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Protective Effects of *Cyphomandra betacea* Extract Against Hydrogen Peroxide-induced Oxidative Stress in 3T3 Cells and Synergistic Anticancer Properties in HepG$_2$ and MDA-MB-231 Cell Lines

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Abstract. Using the MTT 3-(4, 5-dimethylthiazol-2yl-2, 5-diphenyltetrazolium bromide) assay induced by hydrogen peroxide (H_2O_2) and the ability to increase the cytotoxicity of doxorubicin in HepG$_2$ and MDA-MB-231 cells, the protective effects of ethanolic extract of tamarillo were assessed in the current study. The cells were pre-treated with tamarillo ethanolic extract at 10 g/mL for 4 h before being exposed to 0.6 mM H_2O_2 for 3 h showed the greatest protection, with a significant increase (P 0.05) in cell viability of 79% compared to the control (57%). The results support a role that cyto-protective effect of tamarillo and doxorubicin which low in toxicity opens a new possible approach for possible future use in conventional chemotherapeutic drugs and radiation therapy.

Keywords: chemotherapeutic, radiation therapy, hydrogen peroxide, *Cyphomandra betacea*, synergistic

Introduction

Recently, anticancer therapies have gained increasing attention as a potentially significant tool in controlling the disease because it is now possible to examine the mechanisms at the molecular level. Commonly, traditional chemotherapy is still the most predominant way of treating cancer despite the short and long term adverse side effects such as pain, nausea, fatigue, vomiting and anaemia and nerve problems by Hassan (2012). However, currently the concept of chemoprevention is receiving great interest as an alternative to conventional cancer treatment.

Chemoprevention is mainly the use of naturally occurring or synthesised chemicals in pharmacology to postpone, suppress or reverse the development of carcinogenesis, Domenico *et al*. (2012). It is also sometimes referred to as inhibition of tumor angiogenesis and metastasis, as well as direct killing of cancer cells Lewandowska *et al*. (2014). Several studies have been done recently to examine the possible anticancer effects of natural antioxidants, especially dietary polyphenols. For its prospective therapeutic uses as chemo-preventive agents in the treatment of carcinogenesis, the use of natural extract may represent an alluring and affordable strategy.

It is well recognised that plant-derived polyphenols have a variety of pharmacological properties as anti-proliferative, pro-apoptotic and anti-angiogenic activities that could be used in both prevention and therapy of various cancers, Sporn *et al*. (1976). The cyto-protective effect (the cancer blocking effect) of dietary polyphenolic substances on normal cells and their cytotoxic effect on cancer cells may work together to explain their anti-proliferative activity (cancer suppressing action). Besides, polyphenols may work together with one another or with natural or synthetic drugs by additively, synergistically or antagonistically, Colon and Nerin (2016). The combination of the said interactions is looked upon as it may give room for more effective yet less toxic therapeutics, a cost effective alternative as well as minimization of the side effects.

Tamarillo (*Cyphomandra betacea*) is a small plant belonging to the family Solanaceae, originating from

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south America, Lim (2013). There are three types while the most commonly known type is the red fruit, Richardson and Patterson (1993). When ripe, it has a skin that is reddish to orange with thin, green to brown stripes. It has an oval shape and orange-coloured skin. The golden fruit is the second kind. It has bright yellow skin and flesh with barely perceptible longitudinal brown to green stripes that are oval shaped. The other kind is purple, commonly referred to as dark-red or black. It has vividly dark red skin with extremely faint vertical stripes of green. Its flesh is purple and has an oval to circular shape, Morton (1987). The kind that is sold in Malaysia is egg shaped, measuring 9–12 cm in diameter, with thin reddish-brown peel, dark red seed mucilage and orange pulp, Gannasin *et al*. (2015). Tamarillos are an excellent source of carotenoids like carotenes and xanthins, which work as co-factors for enzymes in the metabolism of body and other synthetic processes, Hassan and Abu Bakar (2013), vitamins such as ascorbic acid and tocopherol, which make them a significant immune boosting antioxidant power. The phenolics, flavonoids and anthocyanin present in tamariol fruits in large amounts support its antioxidant function, Athar *et al*. (2003). Tamarillo contained 17 carotenoids and 3 important anthocyanins, Wrolstad and Heatherbell (1974). By giving hydrogen to highly reactive species, especially the peroxyl radicals and anthocyanins function as antioxidants and stop the creation of new radicals, Ignat *et al*. (2010). In order to demonstrate the combined impact of tamarillo with commercial chemotherapy treatment and to give higher anticancer characteristics, and this study intends to demonstrate the protective benefits of tamarillo on hydrogen peroxide-induced cell death.

