B) triggered p53 activity may be linked to their antimicrotubule effects. This is attributed to the phosphorylation and stability of p53 that have been shown to increase after microtubule disruption. In fact, the p53 residue responsible for microtubule disruption induced phosphorylation varies depending on the type of microtubule inhibitor and cell types (Stewart et al., 2001). This is further supported by the fact that p53 activates mitochondria pathway via upregulation of Noxa and Puma and blocking of Bcl2 anti-apoptotic proteins. Therefore, the predominant activation of mitochondria by microtubule targeting agents including jerantinines (A and B) herein may be also mediated via increased p53 activity. However, this is not conclusive based on this study considering that p53 can be activated in response to DNA damage as well.
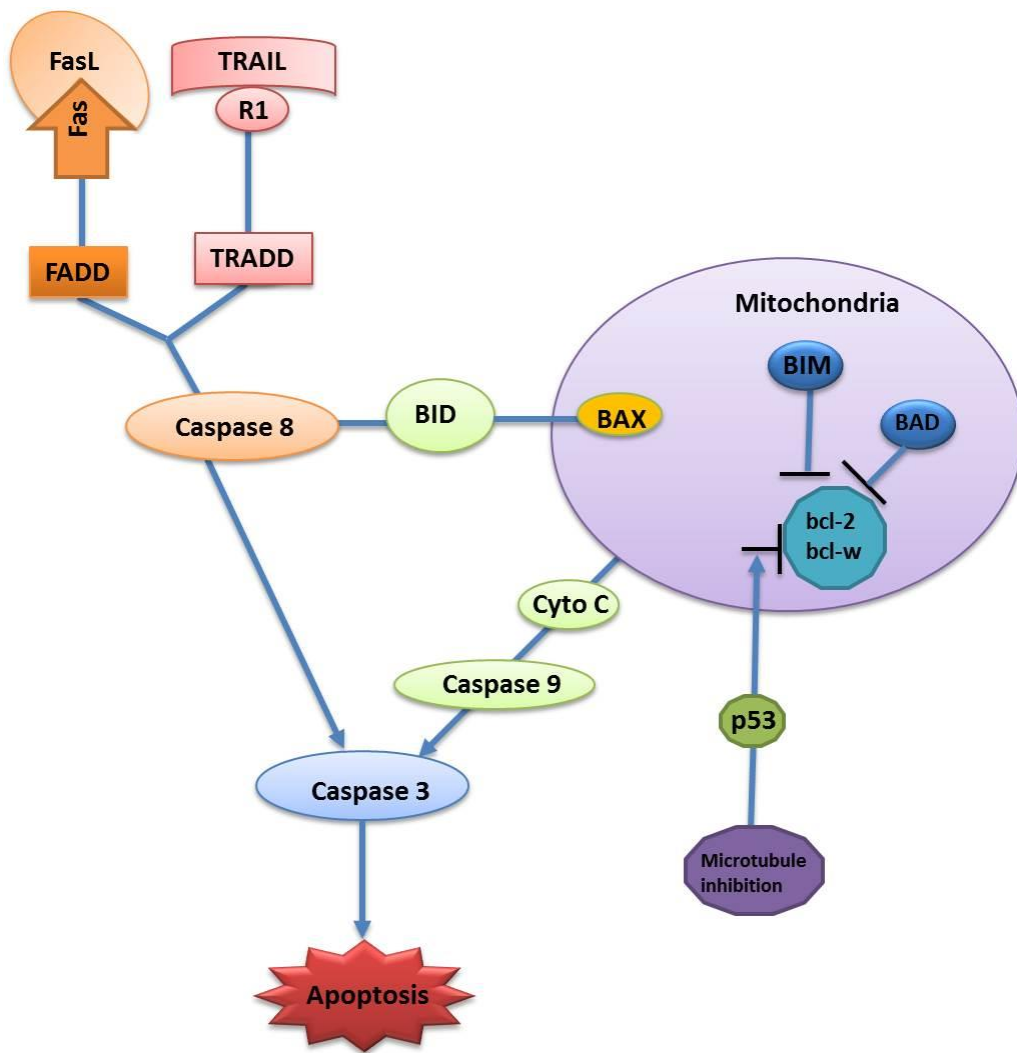
Fig 7.2: Schematic representation on the proposed mechanism of action for jerantinines (A and B). Apoptosis was induced via death receptor (TRAIL and FAS) and mitochondria pathways. The activation of caspase 8 by TRAIL and FAS caused BID activation and subsequent release of cytochrome C from mitochondria to induce apoptosis. Microtubule inhibition also promoted mitochondria mediated pathway of apoptosis via p53 activation.

7.5 Combined treatment confers multi-targeted mechanisms for enhanced therapeutic application

The combined treatment approach at lower dosage adopted in this study was aimed at limiting the need for high-dose tocotrienols and also to reduce the toxicity of jerantinines (A and B) towards non-cancerous MRC5 cells. As stated above and in Chapter Three, the combined treatment caused a reduction in required potent doses and reduced toxicity towards non-cancerous cells. Combined treatments that induced synergistic antiproliferative effects with at least 2-fold selective toxicity between cancer cells (U87MG, A549 and HT-29) and non-cancerous MRC5 cells were selected for further studies. Based on these criteria, the combined treatments were most effective on U87MG cells with three different combinations comparing to the combined treatments for HT-29 (two combinations) and A549 (one combination) cells. Induction of similar multitude of morphological changes, double stranded DNA breaks, caspase enzyme activity and caspase independent apoptosis by individual doses of tocotrienols (delta and gamma) and combined low-dose treatments with jerantinines (A and B) were observed in general. Besides these similarities, the combined low-dose treatment conferred multi-targeted mechanisms that enhanced therapeutic potency compared to individual doses of tocotrienols. Unlike the individual doses of tocotrienols, the combined low-dose treatments caused disruption of microtubule networks on U87MG, HT-29 and A549 cells which might be the surplus effects of jerantinines (A and B). Further mechanistic studies conducted on U87MG cells revealed the activation of both Fas and TRAIL death receptors compared to only TRAIL activation by tocotrienols (delta and gamma). Furthermore, as shown in the proposed mechanism of action (Fig 7.3), combined low-dose treatments activated p53 which may be attributed to microtubule disruption and

