Evaluation of Anti-angiogenic Activity and Biological Safety of Limonoids from Selected Philippine Citrus Fruits

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RESEARCH ARTICLE

Abstract

Background and Objectives: Suha *Citrus maxima* (Burm.) Merr and kalamansi *Citrofortunella microcarpa* (Bunge) Wijnands are citrus fruits common to the Filipino diet and are found to contain bioactive phytochemicals, such as limonoids. Limonoids are triterpenoid bitter principles from *Citrus* fruits, predominantly found in the seeds of citrus fruits. Initially studied for their intrinsic bitterness, this group of phytochemicals was found to have a plethora of health-giving benefits including anti-microbial, anti-carcinogenic, and hepatoprotective, among others. In this study, seed limonoids from suha and kalamansi, along with their major limonoids, limonin and nomilin, were evaluated for their angiogenic activities and biological safety.

Methodology: Limonoids were isolated and characterized from the seeds of suha and kalamansi along with their major limonoids, limonin, and nomilin. A modified duck egg chorioallontoic membrane (CAM) assay was aided by AngioQuant, a digital imaging software used to evaluate angiogenic activity. Inhibition of angiogenesis was evaluated by percent increase or decrease in mean length of blood vessels, mean size of blood vessels, and total number of blood vessel junctions. Zebrafish embryotoxicity assay was utilized to evaluate the toxicity of limonoids. Zebrafish embryos were exposed to the aforementioned limonoids at 100 ppm [maximum concentration for a Category 5 (practically non-toxic) substance] and were observed for 96 hours for the four apical signs of zebrafish lethality.

Results: Analysis with AngioQuant revealed that treatment of the duck egg CAM with limonin, nomilin, and seed limonoid mixtures of suha and kalamansi showed a decrease in the percent mean length and size of blood vessels, and the total number of blood vessel junctions comparable to that of quercetin, a known anti-angiogenic compound ($P<0.0001$). Zebrafish exposed to the same phytochemicals at 100 ppm did not show any of the four apical signs of zebrafish lethality 96 hpf.

Conclusion: Limonin, nomilin, and the seed limonoid mixtures of suha and kalamansi inhibited angiogenesis in a dose-dependent manner, comparable to the anti-angiogenic effect of quercetin. These are bioactive, yet non-toxic phytochemicals.

Keywords: *limonoids, angiogenesis, duck egg chorioallontoic membrane assay, zebrafish embryotoxicity assay*

Introduction

Citrus fruits are regarded as vital components in the human diet. In the Philippine context, *Citrus* fruits such as, kalamansi *(Citrofortunella microcarpa*) (Bunge) Wijnands (Fam. Rutaceae) and suha *(Citrus maxima*) (Burm.) Merr. (Fam. Rutaceae) are popular in the Filipino diet, either as drinks, food ingredient, or dessert. Since ancient times, their nutritive and medicinal values have been established. Several studies have shown that consumption of these fruits is associated with positive health-giving and disease-preventing benefits [1], which have been associated with their phytochemical components, such as, vitamin C, carotenoids, flavanones, and limonoids [2,3].

Limonoids are a large group of highly oxidized triterpenoids found in the families of Meliaceae and Rutaceae, where *Citrus* fruits belong. Limonoids, specifically

limonin was first discovered in 1864 as *Citrus* fruit bitter principle and was studied primarily to ensure that the bitterness of *Citrus* fruit products, such as juices do not exceed the human threshold for bitterness. Later studies showed that limonoids possess health-promoting properties, among which is their cancer chemopreventive properties [4]. Limonoids are found in highest abundance in the seeds of *Citrus* fruits like *suha*. However, seeds usually end up as waste. Aside from limonoids, other health-giving phytochemicals are present in *Citrus* seeds, such as flavonoids [5]. While not an objective of this paper, this study presented a way to exploit byproducts, such as seeds to develop formulations of functional foods.

Studies have shown that limonoids have cancer chemopreventive properties, as shown by the inhibition of cell proliferation in cell lines, such as the pancreatic cell line Panc-28 [6], colon adenocarcinoma cell lines SW480 and Caco-2 [7,8], neuroblastoma cell line SH-SY5Y [9], and the multi-drug resistant human leukemia cell line CEM/ADR5000 [8]. Limonoids were also shown to inhibit the progression of 7,12-dimethylbenz[a]anthracene-induced oral cancer in hamster models [10]. Studies have shown that limonoids inhibit the progression of carcinogenesis by inducing the tumor suppressor protein p53, which implicates that limonoids could inhibit the same by apoptosis, inhibition of inflammation, or cell cycle arrest [1]. Limonoids were also shown to inhibit cell metastasis in both cell culture and animal models [11,12]. However, other targets of tumorigenesis and metastasis, including angiogenesis, have yet to be explored [11].

Tumorigenesis was thought to be dependent on angiogenesis, the *de novo* formation of blood vessels. New vasculature is important in tumor growth and metastatic spread of cancer cells since these provide an adequate supply of oxygen and nutrients to the cells, as well as removal of waste products [13]. Because of this, angiogenesis has been an attractive drug target against carcinogenesis, metastasis, and even chemoresistance [14]. Angiogenesis inhibitors have been approved for use in USA and in 26 other countries, including the Philippines, as treatment for cancer.

The safety of a drug candidate must be established before it is utilized as a potential lead. While studies have shown the relative safety of limonoids both in humans and in animal models, these have been mostly incidental since these studies focused more on the other aspects, such as bioavailability [15,16]. In this study, the toxicity of limonoids was investigated using zebrafish embryotoxicity test. Zebrafish is an emerging and attractive bioassay platform for high-throughput method of toxicity testing, and offers an inexpensive and minimized, yet comparable alternative model to that of rodents [17].