Materials and Methods

Fruit sampling. Cameron Highland, Pahang, Malaysian workers gathered tamarillo fruit in June 2013. The identified herbarium specimens were placed in Borneensis at the University of Malaysia, Sabah in Malaysia. To get rid of any impurities the fruits were cleaned thoroughly before being weighed and sliced into standard sizes. After being freeze-dried, 500g sample was grinded into a powder. The sieved powdered samples were then stored in a freezer (20 °C) pending for further analysis.

Preparation of sample. Tamarillo dry powder was extracted three times with 80% ethanol at room temperature over the course of 24 h. The residue (dried 50 mg) was dissolved in the DMSO (1 mL) after being dried by evapouration at 50 °C to produce a working solution of 1 mg/mL. A 0.45 m nylon membrane syringe filter was used to filter these final extracts before usage.

Cell culture. The cells were raised in RPMI-1640 culture media with 10% FBS and 1% penicillin/streptomycin in 75 cm^2 flasks. Cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. With 0.25% (w/v) trypsin-EDTA, confluent monolayer cells that were 80% confluent were harvested.

Protective effects against H_2O_2 induced oxidative stress. Before assessing the protective impact of tamarillo, dose-response tests were conducted to determine the highest nontoxic concentration of tamarillo that may be used. 96-well plates with 1 105 3T3 cells/mL of seed were incubated for 24 h. Subsequently, various quantities of tamarillo ethanolic extract (0–200µ g/mL) were applied to the plates. The MTT test, which was previously published, was used to count the number of live cells after 24 h.

MTT assay protocol. Discard media from cell cultures; Add 50 µL of serum-free media and 50 µL of MTT solution into each well; Incubate the plate at 37 °C for 3 h; After incubation, add 150 µL of MTT solvent into each well; Wrap plate in foil and shake on an orbital shaker for 15 min; Read absorbance at OD=590 nm.

In 3T3 cells, Park *et al*. (2014). examined the defence-enhancing potential of tamarillo extracts against hydrogen peroxide-induced cell death. In order to identify the major reduction in cell viability, the 3T3 cells were first tested for their cell viability activity with concentrations of H_2O_2 ranging from 0.1 to 1 mM for 3 h, then treated with different exposure times (0 to 5 h). In 96-well plates, 3T3 cells (1 105 cells/mL) were planted. The cells were given different amounts of tamarillo extract (0–200 µg/mL) for 4 h after 24 h of incubation and then each well received the addition of 0.8 mM of H_2O_2. Number of viable cells was counted with the help of MTT assay after 3 h of incubation.

Drug combination studies. ***Measurement of inhibition of $HepG_2$ and MDA-MB-231 cell proliferation in isolation.*** Tamarillo and doxorubicin's effects were assessed using the MTT assay, as previously mentioned. In the 96-well plates, cells (1 105 cells/mL) were planted and given the night to adhere. Several quantities of tamarillo extract (0–200 µg/mL) and doxorubicins (0.1–0.8 µg/mL) were applied to cells either separately

or in combination. The CI_{50} values of the isolated treatments were determined firstly. Based on each CI_{50} value achieved in isolation treatment, a series of concentrations were designed for the combination effect that comprise of a range of Doxorubicin concentration and combined with the CI_{50} values of MDA and $HepG_2$ cells: $0.2 \times CI_{50}$, $0.4 \times CI_{50}$, $0.6 \times CI_{50}$, $0.8 \times CI_{50}$ and $1.6 \times CI_{50}$ (µg/mL).

