Acalypha Wilkesiana Ethyl Acetate Extract Enhances the in *Vitro* Cytotoxic Effects of α-Tocopherol in Human Brain and Lung Cancer Cells

Su-Wen Lim, Hwei-San Loh, Kang-Nee Ting, Tracey D. Bradshaw, and Nazariah A. Zeenathul

***Abstract*—Multi-combinatorial approachesare considered nowadays to enhance the effectiveness of cancer treatment. In this study, α-tocopherol was tested in combination with the ethyl acetate extract from Acalypha wilkesiana for cytotoxicity activity against U87MG and A549 cell lines. The GI50 values for α-tocopherol against U87MG and A549 cells were 0.923±0.411μg/ml and 5.290±1.952μg/ml respectively in cell viability tests; when A. wilkesiana extract was added in adjunct with the treatment of α-tocopherol in minimum inhibitory concentration (MIC), the GI50 values of α-tocopherol improved significantly (p<0.05) to <0.43μg/ml (1μM) for both cell lines tested. Histological staining signified that both α-tocopherol and A. wilkesiana extract treated cancer cell lines exhibited apoptotic morphological characteristics. Single cell gel electrophoresis (SCGE) comet assays revealed that α-tocopherol caused only double strand DNA breaks; whereas A. wilkesiana extract caused both single strand and double strand DNA breaks in U87MG and A549 cells. It is proposed that α-tocopherol and A. wilkesiana extract might trigger apoptosis in both U87MG and A549 cells through different apoptotic pathways that might complement each other to enhance their antiproliferative efficacy against the cancer cells.**

***Index Terms*—α-Tocopherol, acalypha wilkesiana, apoptosis, DNA damage.**

I. Introduction

Vitamin E generally exists in the form of two subgroups namely tocopherol and tocotrienol; each consists of structurally similar compounds: α-, β-, γ- and δ-tocopherols, and α-, β-, γ- and δ-tocotrienols [1], [2]. Tocopherol can be found in abundance in vegetable oils extracted from nuts, corn, sesame, soybean cottonseeds and more [1], [3]. α-Tocopherol is usually present at higher levels in the blood after consumption as compared to the other tocopherols and tocotrienols [1], [4]. In the tocotrienol-rich fraction (TRF) extracted from palm oil, 25% is made up of α-tocopherol and the remaining 75% consists of tocotrienols [5], [6].

As a corollary of that, initially, most of the studies and research done on vitamin E have focused on α-tocopherol; other vitamin E isomers have not received the same amount of attention as compared to α-tocopherol. However, it was found in many studies that, the potent antiproliferative effects of the TRF are actually mediated by the tocotrienols and not α-tocopherol, and further corroborated by the investigation of *in vitro* cytotoxicity of individual isomers [1], [6]. It is still not completely understood why *in vitro* studies indicate that tocotrienols evokemore potent antiproliferative effects than tocopherols, some suggested that it was because greater cellular accumulation of tocotrienols is observed[6]. However, tocotrienols were found degradingin a larger extent as compared to tocopherols *in vitro*owing to theinstability of their unsaturated side chain, as compared to their counterparts with saturated side chain[4]. In fact, α-tocopherol is always found to be present at higher portions in blood plasma due to the preference of specific transfer protein in the liver towards α-tocopherol to selectively transfer α-tocopherol to very low-density lipoproteins (VLDL) [7], [8]; therefore, α-tocopherol is always preferentially secreted into the circulation and is then further transported to the non-hepatic tissues *in vivo* [1], [9]. Compared to α-tocopherol, other tocopherols and tocotrienols are less efficiently transferred to VLDL, therefore smaller portions are found in the blood or tissues as they are mostly excreted in the faeces [1].

Although previous studies have shown that α-tocopherol is not as effective as tocotrienols in its antiproliferative effect *in vitro*; the selectivity and preference of α-tocopherol to be secreted to the circulation and be transported more effectively *in vivo* as compared to the other vitamin E isomers suggest that α-tocopherol might be a more suitable candidate as a potential anticancer agent *in vivo*. However, in order to compensate for the weaker antiproliferative effect of α-tocopherol compared to the tocotrienols, a combinatorial treatment of α-tocopherol with other potent antiproliferative agents might present a solution.