This study, thus, aimed to (1) determine the angiogenic potential of limonoids from seeds of selected Philippine *Citrus* fruits, namely, suha and kalamansi, and major limonoids limonin and nomilin using an *in vivo* duck egg chorioallontoic membrane (CAM) assay assisted by an automated image analyzer, and (2) characterize the toxicity profile of the same using zebrafish embryotoxicity assay.

Materials and Methods

Materials and Reagents

All reagents used were of analytical grade. Acetone, absolute ethanol, ethyl acetate, hexane, hydrochloric acid, methanol, and sulfuric acid were from LAB-SCAN Analytical Sciences. Acetonitrile, dichloromethane, chloroform, dimethylsulfoxide (DMSO), and petroleum ether were from JT Baker. P-dimethylaminobenzaldehyde (p-DMAB) was from Hi-Media Laboratories. Thin layer chromatography (TLC) aluminum sheets pre-coated with silica gel 60 F_{254} and silica gel 60 were from Merck. Quercetin was from Sigma-Aldrich. β-carotene, which was used for angiogenesis, was isolated from carrot and assayed using the method of Rodriguez-Amaya [18] and was stored in mineral oil.

Plant Material

Kalamansi *(Citrofortunella microcarpa)* (Bunge) Wijnands, and suha *C. maxima* (Burm.) Merr were purchased from the local market during their fruiting season. The plant material was authenticated by Prof. Annalee S. Hadsall, curator of the Museum of Natural History, University of the Philippines Los Baños.

General Procedures

The melting points of the purified limonoids were determined using a Fisher-Johns apparatus hot stage melting point apparatus and were uncorrected. Ultraviolet (UV) analysis was performed using Mini 1240 Shimadzu UV Scanning Spectrophotometer. The maximum peaks of absorption of the pure limonoid isolates in acetonitrile were determined by scanning from 300 to 200 nm. The Infrared (IR) spectra of the pure limonoid isolates were recorded on a Shimadzu IR Prestige-21 Fourier Transform IR Spectrometer by

a diffuse reflectance technique. The ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra of the pure limonoid isolates were recorded on a 500 MHz Agilent NMR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively. The chemical shifts were measured in $CDCl_3$ with internal TMS and were expressed in δ (ppm). Thin layer chromatographic analysis was performed using silica gel 60 F_{254} thin layer chromatography plates (Merck, Germany). The plate was developed with ethyl acetate/n-hexane (8:2 v/v) and was visualized by either by spraying with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in ethanol) followed by color development with HCl gas in an enclosed chamber until a definite pink coloration developed, or by spraying with 20% sulfuric acid in methanol followed by charring. Photographic documentation was performed using 12.1 megapixel Sony Cybershot camera.

Extraction and Isolation of Limonoids from Suha and Kalamansi Seeds

Limonoids from suha seeds were extracted using a modified procedure of Jitpukdeebodintra [5]. Previously cleaned, dried, and ground suha seeds (434.4 g) were defatted with petroleum ether overnight. The defatted seed meal was macerated with acetone for 12 hours. The acetone liquor was concentrated *in vacuo* at 35°C to dryness, obtaining an oily, crude extract. Petroleum ether was added to this extract to crystallize limonoids. The off-white limonoid crystals were filtered by suction and washed thrice with cold 95% ethanol to obtain the suha seed limonoid extract. The same procedure was followed for kalamansi seeds but without the subsequent purification procedure.

About 1.7 g of the suha seed limonoid mixture was subjected to column chromatography using silica gel, isocratically eluted using ethyl acetate/dichloromethane (1:7 v/v) in eluates of 10 mL and at a flow rate of 3 drops per second. Eluates were monitored using thin layer chromatography using the same solvent system. Eluates with the same elution profile were pooled to yield two fractions tentatively named as Limonoid-1 (0.5 g) and Limonoid-2 (0.5 g). A fraction containing a combination of Limonoid-1 and -2 was also obtained (0.3 g), but no efforts to purify this fraction was made.

Limonoid-1

White crystals, melting point 278-280°C; ^{1}H NMR ($CDCl_3$): δ 7.40 (1H, d, *J* = 0.8 Hz, H-22, H-23), 6.32 (1H, d, *J* = 0.9 Hz, H-21), 5.44 (1 H, s, H-17), 5.01 (1H, d, *J* = 7.1 Hz, H-1), 3.80 (1H, s, H-15), 3.19 (1H, d, *J* = 15.5 Hz, H-2b), 3.11 (1H, dd, *J* = 15.6 Hz, 7.2 Hz, H-2a), 2.76 (1H, t, *J* = 15.1 Hz, H-6b), 2.60 (1H, dd, *J* = 15.0 Hz, 3.8 Hz, H-6a), 2.57 (1H, dd, *J* = 15.0 Hz, 3.8 Hz, H-5), 2.47 (1H, dd, *J* = 10.4 Hz, 2.6 Hz, H-9), 2.01 (3H, s, $CH_3C=O$), 1.79 (1H, m, H-12b), 1.64 (1H, m, H-11b), 1.61 (1H, m, H-11a), 1.56 (3H, s, H-25b), 1.52 (1H, m, H-12a), 1.47 (3H, s, H-25a), 1.33 (3H, s, H-19), 1.18 (3H, s, H-18), 1.09 (3H, s, H-24); ^{13}C NMR ($CDCl_3$): δ 206.7 (C-7), 169.2 (C-3, $CH_3C=O$), 166.7 (C-16), 143.3 (C-21), 141.0 (C-23), 120.0 (C-20), 109.6 (C-22), 84.3 (C-4), 78.0 (C-17), 70.7 (C-1), 65.4 (C-14), 53.4 (C-15), 52.9 (C-8), 51.0 (C-5), 44.3 (C-10), 44.1 (C-9), 38.8 (C-6), 37.5 (C-13), 35.3 (C-2), 33.4 (C-25a), 32.3 (C-12), 23.4 (C-25b), 20.8 (C-24, $\underline{C}H_3C=O$), 17.2 (C-19), 17.1 (C-18), 16.5 (C-11); UV (acetonitrile): 214 nm; IR (KBr): υ_{max} 1732, 1709, 1375, 1285, 1225, 1157, 1024 cm^{-1}. Based on the spectral data, Limonoid-1 was identified as nomilin (Figure 1A) [19-21].