Combination index for determining addition, synergism and antagonism. For the assessment of synergism, the combination index (CI) was performed, Chou and Talalay (1984) following combination Index equation:

$$\text{Combination index (CI)} = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} + \frac{(D)_1\,(D)_2}{(Dx)_1(Dx)_2} \ldots.$$

where:

$(D)_1$ and $(D)_2$ represents the concentrations of tamarillo and doxorubicin, when administered to the cells concurrently, have the determined effect and $(Dx)_1$ and $(Dx)_2$ are the concentrations of same medications, when administered separately, have the same determined impact. The CI values show an additive effect when = 1, a synergistic effect when 1, and an antagonistic effect when >1.

Statistical analysis. Three copies of each experiment were used, and the findings were presented as mean standard deviation, using SPSS for Windows version 22.0. (S.D.). One-way analysis of variance was employed with Tukey's test to statistically analyse the data. Statistical significance was deemed to exist when $P < 0.05$ was reached.

Results and Discussion

Cyto-protective effect of tamarillo against hydrogen peroxide-induced oxidative stress in 3T3 cells. The US NIEHS, interagency coordinating committee in the validation of alternative methods for determining basal cytotoxicity, National Institute of Environmental Health Sciences, NIEHS (2001) recommends the lowest cytotoxic dose of tamarillo ethanolic extract on non-tumorigenic 3T3 mouse fibroblast, which is identified in the first section of the study. Findings showed that treatment with tamarillo at doses ranging from 0 to 200µg/mL didn't significantly change the viability of cells. Cell viability was about 79% at 200 µg/mL concentration level (Fig. 1). At the greatest dosage of tamarillo ethanolic extract, no harmful effect on 3T3 cells was seen, indicating that tamarillo is a selective anticancer agent. After that, it was determined how well tamarillo ethanolic extracts protected 3T3 cells from oxidative stress brought on by hydrogen peroxide. According to Fig. 2 and 3, after being exposed to 0.6 mM H_2O_2 for three hours, the viability of the cells dropped in a manner; with a significant ($P < 0.05$) decrease in viability relative to the control cells of about 62%. Contrarily, 3T3 cells pre-treated with tamarillo extract for 4 h before being exposed to 0.6 mM H_2O_2 for 3 h had higher vitality than cells that had not received this pre-treatment (Fig. 4). The most protective effect was observed at 10 µg/mL of tamarillo ethanolic extract with a significant increment ($P < 0.05$) in cell viability

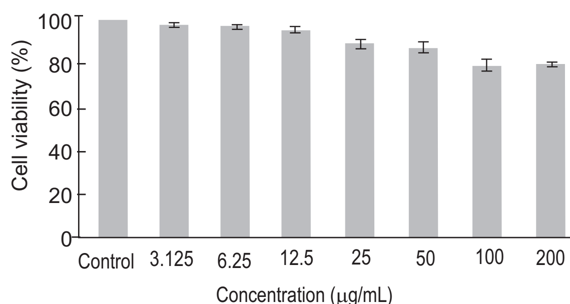

Fig. 1. Cell viability of 3T3 cells exposed to tamarillo ethanolic extract at different doses (0–200 µg/mL). The MTT assay was used to measure cell viability. The results are shown as mean (n = 3) standard deviation of mean.

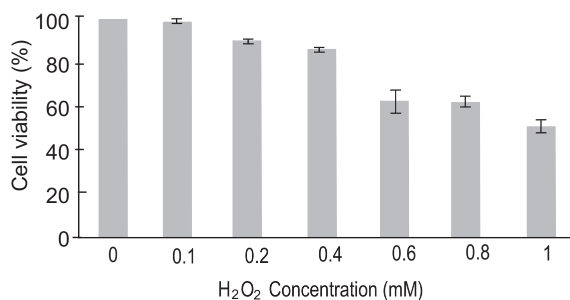

Fig. 2. Viability of 3T3 cells after being exposed to H_2O_2 at varied doses (0.1–1 mM) for three hours. The MTT assay was used to measure cell viability. The results are shown as mean (n = 3) standard deviation of mean. From untreated cells, *P < 0.05.