mitochondria pathway of apoptosis. The initial hypothesis was that the antimicrotubule effect of jerantinines (A and B) even at lower dosage may be mediated via p53 activation which has been further potentiated by tocotrienols. However, considering the fact that tocotrienols do not activate p53 suggests that the antimicrotubule effects could be attributed solely to IC_{20} doses of jerantinines (A and B). p53 protein in response to chemotherapeutic agents also mediate cell cycle arrest at stages preceding cell replication (G1/S), pre-mitotic (G2), chromosome condensation and chromosome partition (Schwartz and Rotter, 1998). p53 trans-activate kinase inhibitor proteins including p21 and p27 to induce cell cycle arrest. Herein, both IC_{50} doses of delta and gamma tocotrienols caused an upregulation of p27 indicative of cell cycle arrest in U87MG cells. p27 is however not limited to regulation of the early G1 phase only (Slingerland and Pagano, 2000). Contrary to individual dose of jerantinines (A and B), the combined low-dose treatments induced predominantly G0/G1 cell cycle arrest. This is consistent with individual doses of tocotrienols (delta and gamma) in this study and previously published studies (Fernandes et al., 2010; S.-W. Lim et al., 2014a; Xu et al., 2009). Therefore, this implies that although antimicrotubule effect is attributable to IC_{20} doses of jerantinines (A and B), the multi-targeted mechanism demonstrated by combined low-dose treatments is undoubtedly potentiated by low-dose tocotrienols. The combined low-dose treatments would be valuable for future anticancer chemotherapeutic regimen.

It is noteworthy to mention that the schematic diagram of the proposed mechanisms of action for individual and combined treatments as shown in Fig. 7.1, 7.2 and 7.3 are representation of only caspase dependent apoptosis. Caspase independent apoptosis is

mediated via lysosomal cathepsin and mitochondrial proteins such as AIF, endonuclease G and SMAC/DIABLO (Bröker et al., 2005; Elmore, 2007; Lockshin and Zakeri, 2004). Indeed all individual and combined treatments demonstrated an induction of caspase independent cell growth inhibition on U87MG, A549 and HT-29 cells (Chapter Five). However, the present was focused on caspase dependent apoptosis and as such did not establish the mechanism mediating the caspase independent apoptosis.
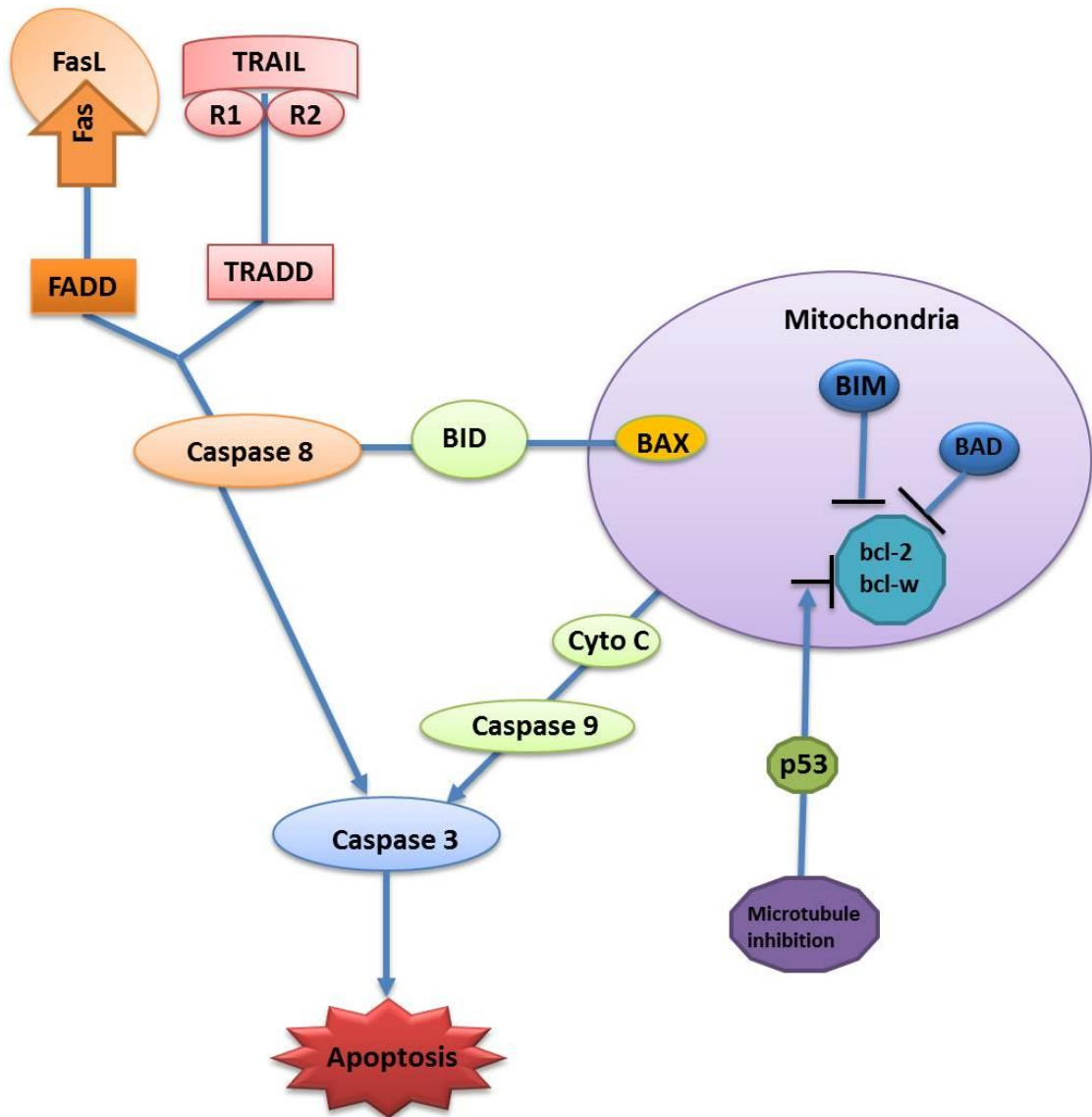
Fig 7.3: Schematic representation on the proposed mechanism of action for combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B). Apoptosis was induced via death receptor (TRAIL and FAS) and mitochondria pathways. The activation of caspase 8 by TRAIL and FAS caused BID activation and subsequent release of cytochrome C from mitochondria to induce apoptosis. Microtubule inhibition also promoted mitochondria mediated pathway of apoptosis via p53 activation.

7.6 Conclusions

Findings from this study confirmed the cytotoxic potency of *Ficus* species against cancer cells thereby corroborating the ethno-medicinal use of *Ficus* species for treatment of cancer. Furthermore, the study demonstrated that *F. fistulosa* and *F. schwarzii* would be most suitable to treat brain and colon cancers and the potent effects observed against these cancer cells are due to the presence of alkaloids in the crude extracts. On the other hand, the findings revealed that the cytotoxic potencies of tocotrienols (delta and gamma) and jerantinines (A and B) on U87MG, A549 and HT-29 cells are in line with recent published studies.