Limonoid-2

White crystals from dichloromethane-isopropyl alcohol, melting point 292-294°C, ^{1}H NMR ($CDCl_3$): δ 7.41 (1H, m, H-

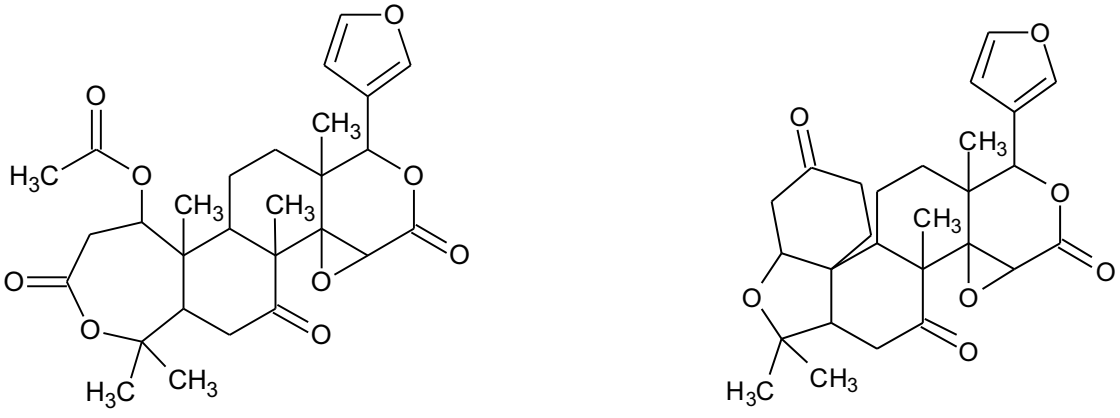

Figure 1. *Structures of two of the most commonly occurring limonoids in Citrus fruits: (A) nomilin; and (B) limonin.*

23), 7.40 (1H, m, H-22), 6.34 (1H, m, H-21), 5.47 (1H, s, H-17), 4.75 (1H, d, *J* = 13.0 Hz, H-19a), 4.45 (1H, d, *J* = 13.0 Hz, H-19b), 4.04 (1H, m, H-1), 4.03 (1H, s, H-15), 2.99 (1H, dd, *J* = 16.8 Hz, 4.0 Hz, H-2b), 2.85 (1H, t, *J* = 15.2 Hz, H-6b), 2.66 (1H, dd, *J* = 16.8 Hz, 1.3 Hz, H-2a), 2.56 (1H, dd, *J* = 12.4 Hz, 2.7 Hz, H-9), 2.48 (1H, dd, *J* = 16.8 Hz, 1.3 Hz, H-6a), 2.21 (1H, dd, *J* = 15.8 Hz, 3.2 Hz, H-5), 1.29 (3H, s, H-25a), 1.18 (3H, s, H-18, H-25b), 1.07 (3H, s, H-24); ^{13}C NMR ($CDCl_3$): δ 206.0 (C-7), 169.0 (C-3), 166.5 (C-16), 143.2 (C-22), 141.1 (C-23), 120.0 (C-20), 109.7 (C-21), 80.3 (C-4), 79.1 (C-1), 77.8 (C-17), 65.6 (C-14), 65.3 (C-19), 60.6 (C-5), 53.8 (C-15), 51.3 (C-8), 48.1 (C-9), 45.9, (C-10), 37.9 (C-13), 36.4 (C-6), 35.6 (C-2), 30.9 (C-12), 30.2 (C-12), 21.4 (C-25a), 21.4 (C-25b), 20.7 (C-18), 18.9 (C-11), 17.6 (C-24); UV (acetonitrile): 213 nm; IR (KBr): υ_{max} 1755, 1709, 1501, 1391, 1281, 1157, 1026 cm^{-1}. Based on the spectral data, Limonoid-2 was identified as limonin (Figure 1B) [20-21,23].

Angiogenesis Assay

The angiogenic activity of limonoids was evaluated using a duck egg chorioallontoic membrane (CAM) assay using 10-day old duck eggs purchased from a trusted duck farm at Barangay Malinta, Los Baños, Laguna [24]. Increasing doses of limonin, nomilin, suha and kalamansi seed limonoid extracts (10, 50, 100, 200, 300 ppm) were used. Quercetin and β-carotene, known anti- and pro-angiogenic compounds, respectively, were used as reference compounds for comparison. All treatments were performed in triplicates.

A small opening was made in the rounded part of the egg, previously cleaned with 70% ethanol. A UV-sterilized filter disc with 5 mm diameter was planted over the duck egg CAM. Ten microliters (10 µL) of the test substance were loaded into the filter paper disc using a tuberculin syringe. The opening was sealed with a UV-sterilized paraffin. The eggs were incubated for 48 hours and maintained at a temperature of 37±1ºC. After 48 hours, the CAM was exposed, and the filter paper was removed. The affected area was photographed. The digital image was converted to a black and white image. Pro- or anti-angiogenic activity was quantified through image analysis using AngioQuant v.1.33 (MATLAB, Inc. Tampere, Finland) [25,26]. Using CAM treated with vehicle control (0.02 M phosphate buffer pH 7.4 for quercetin, 0.02 M phosphate buffer pH 7.4 with mineral oil for β-carotene, and 0.02 M phosphate buffer pH 7.4 with 0.2% DMSO for limonin, nomilin, suha and kalamansi seed limonoid extracts) as baseline, the percent increase/decrease in length, size, and number of junctions of CAM blood vessels were quantified from the images. The percent increase/decrease of the parameter was computed using Equation 1.

