Cytotoxic and Angiosuppressive Potentials of *Zehneria japonica* (Thunb. ex Murray) S.K. Chen (Cucurbitaceae) Crude Leaf Extracts

Marri Jmelou Roldan[1,4]*, Ting-Yu Chin[5], Yun-Chieh Tsai[5], Agnes L. Castillo[1,2,4], Oliver B. Villaflores[1,3,4]

*Corresponding author's email address: mjmeroldan@gmail.com

[1] *The Graduate School, University of Santo Tomas, Manila, Philippines,*
[2] *Faculty of Pharmacy, University of Santo Tomas, Manila, Philippines,*
[3] *Department of Chemistry, College of Science University of Santo Tomas, Manila, Philippines,*
[4] *Research Center for the Natural and Applied Sciences, University of Santo Tomas, Manila, Philippines,*
[5] *Department of Biotechnology, Chung Yuan Christian University, Chungli District, Taoyuan City, Taiwan*

RESEARCH ARTICLE

Abstract

Background and Objectives: *Zehneria japonica* belongs to the Cucurbitaceae family which is one of the most important plant families. It is commonly known as Pipinong-gubat and is widely distributed in Central Luzon regions and at areas along streams and clearings and at low and medium altitudes in the Philippines. This study aims to evaluate the potential cytotoxic and angiosuppressive properties of *Zehneria japonica* (Thunb. ex Murray) S.K. Chen (Cucurbitaceae) leaf extracts.
Methodology: The *Z. japonica* semi-crude extracts were obtained by sequential extraction using hexane, ethyl acetate and n-butanol. 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. Moreover, the cytotoxic effects of the extracts were determined through MTT Assay. Osteosarcoma (U2Os) and hepatocellular carcinoma (HepG2) cells were used as cancer representatives while human umbilical vein endothelial cells (HUVEC) were used as the normal cell control.
Results: Analysis with AngioQuant revealed that treatment of the duck egg CAM with *Z. japonica* semi-crude extracts suppressed angiogenesis with IC_{50} values of 1,810.00 µg/mL, 192.50 µg/mL and 147.70 µg/mL for hexane, ethyl acetate and n-butanol, respectively, with Celecoxib (20 µg/mL) as the positive control. For MTT assay, *Z. japonica* extracts exhibited strong cytotoxic effect against U2Os with an IC_{50} values of 19.65 µg/mL, 9.89 µg/mL and 31.04 µg/mL for the hexane, ethyl acetate and n-butanol extracts, and no cytotoxic effects against HepG2 with IC_{50} values of 770.90 µg/mL, 130.10 µg/mL and 231.60 µg/mL for the hexane, ethyl acetate and n-butanol extracts. Doxorubicin (0.544 µg/mL) was used as the positive control. The extracts also inhibited the growth of the normal cells, with IC_{50} values of 69.46 µg/mL, 42.23 µg/mL and 63.44 µg/mL for the hexane, ethyl acetate and n-butanol extracts. There is no mortality and toxic symptoms observed for 14 days after the administration of the crude butanolic extract of *Z. japonica* in six female Sprague- Dawley rats.
Conclusion: *Z.japonica* crude leaf extracts exhibited angio-suppressive activity through CAM assay. In MTT assay, the extracts exhibited strong cytotoxicity in U2Os (IC_{50} ≤20 µg/mL), no cytotoxic effect in HepG2 (IC_{50} >100 µg/mL) cells and mild cytotoxic effect in HUVEC (IC_{50} 40-60 µg/mL). Phytochemical screening through TLC revealed that the extracts contain alkaloids, anthrones, flavonoids, and sterols.

Keywords: *Zehneria japonica (Thunb. ex Murray) S.K. Chen, Cucurbitaceae, cytotoxic, MTT Assay, angiosuppressive, CAM Assay*

Introduction

The vast increase of new cases of various diseases has been detrimental to one's health and lessens the person's quality of life. One of the notable diseases that causes morbidity and mortality worldwide is cancer. According to the World Cancer Report, there is an approximate of 8.2 million cancer-related deaths and 14 million new cases of cancer in 2012 [1]. Cancer cells grow abnormally and they frequently invade healthy cells in the body [2]. Angiogenesis plays a vital role in the etiology of tumor progression and metastasis [3]. Innovation of new strategies and therapies in the field of oncology has led to the study of angiogenic inhibitors [4]. Synthetic angiogenic inhibitors have been produced like Bevacizumab but investigations involving natural sources or products have limited or few studies available [5].