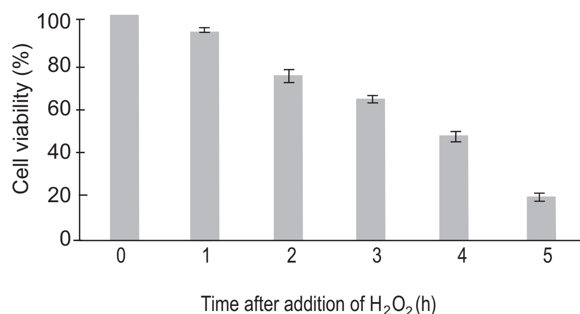

Fig. 3. Cell viability of 3T3 cells exposed to 0.6 mM H_2O_2 for various exposure times (0–5 h). The MTT assay was used to measure cell viability. The results are shown as mean (n = 3) standard deviation of mean. From untreated cells, *P < 0.05.

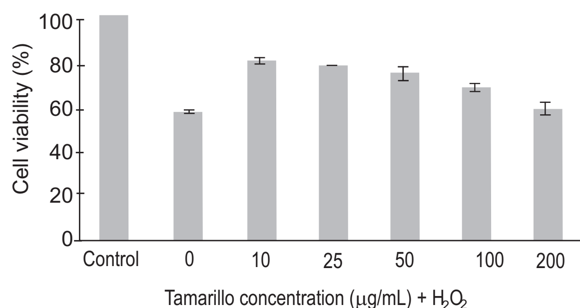

Fig. 4. Tamarillo's defence against H_2O_2-induced toxicity in 3T3 cells. Tamarillo ethanolic extract (0–200 µg/mL) was applied to the cells for 4 h and 0.6 mM H_2O_2 was applied for 3 h. The MTT assay was used to measure cell viability. The results are shown as mean (n = 3) standard deviation of mean. From untreated cells, *P < 0.05.

by 79% compared to the control (57%). It was evaluated that H_2O_2 induced-3T3 cells were treated with different concentrations of the tamarillo ethanolic extracts significantly reversed the H_2O_2-induced cytotoxicity and resulted in the prevention of cell death, which suggests the protective role of tamarillo against oxidant induced damage. It is well recognised that hydrogen peroxide, a precursor of many ROS, can operate as a powerful inducer of ROS and cause cell damage both *in vitro* and *in vivo* by Park *et al*. (2014). It is widely acknowledged that excessive ROS production in cells results in the breakdown of cellular homeostasis, oxidative stress and ultimately the death of organ cell populations. By scavenging free radicals, the antioxidant defence of the cell which consists of naturally occurring antioxidant molecules and enzymes prevents damage brought on by oxidative stress. However it may not be sufficient that will lead to extensive damage to the macromolecules and ultimately leading to cell death and tissue damaged, Halliwell (2011). Therefore, inducing antioxidant defence machinery through dietary polyphenols would be an effective strategy in maintaining the oxidative stress at low levels. Tamarillo ethanolic extracts have earlier been shown to possess good antioxidant activities with chemical antioxidant assays (DPPH scavenging and beta-carotene bleaching assays). Thus, the present study suggests that protective effect of tamarillo is more like its antioxidant potential in cyto-protective activity against hydrogen peroxide-induced toxicity in 3T3 normal cells.

Isolated effects of doxorubicin and tamarillo on $HepG_2$ and MDA-MB-231 cell viability. The viability of the cell was assessed after 72 h of the culture for $HepG_2$ and MDA-MB-231 cell lines, which were exposed to various doses of doxorubicin and tamarillo in isolation. The results showed that in $HepG_2$ and MDA-MB-231 cells, tamarillo and doxorubicin both lowered cell viability in a dose-dependent manner. Most sensitive cell line, $HepG_2$ showed just 40% of the cell viability at highest dose of doxorubicin that was examined (1.6 µg/mL) (Fig. 5). On comparison with untreated cells, statistical significances (P < 0.05) were established in two cell lines examined at doses between 0.4 and 1.6 µg/mL.