The combined treatments of tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) and alkaloid extracts of *F. fistulosa and F. schwarzii* induced synergistic cytotoxic effects on U87MG, A549 and HT-29 cells. In addition, the combined treatments have caused reduction in required potent doses thereby minimizing toxicity to non-cancerous cells. This undoubtedly accomplished the ultimate aims of this study to reduce potent doses and minimize toxicity towards non-cancerous cells. Furthermore, the study revealed that tocotrienols (delta and gamma) induced apoptosis characterized by manifestation of morphological changes, double stranded DNA breaks, G0/G1 cell cycle arrest and caspase enzyme activity which were mediated via TRAIL death receptor and mitochondria pathway. Whereas, in addition to TRAIL death receptor and mitochondria pathway, individual doses of jerantinines (A and B) and the combined low-dose treatment with tocotrienols (delta and gamma) also induced FAS and p53 activation characterized by the disruption of microtubule networks. These results demonstrated that multi-targeted and improved mechanism of action including microtubule disruption and p53 activation to the

existing mechanism of tocotrienols induced apoptosis. Furthermore, the confirmation of induction of double stranded DNA breaks, caspase 8 enzyme activity and potential induction of apoptosis independent of caspase enzymes by jerantinines (A and B) might be the first. Therefore, this study provides a new insight into the mechanistic action of jerantinines (A and B). It also accomplishes one of the specific objectives of this study to investigate the mechanism mediating the apoptotic effects of individual and combined low-dose treatments. Undoubtedly, the combined low-dose treatments of tocotrienols (delta and gamma) and jerantinines (A and B) would be a valuable chemotherapeutic regimen against cancers in future.

7.7 Limitations of current study and recommendations for future investigations

The combined low-dose treatments of tocotrienols with jerantinines (A and B) and *Ficus* extracts induced synergistic effects with improved potency on U87MG, A549 and HT-29 cells as shown in Chapter Three. Stronger antiproliferative potency has been exhibited by the alkaloids that are present in the crude extracts of *F. fistulosa* and *F. schwarzii* compared to ethanol extracts. However, unlike jerantinines (A and B), these are not purified alkaloids which highlight one of the limitations of this study. Nonetheless, this opens a new avenue for further research as such, future bioassay guided phytochemical studies should be conducted to isolate the pure bioactive alkaloidal compounds to allow for proper elucidation of mechanism of action which is responsible for the induction of apoptosis.

Most MTAs are known to be toxic to non-cancerous cells by which the phenomenon was also demonstrated by the individual doses of jerantinines (A and B) in the present study. Undoubtedly, combined low-dose treatments demonstrated an improved

selective toxicity towards cancer cells with negligible harm on non-cancerous cells. However, only one type of normal cells (human lung fibroblast in this case) was tested and future studies should be planned to cover a broader range of non-cancerous cells. Furthermore, *in vivo* toxicological studies should also be conducted on jerantinines considering that MTAs have been shown to be neurotoxic. In addition, future studies focusing on targeted delivery might also minimize the toxicity of jerantinines (A and B).

The individual IC_{50} doses of tocotrienols (delta and gamma), jerantinines (A and B) and combined treatments of low-dose tocotrienols (delta and gamma) with IC_{20} doses of jerantinines (A and B) induced caspase dependent apoptosis and apoptosis independent of caspase activation. However, the present study has been more focused on the mechanisms associating with the caspase dependent apoptosis. Considering the induction of caspase dependent and independent apoptosis have been suggested as potential alternative to improve potency, it is important that future studies enlist the elucidation of the mechanism mediating caspase independent cell death and establish the potential roles of protein such as SMAC/DIABLO, AIF, cathepsins and Endonuclease G amongst others that have been shown to promote caspase independent cell death.

Furthermore, the present study confirmed the activation of p53 by individual doses of jerantinines (A and B) and combined low-dose treatments but not individual IC_{50} doses of tocotrienols (delta and gamma). Although this demonstrates that tocotrienol induced DNA damage was independent of p53, however, future study should also determine the mechanism of p53 activation herein which could be as a result of

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microtubule disruption and its potential role in promoting mitochondria mediated apoptosis.

Oral delivery of high-dose tocotrienol causes overload of the transport protein and subsequent metabolic degradation thus resulting in less available therapeutic dose. Indeed, the combination of tocotrienol with jerantinine or extracts from *Ficus* species caused reduction in the required potent dose for tocotrienols thus eliminating the need for single high doses. However, only *in vitro* study had been carried out. A translational *in vivo* study should be conducted to determine if the combined treatments herein can improve the therapeutic potency and reduce toxicity towards normal cells as evidenced in the current *in vitro* study.

Jerantinines A and B have demonstrated the potential of reversing p-glycoprotein induced drug resistance associated with commercially available microtubule targeting agents such as vinblastine and vincristine. Therefore, understanding the mechanism mediating the reversal of drug resistance as well as harnessing the structure-bioactivity relationship would be invaluable. Indeed, this would champion the development of jerantinine derivatives and mimics for combined treatments with tocotrienols as future potent Microtubule targeting agents against brain, colon and lung cancers.