The plant *Acalypha wilkesiana* has been reported to possess anticancer [10], antibacterial and antifungal [11], [12], anti-diabetic and anti-hypertensive properties [13], [14]. It has also been reported recently that the crude leaf extracts and fractions of this plant exhibit antiparasiticand analgesic properties [15]. With such properties, establishing a combinatorial treatment with both *A. wilkesiana* extract and α-tocopherol, the other medicinal properties of the plant and α-tocopherol (e.g. antioxidant) may offer a value-added advantage of such adjunct treatment. In addition, we wish to examine whether enhancement in antiproliferative effectiveness (GI_{50}) and efficacy (GI_{max}) could be observed in

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combination studies.

II. Materials and Methods

A. Sample Materials and Cell Lines

The plant species (*Acalypha wilkesiana*) under investigation was processed accordingly as described in Lim *et al.* [10]. The resultant ethyl acetate (EA) extract of *A. wilkesiana,* namely UNMC9EA was employed in this study. α-Tocopherol isomer was a kind gift from Davos Life Science Ptd Ltd, Singapore.

Cell lines used in the present study were A549 (human lung carcinoma), U87MG (grade IV human brain glioblastoma), and MRC5 (normal human lung fibroblast), all obtained from the American Tissue Culture Collection (ATCC). The conditions for cell propagation and maintenance have been described previously [10].

B. Preparation of α-Tocopherol and Plant Extract for Cell Treatment

UNMC9EA (100mg) were dissolved in DMSO (<2%v/v) to obtain a concentration of 100mg/ml stock solution. The stock solution was then further diluted to the required concentrations innutrientmedium without serum. Cells were exposed to plant extract at different concentrations ranging from 0.1 μg/ml - 1000 μg/ml for 72 hours.

α-Tocopherol isomer was prepared to a final 43.07mg/ml (100mM) stock solution in DMSO. The stock solution was further diluted to the required concentrations in nutrientmedium without serum. Cells were exposed to α-tocopherol at different concentrations ranging from 0.43 μg/ml - 43.07 μg/ml (1 μM-100 μM) for 72 hours.

Medium and vehicle-control treated cells containing equivalent amounts of DMSO served as vehicle and negative controls. Vinblastine at concentrations ranging from 0.01 μg/ml - 240 μg/ml was used as positive control.

As for combinatorial treatment, the minimum inhibitory concentration (MIC), the concentrations needed to reduce the growth of treated cells by 50% (GI_{50}), 80% (GI_{80}) or 100% (GI_{max}) of plant extracts were added to different concentration ranges of α-tocopherol (0.43 μg/ml - 43.07 μg/ml) for 72 hours.

C. Cell Viability Neutral Red Uptake Assay and Statistical Analysis

A total of 5×10^3 cells were seeded per well in a 96-well plate (Orange Scientific, Belgium) and incubated for 24 hours. Cells were then treated with UNMC9EA, α-tocopherol and the combination of both for 72 hours. Cell viability neutral red uptake assay was carried out according to a published procedure [16] and performed in triplicate in 3 separate experiments.

The results were presented as mean ± standard deviation (SD). Probit or logistic analysis was used to plot the concentration-response curve and the GI_{50} values were then calculated by using Graphpad Prism (version 5) bio-statistical software. The MIC, GI_{80} and GI_{max} values were also derived from the concentration-response curve plotted. Unpaired *t* test and ANOVA (using completely randomized design, CRD) were used to compare between two groups or to compare between groups. The level of statistical significance was set at $p<0.05$.

D. Hematoxylin and Eosin (H and E) Staining

A total of 5×10^3 cells were grown in chamber slides (Lab-Tek, USA). The cells were subjected for subsequenttreatment with UNMC9EA, α-tocopherol, vinblastine, at their individual MIC, GI_{50} and GI_{80} values; and combinatorial treatment of both UNMC9EA and α-tocopherol (MIC of UNMC9EA and GI_{50} of α-tocopherol). After receiving these particular treatments, the cells were fixed and stained following procedures described previously [10]. Slides were observed under a normal light microscope (Nikon, Japan) at different magnifications, photographs of cells were then captured and analyzed.