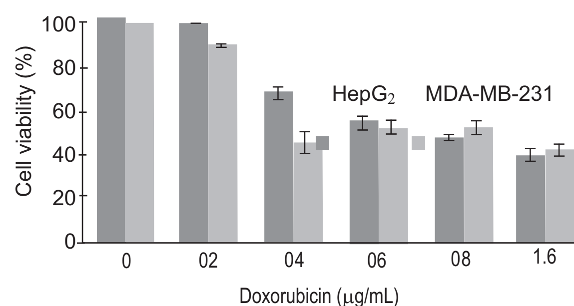

Fig. 5. MTT assay was used to determine the isolated effect of doxorubicin (0.2 - 1.6 µg/mL) on the viability of $HepG_2$ and MDA-MB-231 cells. The results are shown as mean (n = 3) standard deviation of mean. From untreated cells, *P < 0.05.

However, treatment with the tamarillo ethanolic extract resulted in a concentration dependent inhibitory effect on the cell survival, with a striking similarity in the pattern of reaction between $HfepG_2$ and MDA-MB-231 cells (Fig. 6). Most resilient cell type, the $HepG_2$ cell, saw a 33% drop in viability at the higher concentration used (200 µg/mL). When compared to untreated cells, statistically significant values were reported in the two cell lines examined (P < 0.05).

Combined effects of doxorubicin and tamarillo on $HepG_2$ and MDA-MB-231 cell viability. The combination treatment of $HepG_2$ and MDA-MB-231 cells with 30 µg/mL and 80 µg/mL of the tamarillo ethanolic extract respectively, with Doxorubicin (0.2, 0.4, 0.6, 0.8 and 1.6 µg/mL) decreased the cell viability (P < 0.05) when compared to exposure to treatment in isolation (Fig. 7). The $HepG_2$ survival rates were 33%, 17%, 21%, 19% and 15% for this combination, compared to 54%, 42%, 42%, 45% and 44% for MDA-MB-231. Even at the lowest tested dose of doxorubicin (0.2 µg/mL), the $HepG_2$ cell exhibited the greatest sensitivity to the associated medication. When compared to the control group, all combinations are statistically significant (P < 0.05).

Combination index. The MATLAB approach was used to analyse type of interaction (synergistic, additive or antagonistic) between doxorubicin and tamarillo ethanolic extract when they were combined for 72 h on $HepG_2$ and MDA-MB-231 cells, Chou and Talalay (1984). The CI values demonstrate an impact that is additive when it is equal to 1, antagonistic when it is

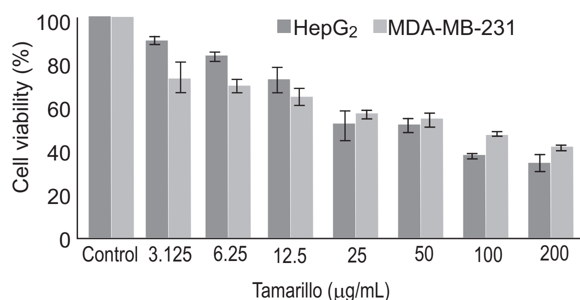

Fig. 6. Using the MTT test, the isolated effects of tamarillo ethanolic extract (0–200 µg/mL) on the viability of $HepG_2$ and MDA-MB-231 cells were measured. The results are shown as mean (n=3) standard deviation of mean. From untreated cells, *P < 0.05.

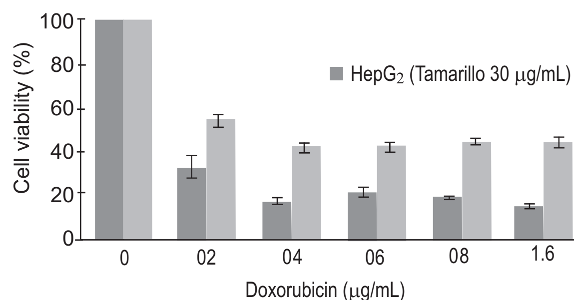

Fig. 7. Using the MTT test, the combined effects of tamarillo ethanolic extract and doxorubicin (0.2 - 1.6 µg/mL) on the viability of $HepG_2$ (30 µg/mL) and MDA-MB-231 (80 µg/mL) cells were determined. The results are shown as mean (n = 3) standard deviation of mean. From untreated cells, *P < 0.05.