Zebrafish Embryotoxicity Assay

Zebrafish embryotoxicity assay, zebrafish maintenance, and husbandry were adopted from the protocol set by OECD Document 236 [27].

Maintenance of zebrafish

Mature male and female zebrafish were obtained from a fish breeder in Pila, Laguna. The zebrafish were kept in separate 10-gallon fish tanks and were fed twice daily with TetraMin and TetraBits (Tetra, Germany) alternately at an amount the fish could consume within five minutes. Uneaten food and other organic waste were removed an hour later, and the tanks were cleaned once weekly.

Egg production and collection

Two male and one female zebrafish genitors were mated in a separate tank the night before the zebrafish eggs would be needed. The tank was fitted with a fine metal mesh at the bottom to prevent the eggs from being eaten. Artificial plants made of inert materials were placed to stimulate spawning. The eggs were collected in the early morning after the zebrafish had spawned. The fishes were returned to their respective tanks. The eggs were collected using gentle suction and were cleaned immediately.

Preparation of solutions

Solutions (100 mg L^{-1}) of limonin, nomilin, suha and kalamansi seed limonoid extracts were dissolved in DMSO and was brought up to volume with egg water (0.06 g L^{-1} Instant Ocean salts in distilled water), bringing DMSO to a final concentration of 0.2%.

Toxicity assay

When the eggs had been cleaned sufficiently, 40 eggs were randomly distributed into Petri dishes containing 100 mg L^{-1} of the test solution, the negative control, or the solvent control, not later than 90 minutes post fertilization. The eggs were screened with a light microscope at 100x magnification. One egg was transferred into 20 wells of an appropriated and labeled 24-well plate within 180 minutes post fertilization. The four remaining wells served as internal plate control, which only contained egg water, to check the validity of the experiment.

Equation 1

$$\% \text{ increase or decrease of parameter} = \frac{\text{score, vehicle control - score, test substance}}{\text{score, vehicle control}}$$

Four apical endpoints: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat (Figure 2) were observed every 24 hours for 96 hours under a light microscope at 100x magnification. Additionally, hatching was recorded in treatment and control groups daily starting a 48 hours post fertilization (hpf). Any positive outcome in one of these endpoints, or failure to hatch signified embryo death is shown in Table 1.

Statistical Analysis

All results were expressed as mean ± SD. Where applicable, statistical analyses were performed using one-way ANOVA followed by Tukey's Multiple Comparison test at 95% confidence ($P<0.05$).

Results

Evalutation of Angiogenic Actvity of Limonoids

Analysis of duck CAM aided by AngioQuant revealed that treatment with limonin and nomilin resulted in percent decrease of mean length, size, and number of junctions of CAM blood vessels, at doses as low as 10 ppm. At this concentration, limonin was able to decrease blood vessel mean length, size, and number of junctions of blood vessels by 7.07±3.60%, 5.71±2.53%, and 13.96±1.27%, respectively. Also at 10 ppm, nomilin was able to decrease blood vessel mean length, size, and number of junctions of blood vessels by 6.19±1.88%, 6.08±3.02%, and 3.57±1.36%, respectively. Nomilin and limonin also effected dose-dependent decrease of the same parameters from 10 to 300 ppm on duck CAM. Duck CAM treated with suha and kalamansi seed limonoid extracts experienced a slight increase in the mean length and number of junctions of CAM blood vessels at 10 ppm, showing 0.79±0.35% increase in mean length of CAM blood vessel and 1.05±0.63% increase in mean total number of CAM blood vessel junctions for suha seed limonoid extracts, and 1.72±0.89% increase in mean length of CAM blood vessel and 3.83±0.45% increase in mean total number of CAM blood vessel junctions for kalamansi seed limonoid extracts. However, from 50 to 300 ppm, duck egg CAM treated with suha and kalamansi seed limonoid extracts experienced dose-dependent decrease in the same parameters, as well as for the mean size of CAM blood vessels. These results were comparable for quercetin ($P<0.0001$), a known anti-angiogenic phytochemical, which at 10 ppm, displayed increase in mean length, size, and

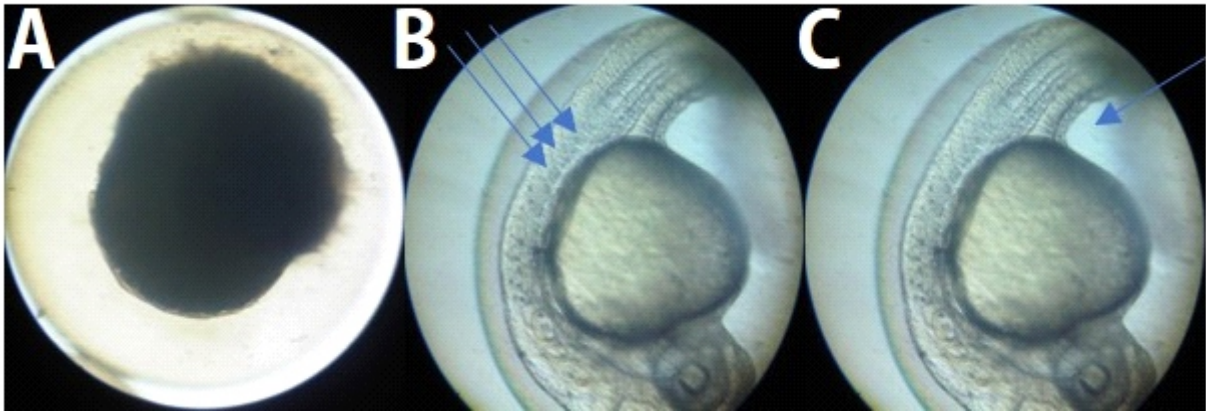

Figure 2. *Three of the four apical endpoints of zebrafish lethality. The zebrafish embryo is deemed as dead when any of these signs are present: coagulation of the embryo (shown in [A]), failure of the somites to form (pointed at by the arrows at [B]), and non-detachment of tail. The lack of heartbeat is, by definition, difficult to represent, and is not shown here.*