E. Fluorescein Diacetate (FDA) and Propidium Iodide (PI) Fluorescence Staining

A total of 5×10^3 cells were grown in chamber slides (Lab-Tek, USA). The cells were then treated with UNMC9EA, α-tocopherol, vinblastine, at their individual MIC, GI_{50} and GI_{80} values; and combinatorial treatment of both UNMC9EA and α-tocopherol (MIC of UNMC9EA and GI_{50} of α-tocopherol). For determination of cell morphology, a mixture of dye combinations was used, namely FDA+PI in the following concentrations: 2.5 μg/ml FDA (Sigma, USA) and 1.5 μg/ml PI (Invitrogen, USA). Treated and untreated control cells were washed three times in PBS, stained in 200 μl of PBS containing dyes and examined immediately using an epifluorescence microscope (Nikon, Japan). A minimum of 400 cells were analyzed in each sample.

F. Single Cell Gel Electrophoresis (SCGE) Comet Assay and Scoring

Cells (5×10^3)were treated with UNMC9EA (0.1 μg/ml, 10 μg/ml and 100 μg/ml), α-tocopherol at GI_{50} values (5 μg/ml for A549 and 0.8 μg/ml for U87MG) or vinblastine (0.01 μg/ml and 10 μg/ml) for 72 hours. SCGE comet assay was carried out in accordance with previously reported protocols [10].

Visual image analyses of DNA damage were adapted from Heaton *et al.* [17]. A total of at least 50 non-overlapping comet images per gel were measured for their tail length and were visually assigned a score on an arbitrary scale of 0 (round and intact without apparent tail; 0-9.9 μm), 1 (minority of the DNA migrated but majority is still intact in the head; 10.0-39.9 μm), 2 (more DNA migrated, tail shows high fluorescence signal; 40.0-69.9 μm), 3 (majority of the DNA migrated, but head is still highly fluorescent; 70.0-99.9 μm), 4 (high amount of DNA migrated, tail shows smearing of huge amount of DNA but head still shows fluorescence signal; 100.0-299.9 μm), and 5 (almost all DNA migrated towards the tail without apparent head; >300.0 μm). A mean DNA damage score for each slide was then tabulated.

III. Results

A. Assessment of Cell Viability

The concentration response curves for the treatments of individual α-tocopherol; UNMC9EA and the combinatorial

effects of both are shown in Fig. 1 and Fig. 2, respectively. Meanwhile, Table I summarizes the GI_{50} values of these treatments. After receiving the treatment with individual α-tocopherol and UNMC9EA for 72 hours, both U87MG and A549 cell numbers decreased drastically in a concentration dependent manner, contrasted to that of the vehicle-control group. Generally, the UNMC9EA is potent for growth inhibition especially towards the U87MG cells; GI_{50} values were given as 1.742±0.299µg/ml for the U87MG cells and 89.63±2.12µg/ml for A549 cells. Interestingly, stimulation of growth in non-cancerous MRC5 cells when treated with UNMC9EA was observed (Fig. 1B). The antiproliferative rank of potency of the tested samples against both U87MG and the A549 cells is vinblastine>α-tocopherol>UNMC9EA. Vinblastine seemed to possess the greatest antiproliferative effect against A549 cells (GI_{50}=0.01±0.711µg/ml) followed by U87MG (GI_{50}=0.05±0.006µg/ml) and non-cancerous MRC5 (GI_{50}=9.507±3.238µg/ml). As opposed to vinblastine, both α-tocopherol and UNMC9EA are more effective towards U87MG rather than A549 (Table I). When both α-tocopherol and UNMC9EA were used concurrently to treat the cells, a significant (p<0.05) decrease on the GI_{50} and GI_{max} values was observed (Fig. 2). Vinblastine seemed to possess significant (p<0.05) growth inhibitory effect towards all cell lines including the non-cancerous MRC5 cells. However, all treatments involving individual or combinatorial application of α-tocopherol and UNMC9EA showed non-significant (p>0.05) inhibitory potential towards the non-cancerous MRC5 cells (GI_{50}>300µg/ml).