larger than 1 and synergistic when it is less than 1. Table 1. lists the combination index (CI_{50}) values computed for $HepG_2$ and MDA-MB-231 cells. Doxorubicin (0.2, 0.4, 0.6, 0.8 and 1.6 µg/mL) and tamarillo (30 µg/mL) together had a synergistic impact on slowing the development of $HepG_2$ cells, with CI_{50} values of 0.86, 0.51, 0.58, 0.54 and 0.45, respectively. The best combination for inhibiting the development of $HepG_2$ cells was 1.6 µg/mL of doxorubicin and 30 µg/mL of tamarillo ethanolic extract (CI values = 0.45) (Table. 1). The dose reduction index 50 (DRI_{50}) indicates percentage of dosage reduction for combination medicine's 50% growth inhibitory impact as compared to each agent alone. Findings showed that DRI_{50} values for doxorubicin and tamarillo in combination for $HepG_2$ cells were 1.55 and 4.64, 2.25 and 16.27, 2.02 and 11.24, 2.15 and 14.03 and 2.45 and 21.60, respectively. However, in MDA-MB-231 cells, no synergistic induction of suppression of cell growth was seen (Table. 1).

These findings imply that the anticancer drugs doxorubicin and tamarillo ethanolic extract jointly limit growth of $HepG_2$ cancer cell line. Chou and Talalay (1984) examined the synergistic effect of tamarillo and doxorubicin on the $HepG_2$ and MDA-MB-231 cell lines. In $HepG_2$ cells with reduced cell viability when compared to each medication administered alone, a synergistic interaction (CI one) was achieved with a combination schedule of doxorubicin and tamarillo ethanolic extract. Most significantly, combining the two at low doses results in a marked reduction in cell

Table 1. Combination index[a] (CI) and dose reduction index (DRI) values of Doxorubicin and tamarillo combination.

Cell lines	Doxorubicin (µg/mL)	Tamarillo (CI50 µg/mL)	CI_{50}	DRI_{50}	Interpretation
$HepG_2$	0.2	30.0	0.86	Doxorubicin: 1.55; Tamarillo: 4.64	Synergism
	0.4	30.0	0.51	Doxorubicin: 2.25; Tamarillo: 16.27	Synergism
	0.6	30.0	0.58	Doxorubicin: 2.02; Tamarillo: 11.24	Synergism
	0.8	30.0	0.54	Doxorubicin: 2.15; Tamarillo: 14.03	Synergism
	1.6	30.0	0.45	Doxorubicin: 2.45; Tamarillo: 21.60	Synergism
MDA-MB-231	0.2	80.0	3.07	Doxorubicin: 2.18; Tamarillo: 0.38	Antagonism
	0.4	80.0	1.02	Doxorubicin: 3.38; Tamarillo: 1.39	Antagonism
	0.6	80.0	1.04	Doxorubicin: 3.34; Tamarillo: 1.35	Antagonism
	0.8	80.0	1.33	Doxorubicin: 3.02; Tamarillo: 1.00	Antagonism
	1.6	80.0	1.22	Doxorubicin: 3.13; Tamarillo: 1.11	Antagonism

aCI_{50} is a combination index for 50% effect, used for quantifying synergism, additivity and antagonism.

viability. To date, chemotherapy is still remains the most popular way of treating cancer, despite many side effects, Sak (2012). However, an alternative approach with use of the polyphenolic compound in treatment of different types of the cancer holds promise for better anticancer competencies, Aggarwal *et al*. (2013). The combination of common cancer therapies with chemo-preventive agents may enhance the anticancer activity through synergic action, Sarkar and Li (2006). The combination therapeutic synergy between the individual compounds that works at physiologically relevant doses may result in the reduction of the individual concentrations and is important to minimize the undesirable dose-related toxicity, this type of tamarillo extract does not present toxicity, Suarez *et al*. (2021).

Conclusion

The cyto-protective effect of tamarillo ethanolic extract with its low toxicity which fosters further evaluation on its potential as a promising active ingredient for possible future use in conventional chemotherapeutic drugs and radiation therapy. Although this is a preliminary study that involves a synergistic action between tamarillo and Doxorubicin, the chemo-preventive phytochemicals present in tamarillo demonstrated significant effectiveness with minimized toxicity, less expensive and more environmental friendly than the synthetic drugs.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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