Through the years, plants have been utilized as sources of useful antitumor compounds and have immensely contributed in the fight and prevention against cancer and are proved to be promising. The Philippines has a diverse collection of plants but these resources are not yet well explored in pursuit of novel antiangiogenic agents [6]. A keen interest in the discovery for alternative source of anti-cancer agents from medicinal plants had led to the study of the Cucurbitaceae family.

Zehneria is one of 125 genera in the family Cucurbitaceae. Zehneria species have been utilized in traditional medicine to treat fever, stomach pain, diarrhea, skin diseases, jaundice, and kidney dysfunction [7,8]. Secondary metabolites reported from this genus includes dammarane-type glycosides (gypenosides) and sphingolipids with antimicrobial and antitumor activities [8,9].

Zehneria japonica (Thunb. ex Murray) S.K. Chen is widely distributed in Central Luzon region in the Philippines, where it is locally known as "Pipinong-gubat". No study has been made to explore the anti-angiogenic and cytotoxic properties of this plant though relatives of this plant under the same family were proven to possess the anticipated pharmacological activities.

This study aims to evaluate the cytotoxic and angiosuppressive property of *Z. japonica* which may be a potential source of phytochemical active compounds with the use of the duck chorioallantoic membrane (CAM) and MTT colorimetric assay, respectively.

Methodology

Instrumentation

Spectrophotometric measurements were performed by using Shimadzu RF-1501 UVVIS (ultraviolet- visible spectrophotometer). Cytotoxic activity of the crude extracts were determined using MTT assay and were measured spectrophotometrically (Biotek® ELX808 Absorbance Microplate Reader). Inverted microscope (Olympus CKX41 microscope), centrifuge (Eppendorf 5810R), CO^2 incubator (5% CO^2, 95% humidity and 37^oC; Thermo Scientific TM 8000 incubator) were also used. INCUBOX™ automatic humidified incubator was used for CAM assay.

Chemicals

Technical grade reagents bought from Belman laboratories were used for extraction. Analytical grade reagents were used for the phytochemical screening and biological assays of the air-dried, ground leaves of *Z. japonica*. Celecoxib was used for the positive control group while dimethylsulfoxide (DMSO) was used for the negative group in the CAM assay.

Plant Material

Fresh leaves of *Zehneria japonica* was collected in March 2016 at Basista, Pangasinan. The plant sample was identified and authenticated by Asst. Prof. Ophelia Laurente, curator at the University of Santo Tomas Herbarium, where a voucher specimen (No. USTH- 013124) has been deposited. The leaves were air-dried under shade for three weeks until they become brittle. The leaves were pulverized in a Wiley mill grinder and weighed.

Test animals

A total of six female, non-pregnant, nulliparous Sprague-Dawley rats, aged 8–12 weeks, weighing 100–160 g were selected for the toxicity test. The test animals were procured from the Food and Drug Administration at Alabang, Muntinlupa City, Philippines and were kept at animal house facility, Research Center of Natural and Applied Sciences, University of Santo Tomas. The animals were subjected to a 7-day acclimatization. An approval from the Institutional Animal Care and Use Committee was obtained as a requirement for the animal testing.

Egg Preparation

Fertile duck eggs were obtained from an egg farm in Baliuag, Bulacan, Philippines. A total of twelve groups composed of five zero-day old eggs were used in the assay. The eggs were incubated with the use of the Incubox automatic turning model with a 1-hour rolling interval at (38 ± 1) °C in humidified incubator until Embryonic Development Day (EDD) 8. The eggs were opened on either Day 8 - Day 10 of incubation. Tap water was placed beneath the egg trays which had a wire mesh bottom to prevent excessive humidity that might allow surface organisms to penetrate the shell [10, 11].