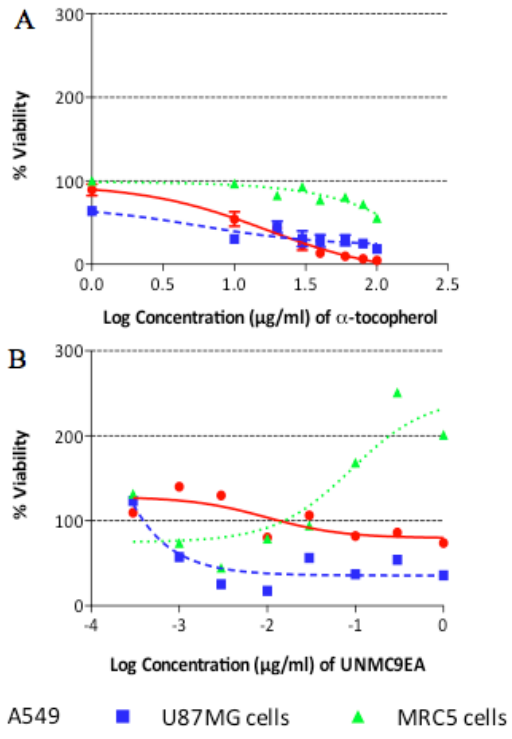

Fig. 1. The cell viability curves of (A) α-tocopherol and (B) UNMC9EA on A549, U87MG, and MRC5 cell lines. Cell viability is presented as percentage where vehicle-treated cells were regarded as 100% viable. Data presented as mean ± SD, n=3.

A. Morphological Observation: Histological and Fluorescence Staining

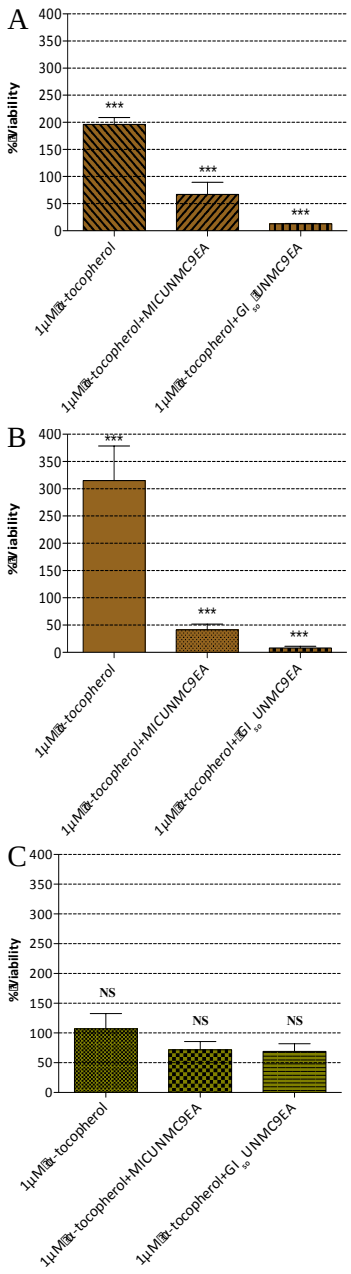

Fig. 2. The effects of combinatorial treatment of α-tocopherol and UNMC9EA on (A) A549, (B) U87MG and (C) MRC5 cell lines. Cell viability is presented as percentage where vehicle-treated cells were regarded as 100% viable. One-way ANOVA was used to compare the significant difference between groups, ***- p<0.0001, NS- non significant. Data presented as mean ± SD, n=3

H & E histological staining and FDA & PI fluorescence staining provided the evidence of the morphological characteristic of cellular apoptotic death in both α-tocopherol and UNMC9EA treated U87MG and A549. Both U87MG and A549 cells treated with α-tocopherol showed morphological characteristics of apoptosis such as cytoplasmic extension, chromatin condensation and the formation of apoptotic bodies at concentrations as low as 0.8µg/ml and 5µg/ml, respectively (Fig. 3). Cytoplasmic extension and multinucleated cells were also found in U87MG (Fig. 3 (B) and 3 (C)) and A549 cells treated with UNMC9EA at concentration as low as 10µg/ml. When the concentration of UNMC9EA was increased to 100µg/ml, apoptotic bodies were observed in both A549 and U87MG cancer cells. Cancer cells exhibited apoptotic morphology in

the presence of α-tocopherol and UNMC9EA at much lower concentrations (Fig. 3 D). Non-cancerous MRC5 cells treated with both α-tocopherol and UNMC9EA at all concentrations showed normal morphology and good viability; except when the GI_{max} of both samples were applied, vacuole formation was observed in the living cells. All cells treated with vinblastine including the non-cancerous MRC5 cells showed apoptotic characteristics at concentration as low as 5μg/ml. No cells survived when the concentration of vinblastine was increased to 50μg/ml.