Table 1. *Apical observations of acute toxicity in zebrafish embryos 24-96 hours post fertilization (OECD, 2013)*

Irritation Endpoints	Score at Different Observation Times			
	24 hpf	48 hpf	72hpf	96 hpf
Coagulated embryos	+	+	+	+
Lack of somite formation	+	+	+	+
Non-detachment of the tail	+	+	+	+
Lack of heartbeat		+	+	+

number of blood vessels of duck egg CAM, but displayed dose-dependent decrease of the same parameters from 50 to 300 ppm. On the other hand, duck egg CAM treated with β-carotene, a known pro-angiogenic phytochemical, displayed increase in mean size and number of junctions in the duck egg CAM blood vessels as low as 10 ppm. At this concentration, duck egg CAM treated with β-carotene experienced an increase in mean size and number of junctions of CAM blood vessels at 10 ppm, showing 2.75±0.07% increase in mean size of CAM blood vessel and 9.67±1.36% increase in mean total number of CAM blood vessel junctions. A decrease in mean length of CAM blood vessels was observed at 10 ppm, from 50 to 300 ppm, duck egg CAM treated with β-carotene experienced progressive, dose-dependent increase in mean length, size, and number of blood vessel junctions. Results are shown in Figures 3-5. Skeletonized black and white digital photographs of duck egg CAM exposed to different treatments are shown in Figure 6. Taken together, *Citrus* limonoids effected suppression of the formation and growth of new blood vessels in duck egg CAM in a dose-dependent manner, comparable to the anti-angiogenic action of quercetin. On the other hand, β-carotene promoted the dose-dependent growth of new blood vessels in duck egg CAM.

Evaluation of Biosafety of Limonoids

Zebrafish embryos (n=20 per treatment) treated with limonin, nomilin, suha and kalamansi seed limonoid extracts did not display any of the apical points of zebrafish lethality, which are described in Table 1. They also hatched within 48 hours post fertilization (hpf), and developed naturally until the end of the experiment, at 96 hpf. After 96 hours post fertilization (hpf), no zebrafish embryo death was observed across all treatments. No apical points of zebrafish lethality, or death in the zebrafish embryo controls were observed in the internal plate controls in the same observation period, thus, validating the results of the experiment. The results are summarized in Table 2.

Discussion

Seeds of *Citrus* fruits like suha, usually end up as waste, yet contain high amounts of various health-associated secondary metabolites, such as limonoids [28,29]. Limonoids have been shown to demonstrate anti-bacterial [30], anti-parasitic [31], chemopreventive, hypoglycemic, and hepatoprotective activities, among others [32]. Across most *Citrus* juices and seeds, limonin and nomilin have been

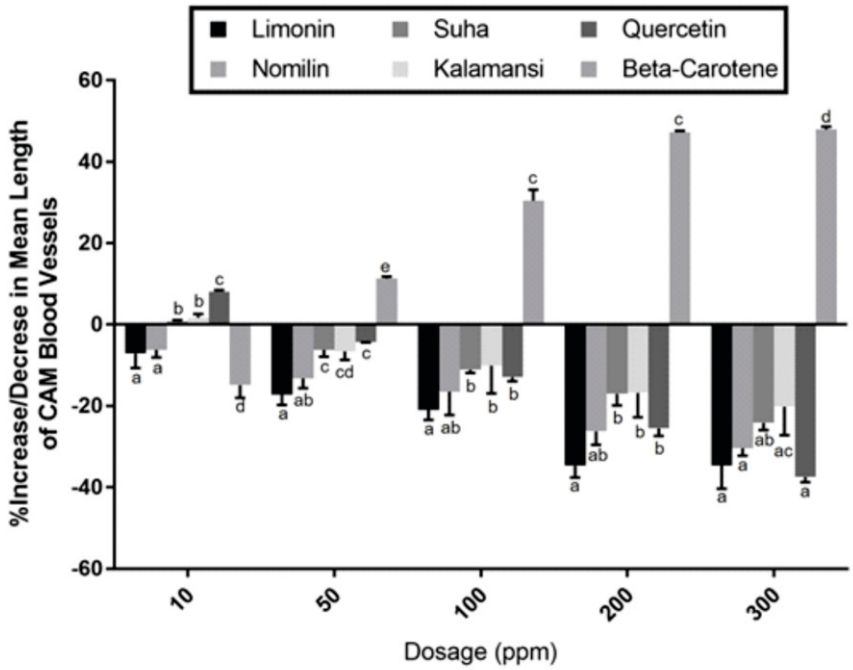

Figure 3. *Percent increase in the mean length of duck embryo CAM blood vessels treated with β-carotene, quercetin, suha and kalamansi seed limonoid extracts, nomilin and, limonin at 48 hours. Values are expressed as mean + SD (n=3). Values with the same letter in the same dosage do not differ significantly (P ≤ 0.05) by one-way ANOVA followed by Tukey's Multiple Comparison Test.*