Cell Lines, Culture Treatments

Osteosarcoma (U2Os), Hepatocellular carcinoma (HepG2) and HUVEC lines cultured by recommended protocols of ATCC were obtained from the Department of Biotechnology in Chung Yuan Christian University, Taoyuan, Taiwan and were applied to the plant hexane, ethyl acetate and n-butanol extracts to determine their cytotoxic activity by the MTT colorimetric assay. The cells were seeded at 104 cells/100 µL into each well of 96-well plates and incubated for 24 hours at 37^oC and in 5% CO^2. The culture medium was removed and the extracts were added to the wells in different concentrations (2, 10, 20, 100, 500 µg/mL

and positive control Doxorubicin 0.544 µg/mL).

Preparation of Plant Extracts

A total of 1.17 kg of the ground leaves was obtained. The powder was extracted sequentially using solvents of increasing polarities. One kilogram of powdered leaves was soaked in 4L hexane (non-polar solvent) for 24 hours at room temperature. After one-day, the hexane extract was collected and the procedure was repeated thrice. The marc/residue was dried before the addition of the next solvent. Then, subsequent extractions with ethyl acetate (semi-polar solvent) and n-butanol (polar solvent) were done in the same sample. The three semi-crude extracts were concentrated using a rotary evaporator and water bath (40^{o}C) to remove the excess solvent. The extracts were properly labeled and stored at 4°C for further use.

Phytochemical Screening

Prepared extracts were subjected to preliminary phytochemical screening in order to qualitatively determine some of the secondary metabolites: saponins, terpenoids, flavonoids, tannins, alkaloids, quinones, anthraquinones and reducing sugar using appropriate methods [12,13,14].

Phytochemical screening was done by using thin layer chromatography sprayed with color reaction reagents. The sample was applied by using a capillary tube on a strip of pre-coated silica plates (Merck EMD TLC). After the sheet was air-dried, it was developed in a chamber that was a beaker containing the most suitable solvent covered with a watch glass. For the *Z.japonica* Hexane (ZjH) a solvent system of hexane and ethyl acetate (7:3) was used. On the other hand, *Z. japonica* Ethyl acetate (ZjEA), was developed with hexane and ethyl acetate (5:5) while *Z.japonica* n-Butanol (ZjB) was developed with dichloromethane and methanol (9:1). The developed chromatogram was air-dried and visualized by spraying with a suitable reagent for the desired constituents.

Toxicity Test

The ZjB was assessed for toxicity by using the acute oral toxicity test according to the Organization for Economic Cooperation and Development Guideline 425 (10). One dose level of 2 000 mg/kg body weight was performed. Initially, the animals were selected randomly and equally divided into two groups designated as Group A (1 rat) and Group B (4 rats). The rats were marked for proper identification and were subjected to a 7-day acclimatization. Prior to dosing, the rats were fasted overnight (12 h) by withholding food but not water. The fasted weight was recorded and the suitable dose for each rat was computed. The ZjB to be administered was diluted with water. One dose level of 2 000 mg/kg body weight was given to Group A via oral gavage. General toxicity signs and behavioral changes were observed daily for a total of 14 days and were noted. With no mortality in a 5-day observation period, Group B was given a one dose level of 2,000 mg/kg body weight. Same observation done on Group A was executed for Group B. After the testing period, the rats were sacrificed through asphyxia using carbon dioxide and were subjected to gross necropsy with the assistance of a Veterinarian, Dr. Joy Cabigan.

Chorioallantoic Membrane Assay

Chorioallantoic Membrane assay was used to determine the angiosuppressive activity of the plant extracts. It was performed based on the method of West *et al.* [11,15], with minor modifications on the choice of control agents. Celecoxib was used as a positive control agent and DMSO as vehicle control.

On 8th day of incubation, the surface of the egg was cleaned with 70% alcohol. A small window was made at the side of the egg using a drill bit and forceps. The filter discs with the test solution (10, 100 and 1000 µg/mL of hexane, ethyl acetate and n-butanol crude extracts and the controls: DMSO and Celecoxib 20 µg/mL) was put on top of the CAM and the window is sealed using a clear tape and is returned to the incubator for 48 h [16,17]. After 48 hours, the egg was opened and the general condition of the embryo and CAM were photographed and quantified using the software, AngioQuant™ [11]. The same procedure was applied for the rest of the eggs. The percent inhibition was determined in each test group using the formula:

$$\% \text{ inhibition} = \frac{\text{no. of branch points (treated)} - \text{no. of branch points (control)}}{\text{no. of branch points (control)}} \times 100$$

MTT Cytotoxicity Assay

MTT assay was performed to determine the cell viability which was measured by the reduction of MTT to a purple colored formazan product [18,19]. After 24 hours of treatment, 20 µL MTT reagent was added to each well. After incubation for 4 hours at 37^{o}C in the dark, the supernatants of culture media was aspirated and 150 µL DMSO was added to each well to dissolve the formazan crystals. Then, the absorbance was taken at 590nm using a microplate reader (Biotek® ELX808).