TABLE I: GROWTH INHIBITORY EFFECTS OF α-TOCOPHEROL AND UNMC9EA

Samples	Cell lines, GI_{50} values (μg/ml)		
	A549	U87MG	MRC5
α-Tocopherol	5.29 ±1.952	0.92 ±0.411	> 43.07#
UNMC9EA	89.63 ±2.120	1.74 ±0.299	> 300.00
Vinblastine	0.01 ±0.711	0.05 ±0.006	9.51 ±3.238
α-Tocopherol + MIC UNMC9EA	< 0.43*	< 0.43*	20.20 ±0.237
α-Tocopherol + GI_{50} UNMC9EA	< 0.43*	< 0.43*	19.33 ±2.571

The concentration used for α-tocopherol treatment ranges from 1μM - 100μM (0.43μg/ml – 43.07μg/ml). * GI_{50} value is smaller than the minimum concentration range used for the test. # GI_{50} value is greater than the maximum concentration range used for the test. Values presented as mean ± SD, *n*=3

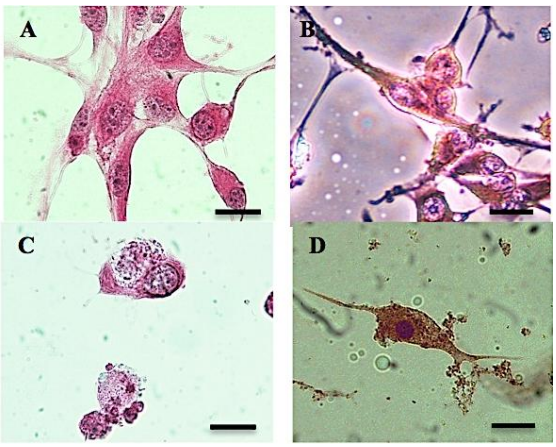

Fig. 3. Morphology of U87MG cells treated with (A) vehicle control, (B) 10μg/ml of UNMC9EA, (C) 0.8μg/ml of α-tocopherol, and (D) combinatorial treatment of both α-tocopherol and UNMC9EA at 0.4μg/ml and 1μg/ml, respectively.H & E stain. Bar: 10μm.

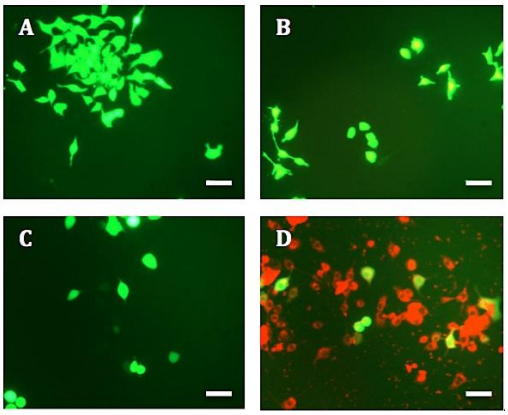

Fig. 4. Morphology of A549 cells treated with (A) vehicle-control, (B) 2μg/ml of UNMC9EA, (C) 5μg/ml of α-tocopherol and (D) combinatorial treatment of 0.8μg/ml α-tocopherol and 1μg/ml of UNMC9EA. FDA & PI stain. Bar: 50μm.

FDA and PI staining was used to analyse the cellular morphological changes. Generally, the number of cells with altered morphology largely depended on the concentration of the α-tocopherol and UNMC9EA applied. Vehicle-control treated cells showed morphology with frequency of dead cells of not more than 1% at the end of the experiment (Fig. 4 (A)). Overall, necrotic cells were not found in all of the treatments. Greater cell death was found in both U87MG and A549 cancer cells when both α-tocopherol and UNMC9EA were applied together (Fig. 4 (D)).

B. Determination of DNA Damage

Cancer cells exposed to growth inhibitory concentrations of α-tocopherol and UNMC9EA were tested for their DNA damage pattern using SCGE comet assay. The DNA damage scores for both neutral and alkaline comet assays are shown in Table II. Overall results obtained from SCGE comet assays suggested that all cells treated with UNMC9EA regardless of the concentrations used (0.1μg/ml, 10μg/ml and 100μg/ml), produced both single strand (SSBs) (alkaline comet) and double strand breaks (DSBs) (neutral comet) (Table II and Fig. 5). The mean DNA damage scores for SSBs in all UNMC9EA treated cells were higher as compared to the DSBs damage scores (Table II and Fig. 5). All cells treated with α-tocopherol produced only DSBs (neutral comet) and not SSBs (alkaline comet) (Table II and Fig. 5). Nonetheless, the mean DNA damage scores of DSBs obtained in all α-tocopherol treated cells were similar to the mean DSBs scores produced in UNMC9EA and vinblastine treated cells. Cells of all 3 cell lines treated with vinblastine showed only DSBs and not SSBs (Table II).