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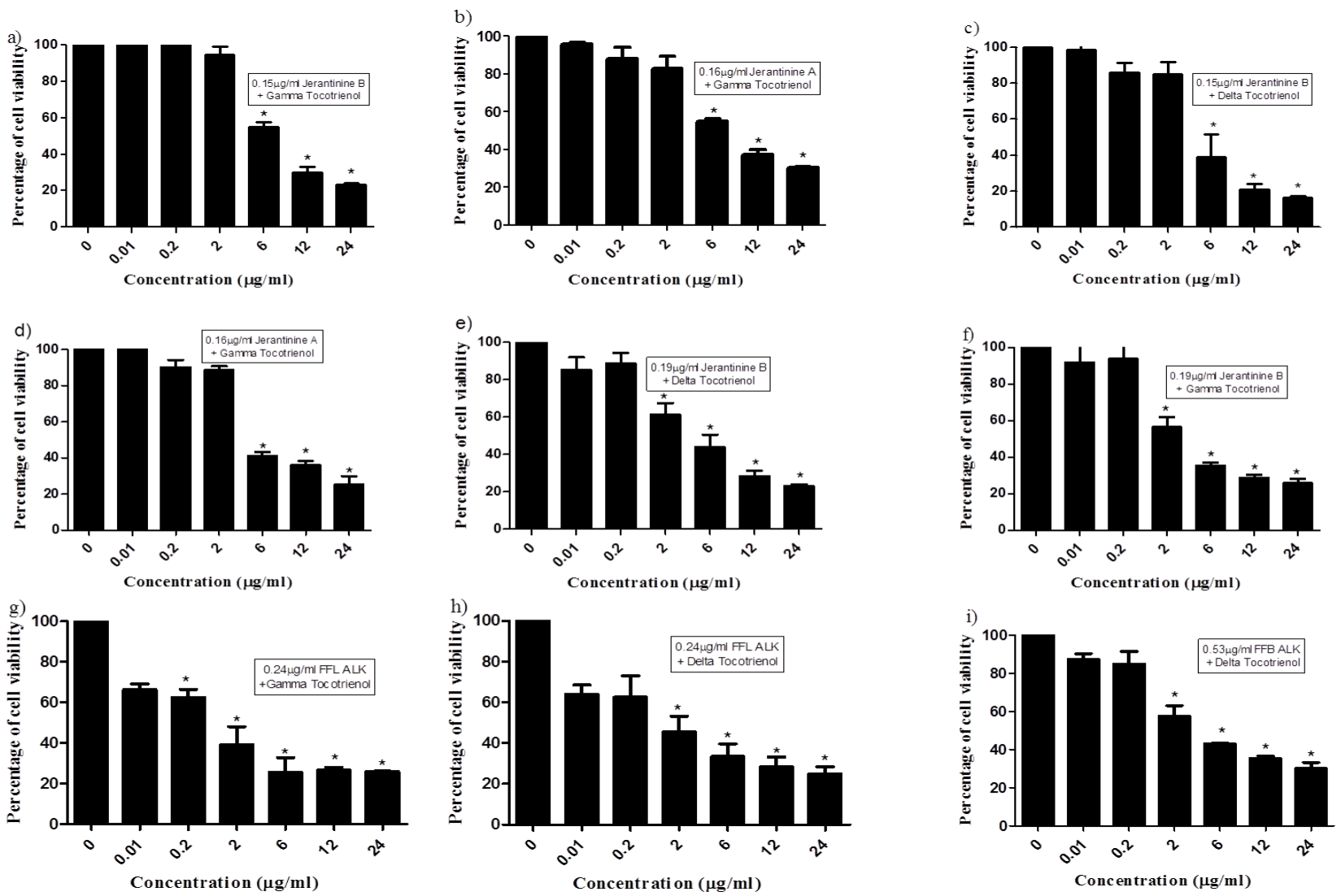
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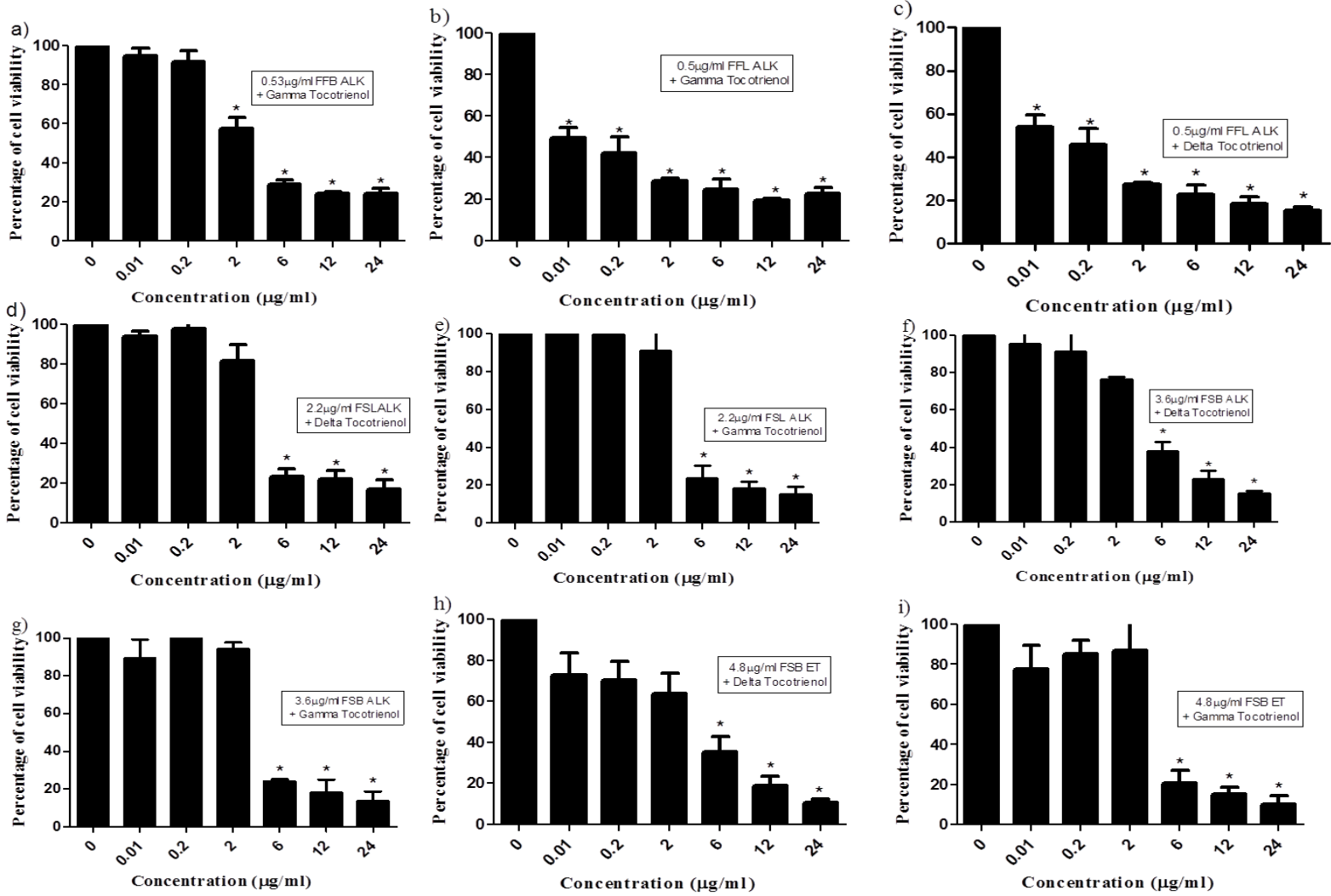
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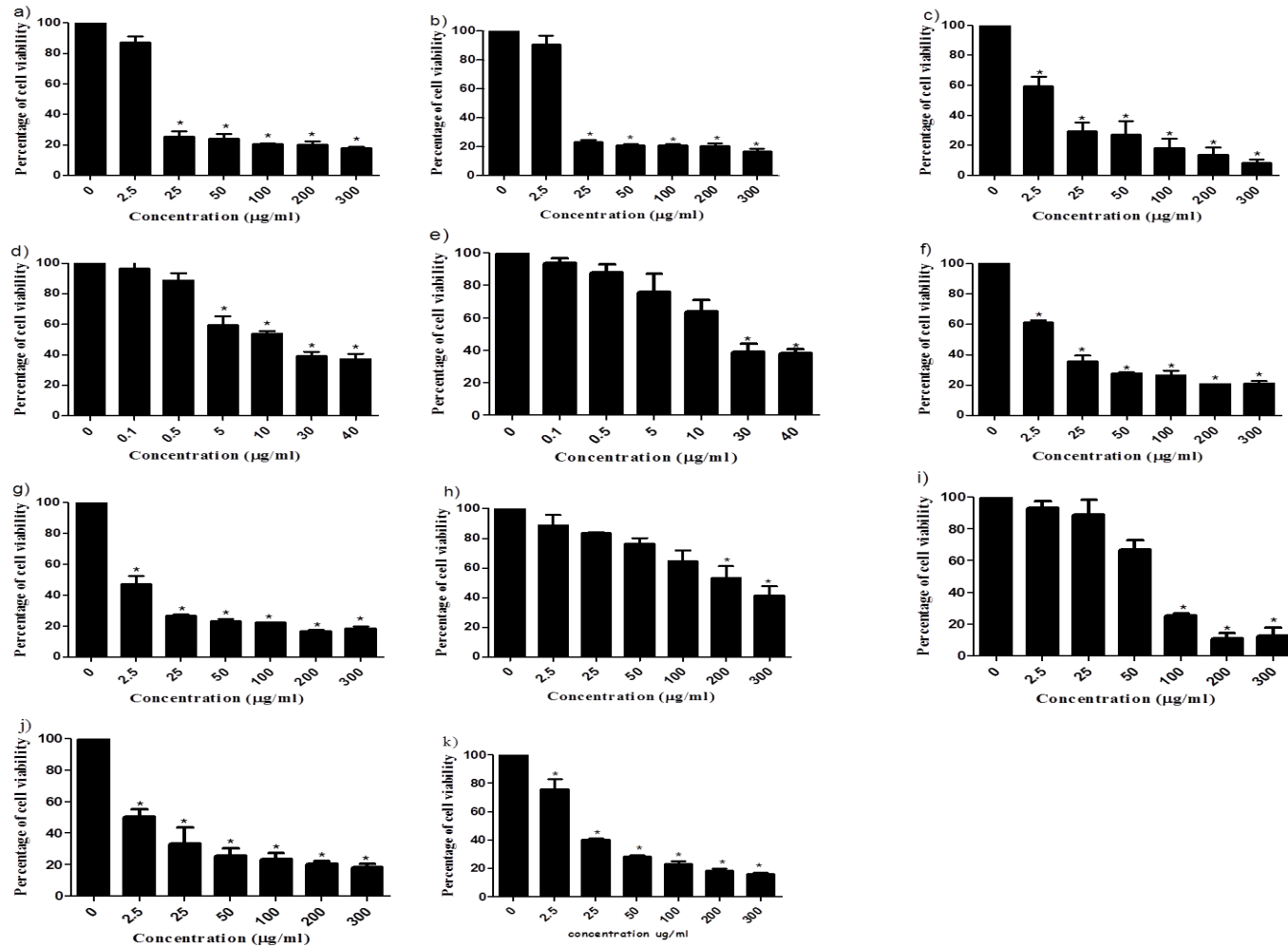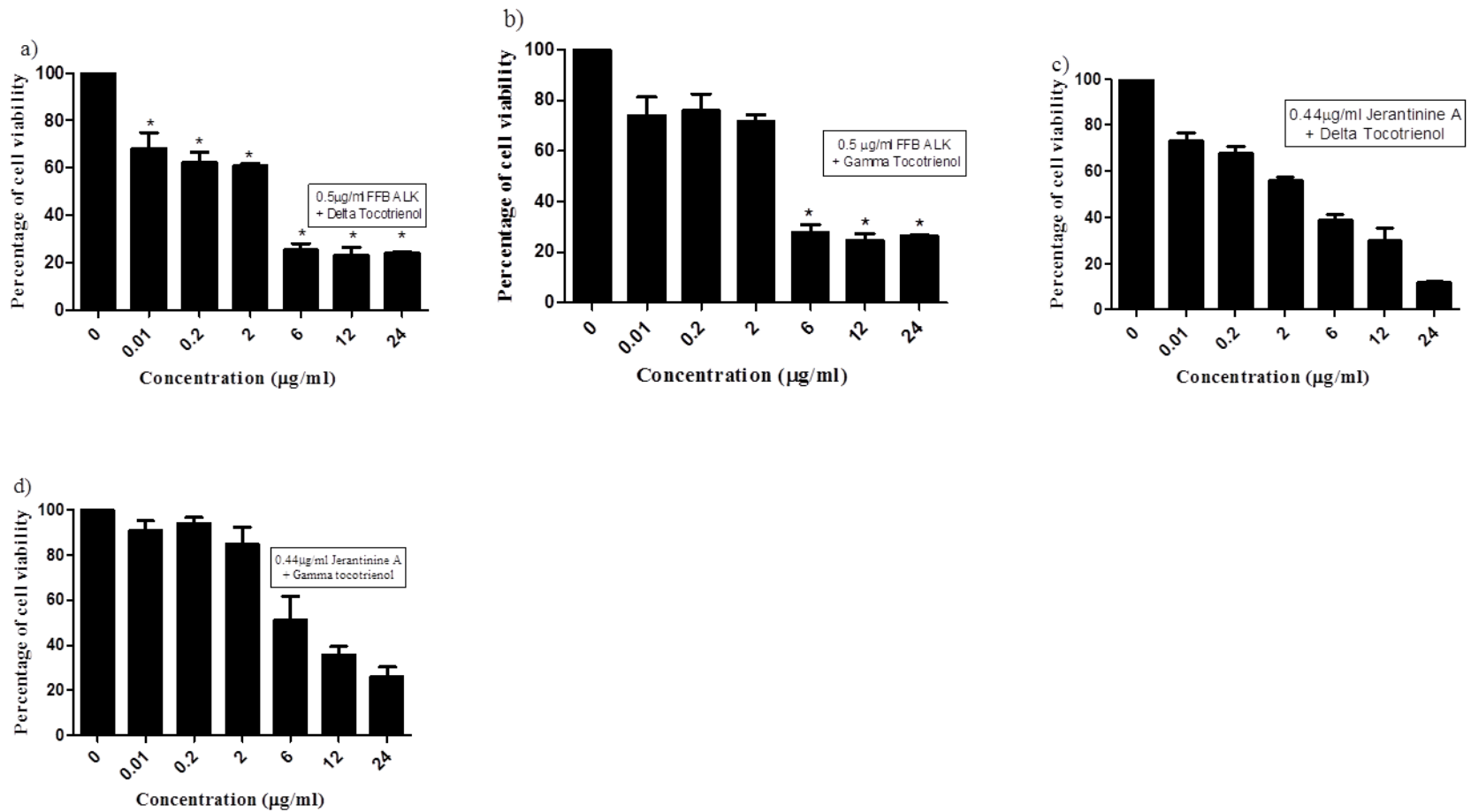
Appendix 1a: Histograms profiles of the growth inhibitory effect of single treatments on MRC5 normal lung fibroblast cell lines a) Delta tocotrienol, b) gamma tocotrienol, c) vinblastine, d) Jerantinine A, e) Jerantinine B, f) *Ficus fistulosa* bark, g) *Ficus fistulosa* leaf, h) *Ficus Schwarzii* bark, i) *Ficus schwarzii* leaf, j) *Ficus hispida* leaf, k) *Ficus hispida* bark after 72-hr treatment. Results are mean ±SEM. N=9 *P<0.001