Statistical method

Means and its standard error were used to summarize the percentage inhibition of hexane, ethyl acetate and n-butanol at different concentrations in CAM and MTT Assay. Single-factor analysis of variance was used to determine the effect of different concentrations of each extract in the percentage inhibition with Tukey's HSD for Post Hoc analysis (MTT). Celecoxib was further compared with each of the concentrations (CAM). Meanwhile, estimates of IC_{50} were determined using four parameter logistic regression model. All these tests were performed using SPSS ver. 20.0 and GraphPad Prism 6.0. All tests were set at 5% level of significance.

Results and Discussion

This study determines and evaluates the cytotoxic and angiosuppressive potentials of *Zehneria japonica* (Thunb. ex Murray) S.K. Chen hexane, ethyl acetate and n-butanol crude leaf extracts.

Plant Extraction

The dried leaves of *Z. japonica* weighing 1.17 kg obtained a percentage yield of 1.68% for hexane crude extract, 2.56% for ethyl acetate crude extract and 0.53% for n-butanol crude extract.

Phytochemical Screening

In the preliminary phytochemical screening using thin layer chromatography, the leaves of *Z. japonica* crude extracts revealed the presence of various secondary metabolites (Table 1). Among the detected in the crude extracts were alkaloids, anthrones, flavonoids, and phenols.

Acute oral toxicity test

The animals showed no mortality and no change in behavior for 14 days after the administration of the crude butanolic extract of *Z. japonica*. Gross necropsy showed no significant findings observed on all limit test animals upon opening and examining its body cavities (Figure 1). Results obtained from the acute oral toxicity test showed that *Z. japonica* was practically non-toxic at a dose of 2,000mg/kg BW.

Chorioallantoic Membrane Assay

Difference in the vascularization of the egg treated with the different semi crude extracts of the plant was assessed at a

Table 1. *Phytochemical screening of Z. japonica crude extracts*

	ZjH	ZjEA	ZjB
Alkaloids	+	++	+
Anthrones	++	+	++
Flavonoids	++	++	+
Phenols, triterpenes, sterols	+	++	++
Sugar	-	+	+