IV. DISCUSSION

Cancer Research UK (CRUK) reveals that the 5 year survival rate of brain cancer patients is only 20%, whereas non-small cell lung cancer (NSCLC) patients' 5 years survival is only 10% [18]. Continued efforts to discover novel molecules and/or treatment regimens are critical. Compounds or molecules that possess high selectivity towards cancer cells but with only minimum toxic effect on non-cancerous cells are desirable. The combination of α-tocopherol and UNMC9EA might be a potential treatment that would fulfil this requirement.

TABLE II: MEAN DNA DAMAGE SCORES OF NEUTRAL AND ALKALI COMET ASSAYS FOR U87MG, A549 AND MRC5 CELLS

		DNA damage scores (Grade 0-5)	
Samples	Cell Lines	Neutral Comet Assay (Double Strand Breaks)	Alkaline Comet Assay (Single Strand Breaks)
UNMC9EA (10μg/ml)	A549	3	4
	U87MG	2	4
	MRC5	1	3
α-Tocopherol (5μg/ml)	A549	2	0
	U87MG	3	0
	MRC5	1	0
Vinblastine (10μg/ml)	A549	3	0
	U87MG	2	0
	MRC5	2	0

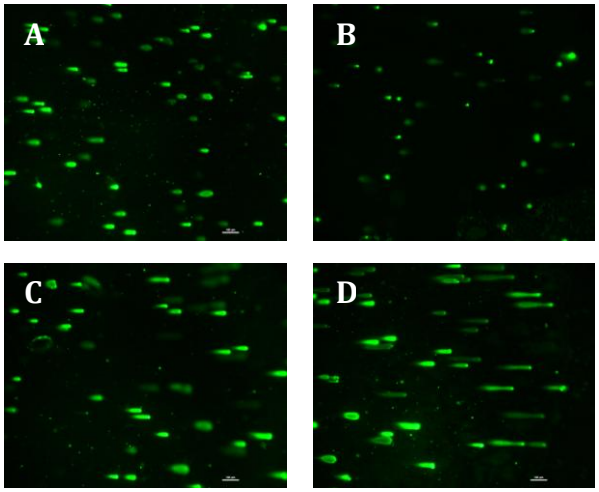

Fig. 5. Representative slides of comet profiles for A549 cells receiving different treatments. (A) Neutral comet profiles of A549 cells treated with 5μg/ml of α-tocopherol. (B) Alkaline comet profiles of A549 cells treated with 5μg/ml of α-tocopherol. (C) Neutral comet profiles of A549 cells treated with 10μg/ml of UNMC9EA. (D) Alkaline comet profiles of A549 cells treated with 10μg/ml of UNMC9EA.

Generally, results obtained suggest that α-tocopherol and the ethyl acetate extract from *A. wilkesiana* possess impressive antiproliferative activity against both cancer cell lines under study. Both α-tocopherol and UNMC9EA showed more potent antiproliferative effects against U87MG cells. α-Tocopherol is approximately 5-fold more potent in U87MG, whereas UNMC9EA is approximately 50-fold more potent in U87MG when compared to A549 cells. Both α-tocopherol and UNMC9EA showed non-significant (p>0.05) growth inhibitory effects on the non-cancerous MRC5 cells. In fact, UNMC9EA stimulated the cell growth of MRC5 in a dose-dependent manner (Fig. 1 (B)). It could be that UNMC9EA might possess mitogenic and/or cytoprotective properties towards the non-cancerous cells in addition to its antiproliferative effects on the cancer cells. In contrast, vinblastine exhibited potent antiproliferative properties towards A549 rather than U87MG cells. Vinblastine is about 5 times more potent in A549 than U87MG cells. The cytotoxic effect of vinblastine however was not selective; the non-cancerous MRC5 cells were equally affected.