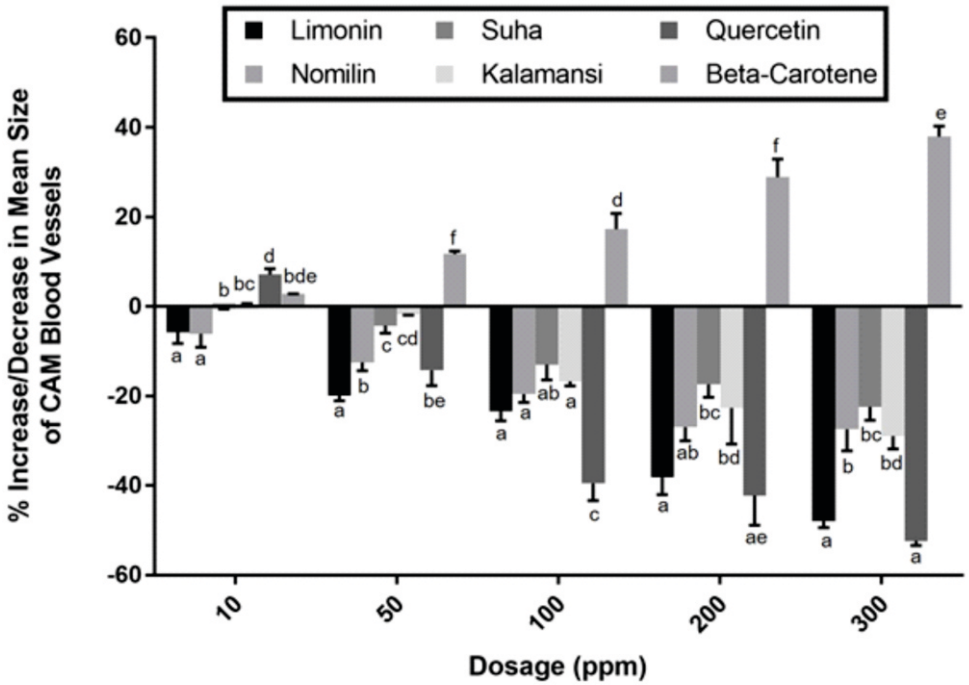

Figure 4. *Percent increase in the mean size of duck embryo CAM blood vessels treated with β-carotene, quercetin, suha and kalamansi seed limonoid extracts, nomilin and, limonin at 48 hours. Values are expressed as mean + SD (n=3). Values with the same letter in the same dosage do not differ significantly (P ≤ 0.05) by one-way ANOVA followed by Tukey's Multiple Comparison Test*

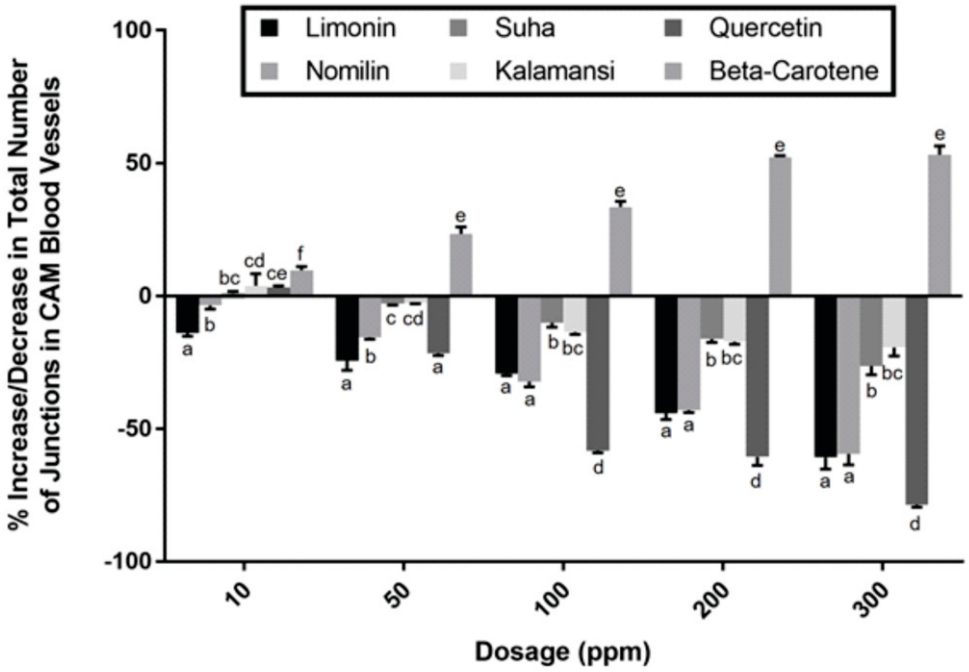

Figure 5. *Percent increase in the total number of junctions of duck embryo CAM blood vessels treated with β-carotene, quercetin, suha and kalamansi seed limonoid extracts, nomilin and, limonin at 48 hours. Values are expressed as mean + SD (n=3). Values with the same letter in the same dosage do not differ significantly (P ≤ 0.05) by one-way ANOVA followed by Tukey's Multiple Comparison Test*