LEGEND: ++: strong positive test; +: low positive test; -: negative test

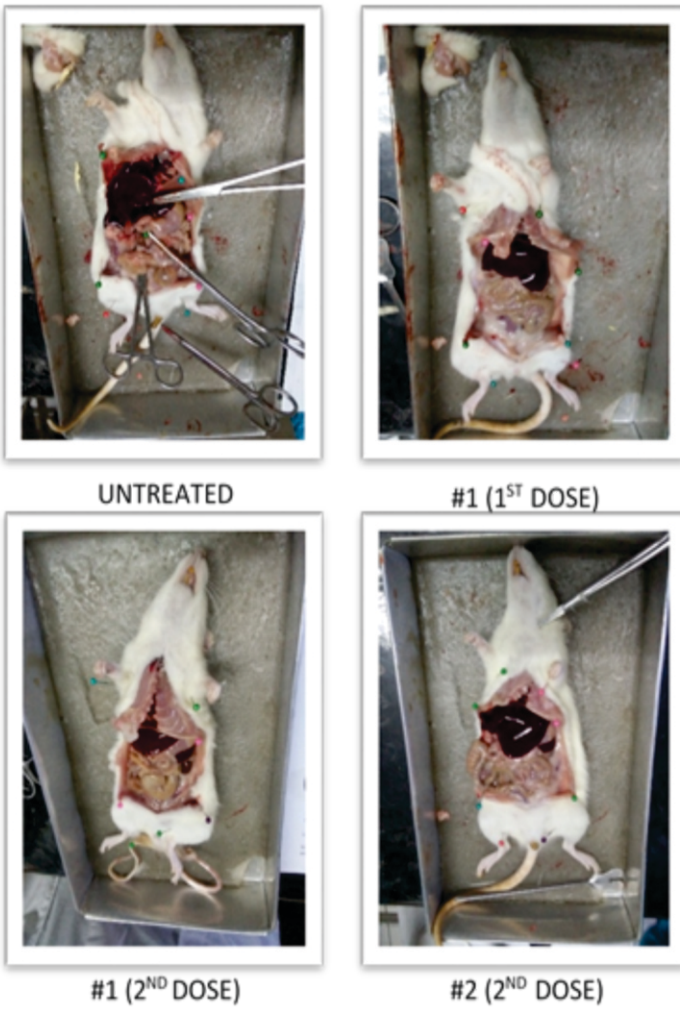


Figure 1. *Gross Necropsy of the female Sprague - Dawley rats for Acute Oral Toxicity*

macroscopic level in Figure 2. Representative chorioallantoic membrane of the samples incubated for 10 days at 38°C +/- 1°C of a humidified incubator with 1000 µg/disc concentration were chosen because at this dose level, the highest anti-angiogenic response was observed. There was an evident reduction in the number of blood vessels as compared with the negative control group, which indicated that the growth of new blood vessels had been prevented.

The means of groups were compared and analyzed using single factor analysis of variance (ANOVA). The treatment groups revealed different responses based on the mean blood vessel count as presented in Figure 3. The p-values of all the extracts are >0.05 thus considered as statistically insignificant (Table 2). This means that the three extracts have comparable angiosuppressive properties at 1000, 100 and 10 µg/mL respectively.

The data obtained from the analysis showed that all the extract exhibited a response that is comparable to the positive control, Celecoxib. Thus, the ZjH, ZjEA and ZjB in all the given concentrations showed anti-angiogenic effect. The graph in Figure 4 reveals a dose dependent activity for the percent inhibition of the three (3) extracts. This indicates a direct proportional relationship that implies that as the dose of a drug increases, the activity also increases. In the same figure, it has revealed that ZjB at 1,000 µg/mL/disc yielded the highest activity with 61.34% inhibition which is comparable with Celecoxib at 20 µg/mL/disc, with a 61.90% inhibition. At 1,000 µg/mL/disc concentration of all the extracts, ZjH indicated the least activity with 47.32% inhibition.

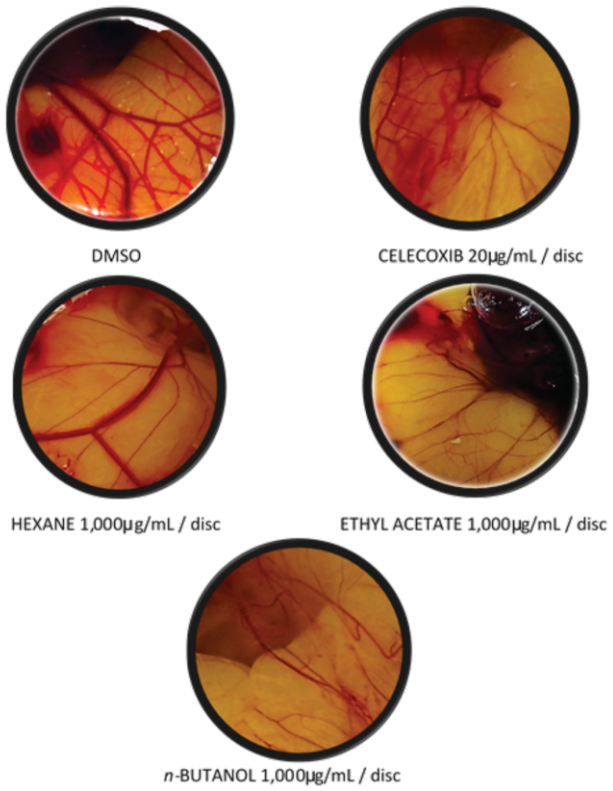
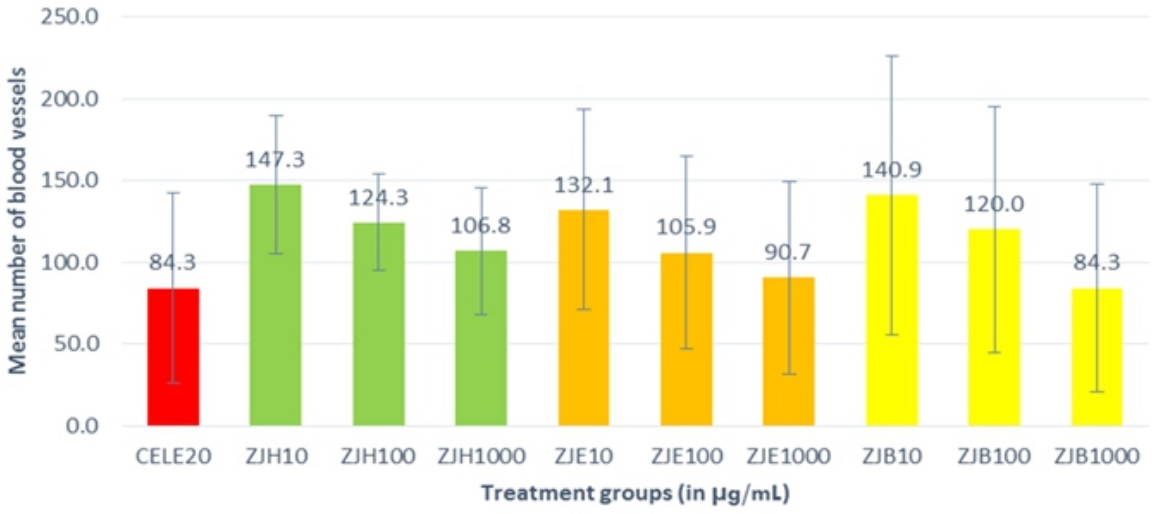