a) 0.15μg/ml Jerantinine B + Gamma Tocotrienol

b) 0.16μg/ml Jerantinine A + Gamma Tocotrienol

c) 0.15μg/ml Jerantinine B + Delta Tocotrienol

d) 0.16μg/ml Jerantinine A + Gamma Tocotrienol

e) 0.19μg/ml Jerantinine B + Delta Tocotrienol

f) 0.19μg/ml Jerantinine B + Gamma Tocotrienol

g) 0.24μg/ml FFL ALK + Gamma Tocotrienol

h) 0.24μg/ml FFL ALK + Delta Tocotrienol

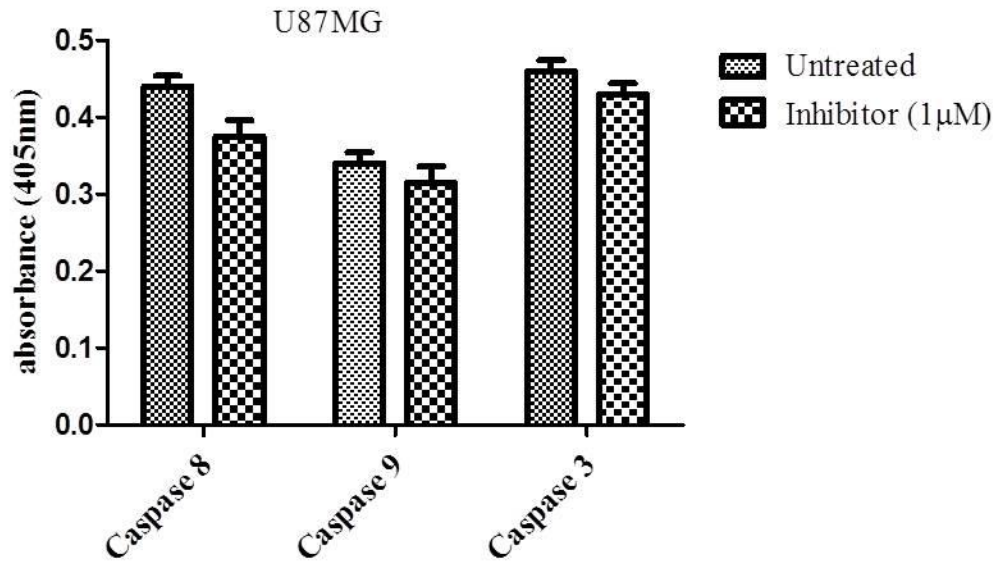i) 0.53μg/ml FFB ALK + Delta Tocotrienol

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Appendix 1b: Histograms profiles of the growth inhibitory effect for combined low-dose treatments on MRC5 cells