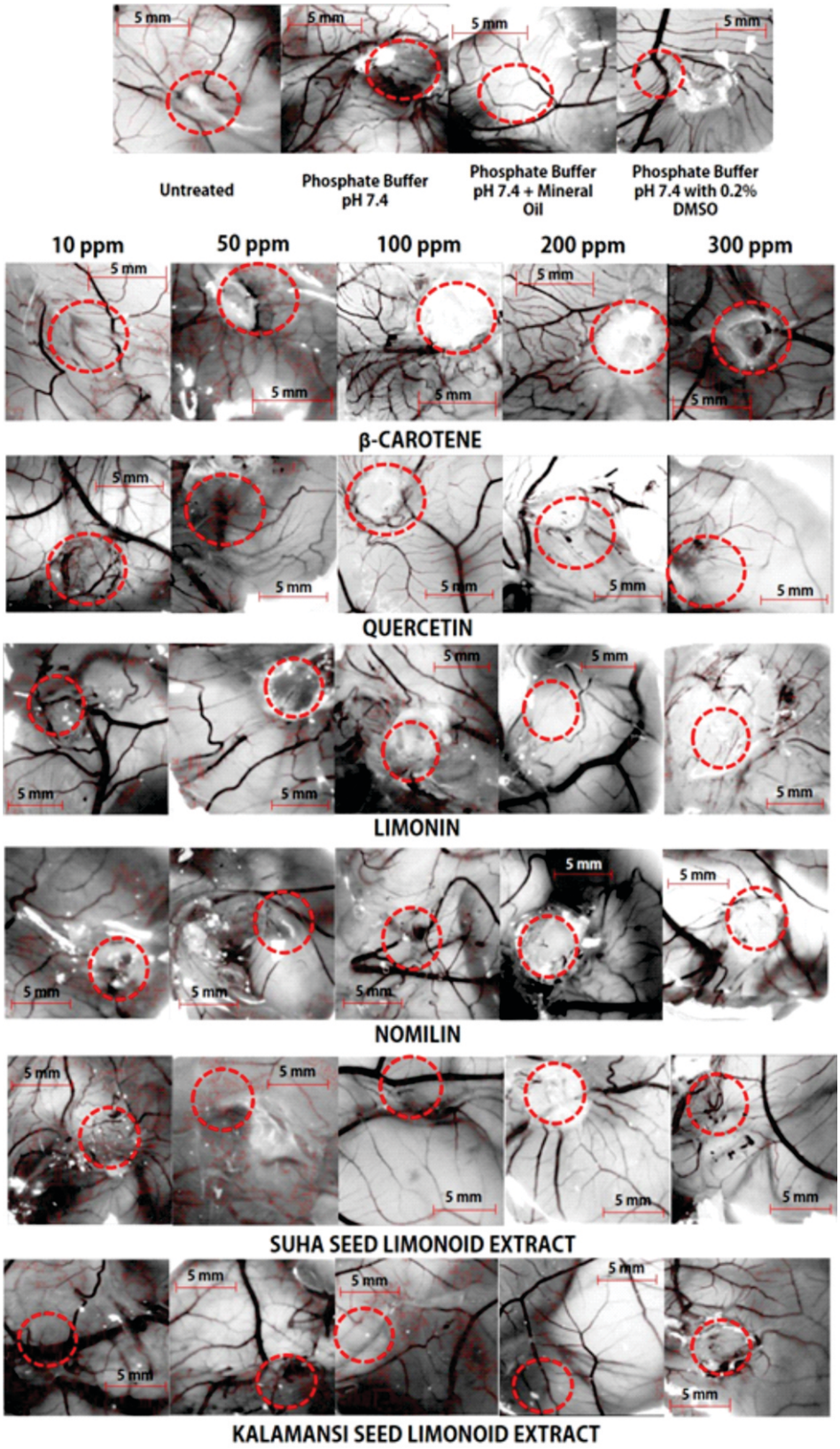

Figure 6. *Skeletonized images of duck CAM eggs treated with different test agents and solvent controls 48 hours post treatment. The treated area is indicated by a dashed circle.*

Table 2. *Score, survival rate, LC50, and toxicity of the limonoids tested on zebrafish embryo**

Test Substance	Irritation Endpoints	Score at Different Observation Times				Survival Rate after 96 hpf, %	Lc_{50}, mg L^{-1}	Toxicity
		24 hpf	48 hpf	72 hpf	96 hpf			
Limonin	Coagulated embryos Lack of somite formation Non-detachment of the tail Lack of heartbeat	- - -	-- - - -	- - - -	- - - -	100	>100	Practically non-toxic
Nomilin	Coagulated embryos Lack of somite formation Non-detachment of the tail Lack of heartbeat	- - -	-- - - -	- - - -	- - - -	100	>100	Practically non-toxic
Suha seed limonoid extract	Coagulated embryos Lack of somite formation Non-detachment of the tail Lack of heartbeat	- - -	-- - - -	- - - -	- - - -	100	>100	Practically non-toxic
Kalamansi seed limonoid extract	Coagulated embryos Lack of somite formation Non-detachment of the tail Lack of heartbeat	- - -	-- - - -	- - - -	- - - -	100	>100	Practically non-toxic

*Legend: + = observance of apical endpoint, - = non-observance of apical endpoint

shown to occur in highest abundance among *Citrus* limonoids [33], which were investigated in this study. Chromatographic and spectroscopic evidences from this study (Figure 7) showed the presence of limonin and nomilin in the seeds of *Citrus* fruits from both suha and kalamansi, whose angiogenic activity and biological safety were evaluated in this study.

The duck egg chorioallontoic membrane is an ideal angiogenesis model. At day 11 or 12 of chick embryo development, the embryo undergoes maturation through constant development of new blood vessels, and is therefore, angiogenic. Between days 8 and 10, the developing blood vessel system of the chorioallontoic membrane readily responds to any pro- or anti-angiogenic stimuli. The test solution is usually localized on a well-defined area in the chorioallontoic membrane, usually by using sterilized paper filter disks. Blood vasculature in response to pro-angiogenic compounds or stimuli would appear converging towards the disk in a wheel-spoke pattern, while lack of blood vessel formation and sometimes disappearance of pre-existing blood vessels are observed with anti-angiogenic compounds or stimuli [34].

The angiogenic activity of limonoids was evaluated using duck egg CAM assay, and quantitatively evaluated using a digital image analysis software, AngioQuant, a freeware that analyzes angiogenesis in co-culutres of endothelial cells with fibroblasts. Rodriguez *et al.* [25] adapted this procedure for analysis of angiogenesis in duck egg CAM. The software generates a skeleton network of blood vessels from an enhanced black and white digital image of the CAM. From the skeletonized image, the software analyzes three parameters: length, size, and number of junctions of blood vessels. The angiogenic activity of limonoids is evaluated using these three parameters, which are quantifiable physical manifestations of the appearance or disappearance of CAM blood vessels [26]. Results are recorded as percent increase/decrease in mean length of blood vessels, percent increase/decrease in mean size of blood vessels, and percent increase/decrease in total number of junctions of blood vessels. Taken together, results showed that the major *Citrus* limonoids nomilin and limonin, and the *Citrus* seed limonoids of kalamansi and suha suppressed the formation and growth of new blood vessels in a dose-dependent manner, comparable to the anti-angiogenic action of quercetin. On the other hand, β-carotene promoted the dose-dependent growth of new blood vessels.