Figure 2. *Representative chorioallantoic membrane of the samples incubated for 10 days at 38°C +/- 1°C of a humidified incubator with 1000 µg/disc concentration.Sprouting of secondary blood vessels lessened and become thinner in the 1000 µg/disc of the extracts and in the positive control, Celecoxib when compared to DMSO. The resolution of the camera has a great impact in quantifying the blood vessels when using the AngioQuant™ to determine the Mean ± SD blood vessels.*



Legend: CELE - Celecoxib; ZJH - *Z. japonica* Hexane Extract; ZJE - *Z. japonica* Ethyl acetate Extract; ZJB - *Z. japonica* Butanol Extract

Figure 3. *The anti-angiogenic activity of Z. japonica extracts represented by mean blood vessel count using AngioQuant™*

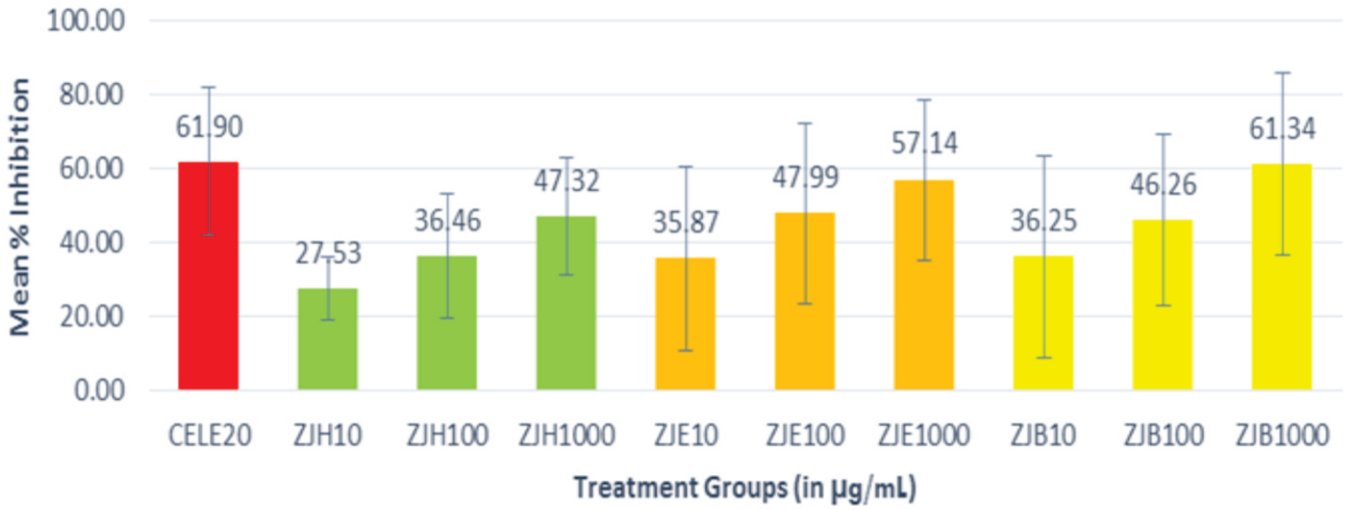
Table 2. *Percentage Inhibition of the Z. japonica extracts at Different Concentrations in CAM Assay.*