Morphological results indicate that both U87MG and A549 cells treated with both α-tocopherol and UNMC9EA underwent apoptosis. Morphological hallmarks of apoptosis such as cytoplasmic extension, chromatin condensation, multinucleation, and the formation of vacuoles and apoptotic bodies were evident in both U87MG and A549 cells treated with α-tocopherol and UNMC9EA (Fig. 3). Apoptosis is a process that engages at least two stages of DNA cleavage, i.e. an early part with the production of DNA fractions around 200-300kbp and 30 - 50 kbp; and a later episode with the production of characteristic internucleosomal cleavage or DNA fragmentation [19]. The latter occurrence can be usually detected by a simple DNA laddering experiment [20]. However, DNA fragmentation detection assay limitations include the inability to differentiate between DNA fragments cleaved by apoptotic enzymes (which occur duringexecution of apoptosis) and the DNA fragmented as the outcome of treatment with DNA damaging agents or drugs [21]. In such cases, the SCGE comet assay would be a more appropriate and powerful tool for detection of DNA SSBs or DSBs and the determination of cell death mechanism (apoptotic or necrotic) [19].

In this study, SCGE comet profiles suggest that DNA damage observed following treatment of cells with UNMC9EA or α-tocopherol is a consequence of apoptosis and not necrosis. Fig. 5 and Table II show that the majority of DNA content migrated towards the tail of the comet after treatment. This would not be the case for necrosis where the majority of damage would remain in the head of the comet [22]. With supportive morphological findings, evidence is convincing that cellular DNA damage is caused by apoptosis. Our previous paper had shown that *A. wilkesiana* extracts might simultaneously activate several modes of action causing both DNA SSBs and DSBs, leading to apoptosis [10]. This hypothesis is supported by the different responses of U87MG and A549 cells to UNMC9EA treatment. More severe DNA DSBs were detected in A549 cells than in the U87MG cells and MRC5 cells (Table II). α-Tocopherol was found to cause only DNA DSBs in all the treated cancer cell lines. The treatment of α-tocopherol did not seem to trigger apoptosis through SSBs. Since vinblastine, which has been known to trigger apoptosis through inhibiting the formation of microtubules [22] clearly evoked DNA DSBs (Table II), it could be possible that α-tocopherol which induced similar DSBs acts also on microtubules or causes topoisomerase II inhibition in both U87MG and A549 cells. Many secondary metabolites isolated from natural sources such as plants are found to be topoisomerase II inhibitors including terpenoids, lignans, triterpenes and anthracenone [23].

However, a few studies have discovered that distinguished vitamin E isomers cause apoptosis by activating caspases-8 through the death receptor pathway following the recruitment of procaspase to the death-inducing signaling complex [24]. There are also studies, which have found that certain vitamin E isomers induce apoptosis through the mitochondrial pathway involving the activation of proapoptotic proteins (especially Bax) from the Bcl-2 family in turn triggeringdownstream effector stage caspases (caspases-9 and caspases-3) [25]. Since SSBs are not found in α-tocopherol treated cells, the probability of involvement of p53 and p21 pathways,usually activated by DNA SSBs is low. Nevertheless, more experiments are needed to elucidate the mechanism of action involved inα-tocopherol-inducedapoptosisin brain and lungcancers.

Irrefutably, the addition of UNMC9EA extract into the treatment of α-tocopherol against the cancer cell lines did enhance their antiproliferative power (Fig. 1-Fig. 4). When applying the adjunct UNMC9EA and α-tocopherol treatment, the entire concentration-response curve shifted to the left and hence lowered the $GI_{50}$and GI_{max}values, increasing antiproliferative effectiveness. In some cases, combinatorial application of drugs is worrisome as the synergistic effects might be too powerful; and in consequence might be harmful at the same time towards the non-cancerous cells. However, this phenomenon is not found:when the combination ofUNMC9EA and α-tocopherol was applied to

α-tocopherol was applied to non-cancerous MRC5 cells, non-significant (p>0.05) growth inhibitory effectswere shown (GI_{50}>300μg/ml) (Fig. 2) and the cells survived throughout the entire course of study.

The combinatorial application of α-tocopherol and UNMC9EA has shown very potent cytotoxic effects towards the cancer cell lines under study. This report offers new insights into the medicinal potentials of α-tocopherol and the ornamental plant, *A. wilkesiana*by revealing their *in vitro* anticancer properties. Therefore, *A. wilkesiana* ethyl acetate extract and α-tocopherol might be potential drugs for anticancer treatment for lung and brain cancers.

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