The results of this study indicate that limonoids may be used to modulate angiogenesis and may play an important role in cancer and metastasis prevention. Angiogenesis is

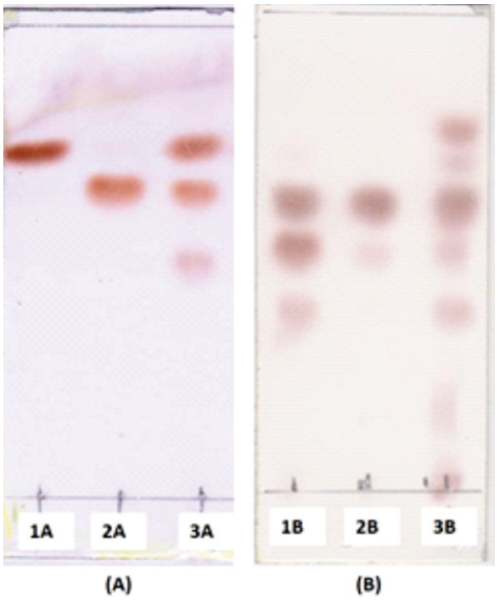

Figure 7. *(A) Thin layer chromatogram of pure samples of limonin (in Lane 1A) and nomilin (in Lane 2A) compared against limonoids in suha (lane 3A). (B) Thin layer chromatogram of limonoid profiles in suha (Lane 1B) and kalamansi (Lane 3B). Dalanghita, which was not investigated in this study, is included in Lane 2B. Extrapolating from suha limonoid profile, limonin and nomilin, among others, were obtained from kalamansi seeds.*

required for tumor growth and metastasis. Proliferation and metastatic spread of cancer cells depend on an adequate supply of oxygen and nutrients, and removal of waste products [13,35]. Limonoids have been identified for their anti-carcinogenic activity in cell culture and animal models [11]. The group of Guthrie *et al.* [36], in their study of nude mice injected with breast cancer cells into their mammary fat pad, and given orange or grapefruit juice, presumed that the limonoids were responsible for the inhibition of growth and metastasis of breast cancer. Kim *et al.* [11] suggests that a possible mechanism of anti-cancer activity of limonoids is through apoptosis mediated by downregulation by inducible nitric oxide synthase (iNOS), which is upregulated in angiogenesis [37]. Nomilin has been shown to inhibit tumor-specific angiogenesis by downregulating nitric oxide in melanoma-induced mice [38].

To determine any adverse effects of limonoids, the acute toxicity of limonin, nomilin and suha and kalamansi seed limonoid extracts were determined using zebrafish embryotoxicity assay, adopting OECD document 236 [27]. The principle of the assay is that newly-fertilized zebrafish eggs are exposed to the test substance. Four observation points, namely, coagulation, tail detachment, somite formation, and heartbeat were observed every 24 hours for 96 hours. Coagulation of embryo, non-detachment of the tail, failure of somite formation, and lack of heartbeat within 96 hours indicates lethality. Failure to hatch within 48 hpf also indicates lethality. The zebrafish embryos were exposed to 100 mg L^{-1} of the previously mentioned limonoids. The concentration of 100 mg L^{-1} is the upper toxic limit of Category 4 (Slightly toxic) substances [39]. If no death is observed within 96 hours post treatment, the LC_{50} of the test substance is noted as >100 mg L^{-1}, and can be classified as a Category 5 (Practically non-toxic) substance by extrapolation. The absence of any of the apical endpoints of zebrafish mortality during the 96-hour observation period indicates that the limonoids in study were practically non-toxic substances with no potential adverse effects on health.

Zebrafish embryo as an alternative model system is gaining attention due to several inherent advantages. It is small, inexpensive to maintain, and easily bred in large numbers; therefore, a large number of compounds can be screened at a given time. Also, a statistically significant number of animals can be used at a given time. In comparison to other model organisms like the fruit fly (*Drosophila melanogaster*) or the worm (*C. elegans*), there is a strong genetic, physiological, and pharmacological similarity between humans and zebrafish, which makes them well-suited for studying complex biological processes like toxicity response. Moreover, drug toxicity response is comparable to mammals, validating its suitability as a bioassay model for toxicity [17].

Evaluating the safety of phytochemicals is vital to ensure that they do not contain unwanted effects. While different experiments have established limonoids in general to be non-toxic, most studies only touch on this peripherally [15, 16]. In this study, it was established using the zebrafish embryo model that limonoids from *Citrus* fruits are non-toxic phytochemicals. However, further studies are needed to determine other modes of toxicity, such as organ-specific toxicity and the like.

Conclusions

Treatment of the duck egg chorioallontoic membrane with limonin, nomilin, and the seed limonoid mixtures of suha and kalamansi at concentrations of 10 to 300 ppm inhibited angiogenesis in a dose-dependent manner, comparable to the anti-angiogenic effect of quercetin, a known anti-angiogenic compound. Zebrafish exposed to the same phytochemicals at a dose of 100 mg L^{-1} did not show any of the four apical signs of zebrafish lethality at 96 hpf, hatched by 48 hpf, and developed normally to the end of the experiment, indicating that limonoids from suha and kalamansi seeds are bioactive, yet non-toxic phytochemicals.

Acknowledgments

The authors acknowledge the financial support given by the Advanced Science and Technology Human Resource Development Program of SEI-DOST, and the Philippine Society of Biochemistry and Molecular Biology. The authors also thank the able assistance of Forest Products Research and Development Institute, DOST Region IV, and the NMR Laboratory of Institute of Chemistry UP Diliman, where IR and NMR analyses were performed, respectively. RFBY would like to thank Mr. David Joram R. Mendoza for processing the CAM digital photographs for AngioQuant.

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