Concentration					
	10 ug/mL	100 ug/mL	1000 ug/mL	F stat	p-value
Hexane	27.53±4.29	36.46±8.50	47.32±7.88	1.929	0.201
Ethyl acetate	35.87±12.39	47.99± 12.29	57.14±10.85	0.808	0.476
Butanol	36.25±13.72	46.26±11.63	61.34±12.24	1.011	0.402

There is no significant differences in the mean percentage inhibition of hexane [F2,9 = 1.929, p=0.201], ethyl acetate [F2,9 = 0.808, p=0.476], and butanol [F2,9 = 1.011, p=0.402] at different concentrations. Moreover, all the mean percentage inhibitions of hexane [F2,9=1.929, p = 0.201, ethyl acetate [F2,9 = 0.808, p = 0.476], and butanol [F2,9=1.011, p = 0.402] at different concentrations did not differ with celecoxib.Values expressed as mean ± SEM, n = 4.

Table 3. *The IC_{50} values of the different extracts based on the four-parameter logistic regression model.*

Estimate			
	IC_{50}	95% Confidence Interval	R^2
Hexane	1810.0	156.7 – 20,907	0.922
Ethyl acetate	192.5	18.5 – 2,008	0.805
Butanol	147.7	19.0 – 1,148	0.789



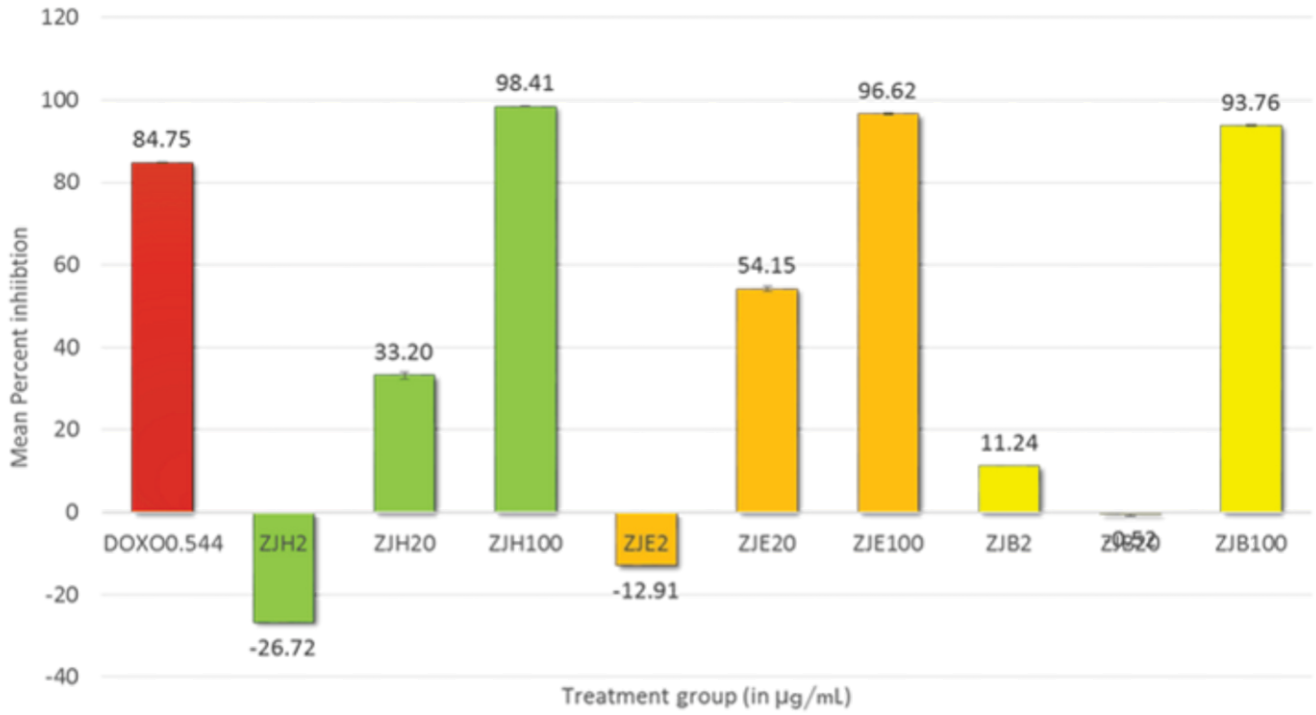
Legend: CELE - Celecoxib; ZJH - *Z. japonica* Hexane Extract; ZJE - *Z. japonica* Ethyl acetate Extract; ZJB - *Z. japonica* Butanol Extract