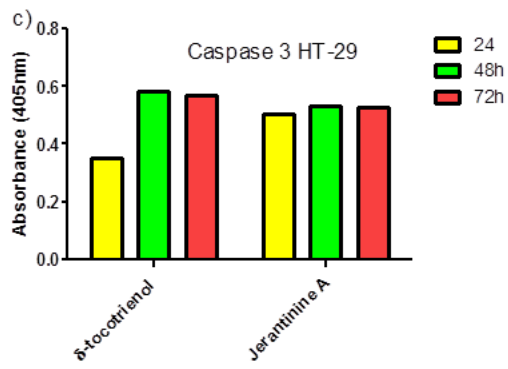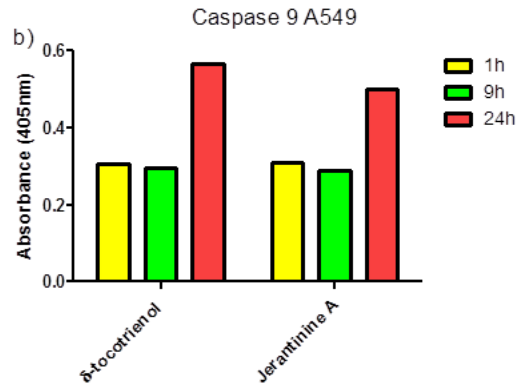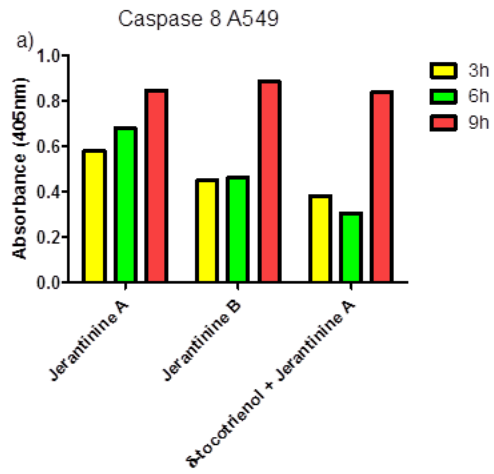
Appendix 2: Kinetic study for caspase enzyme activity
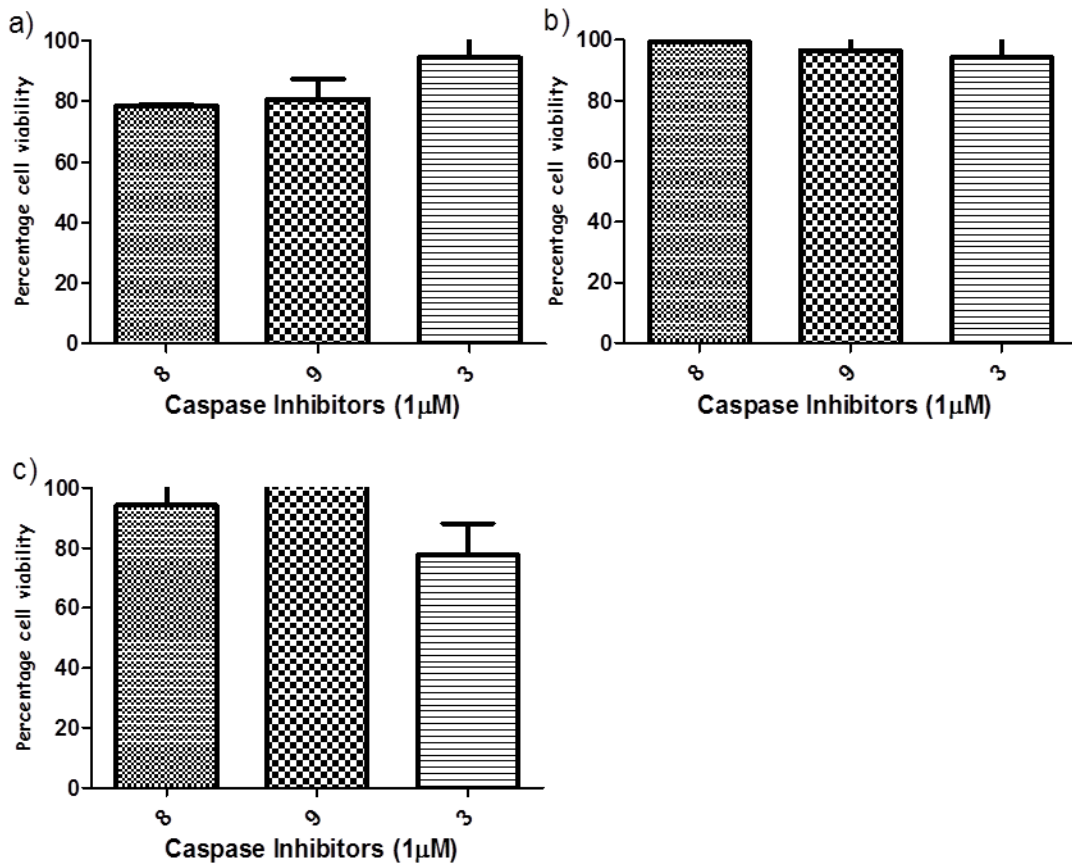
Appendix 3a: Screening of toxicity for caspase specific inhibitors following treatments for 72h with 1µM against a) A549 b) U87MG c) HT-29 cancer cells
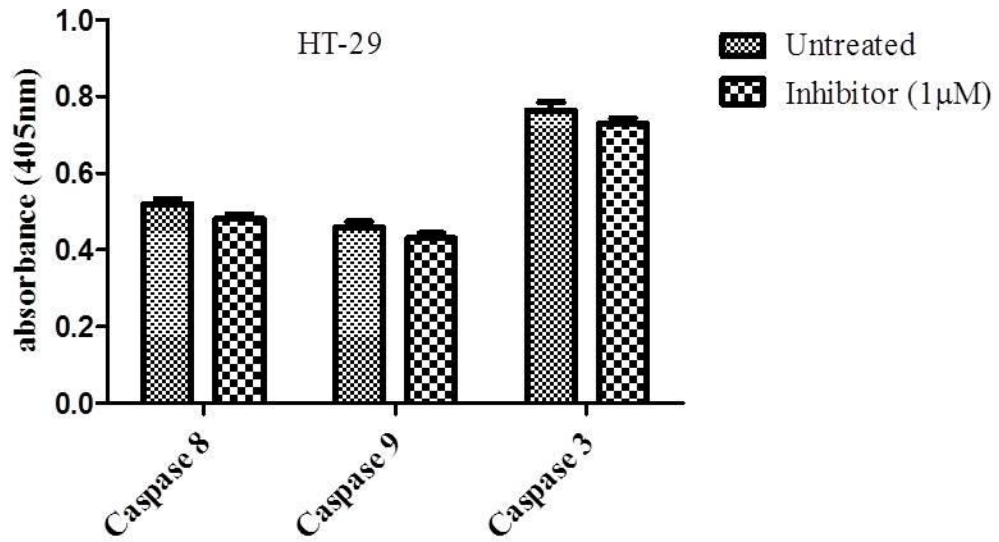
Appendix 3b: Screening on the effect of caspase specific inhibitors (1µM) on basal caspase level

a) Protein calibration curve

b) Interpolation of data from calibration curve
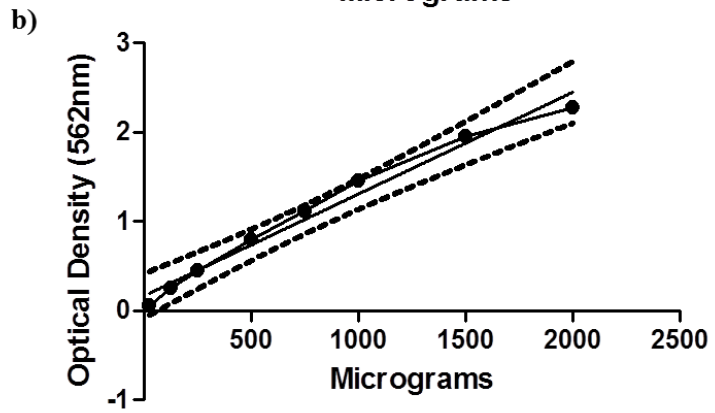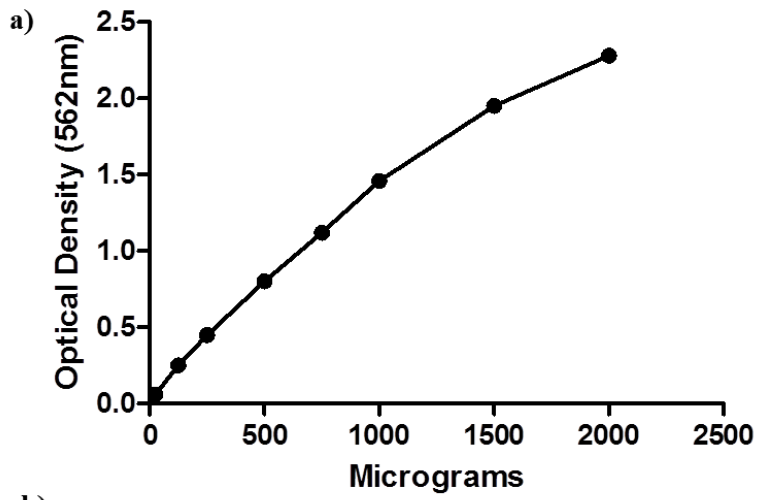
Appendix 4: a) Protein calibration curve b) Interpolation of data from calibration curve
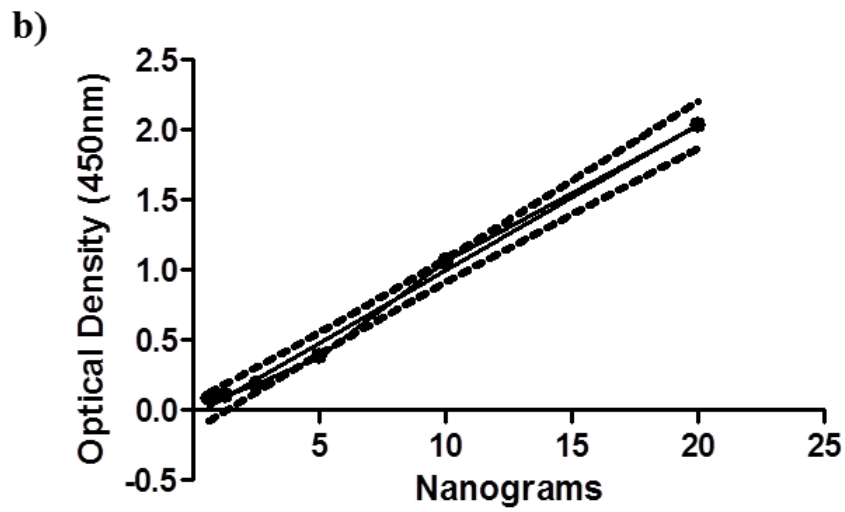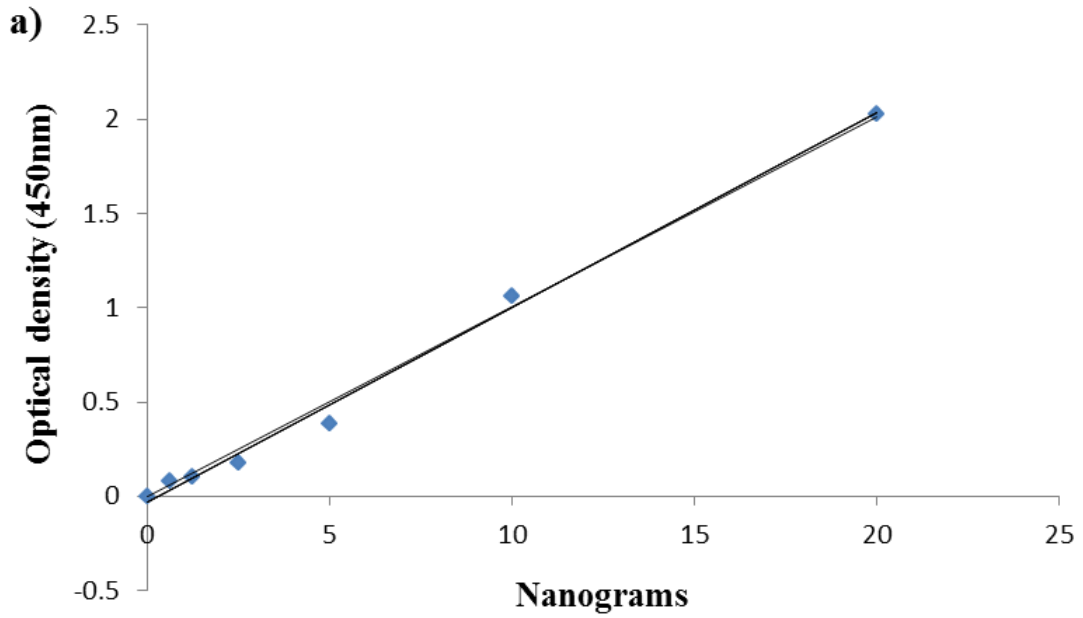
Appendix 5: a) Cytochrome c standards calibration curve b) Interpolation of sample concentration

<div align="center">Appendix 6</div>

a) Recipe for Gill's (1) hematoxylin solution

s/no	Reagents	Volume (ml)/weight (g)
1	Ethylene glycol	250ml
2	Distilled water	750ml
3	Hematoxylin	2g
4	Sodium iodate	0.2g
5	Ammonium aluminium sulfate	20g
6	Glacial acetic acid	20ml

Ethyelene glycol was dissolved in distilled water and subsequently, hematoxylin, sodium iodate, ammonium aluminium sulfate and glacial acetic acid were added respectively with continuous stirring for one hour at room temperature. Thereafter, the solution was filtered and stored at room temperature away from light.

b) Eosin Stock preparation

1% eosin Y stock solution was prepared by dissolving 10g of eosin Y in 800ml of 95% ethanol and 200ml of distilled water. The solution was mixed vigorously to dissolve and stored at room temperature. Subsequently a 0.25% working solution of (1L) was prepared by diluting 250ml of stock in 750ml of 80% ethanol. Finally 5ml of concentrated glacial acetic acid was added to improve the reddish colour of the solution.

c) Towbin Transfer buffer

10x towbin buffer was prepared without SDS based on the following composition

s/no	Reagents	Volume (ml)/weight (g)
1	TrisBase	3.03g (25mM)
2	Glycine	14.4g (192mM)
3	dH_2O	700ml

After mixture, the solution was made up to 1L with dH_2O. 1x was subsequently with the addition of 20% methanol.

d) Tris-buffered saline with Tween-20 (1L)

s/no	Reagents	Concentration
1	Tris	20mM
2	NACL	150mM
3	Tween 20	0.1%

e) Running buffer (Tris/Glycine/SDS) (1L)

s/no	Reagents	Concentration
1	TrisBase	25mM
2	Glycine	192mM
3	SDS	0.1%