Figure 4. *The effect of Z. japonica extracts on angiogenesis "expressed as Mean Percent Inhibition".*

The IC_{50} estimates for the extracts were obtained using the four-parameter logistic regression model as shown in Table 3. Among the different extracts, hexane is the least potent with 1,810 µg/mL as compared to ethyl acetate (192.5 µg/mL) and n-butanol (147.7 µg/mL) which is the most potent.

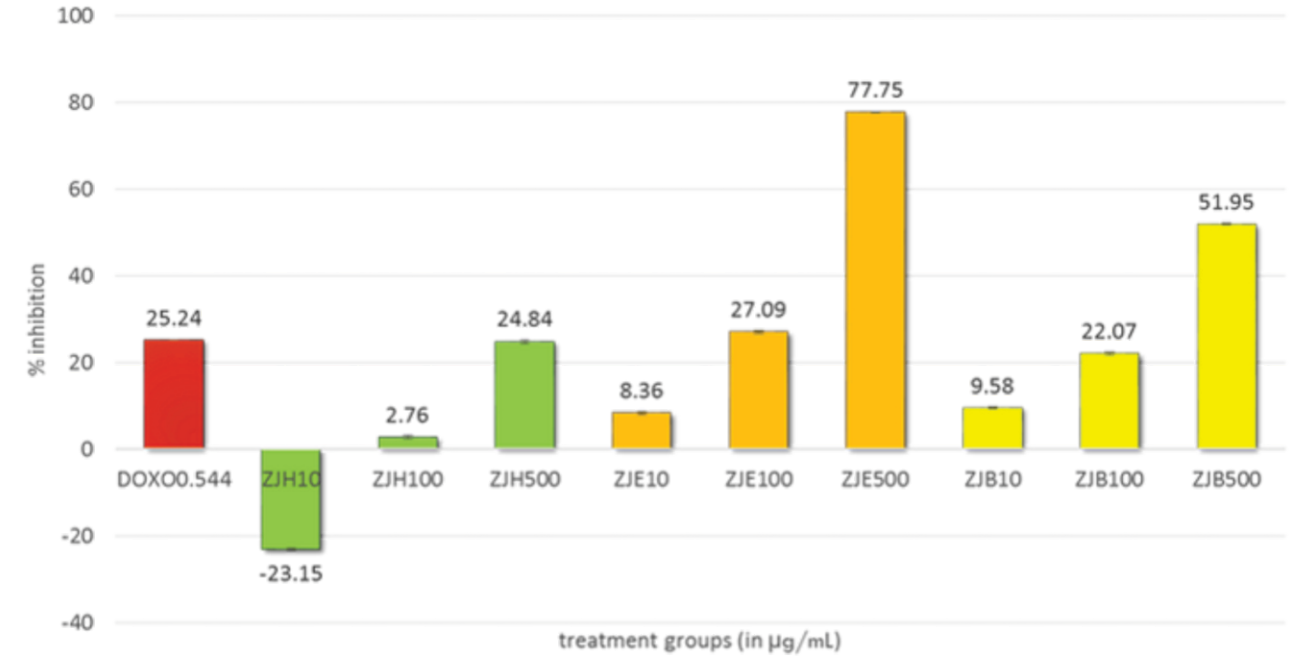
MTT Cytotoxicity Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is based on the principle that only viable and metabolically active cells can cleave the MTT dye to produce formazan crystals, which is dissolved and quantified using a spectrophotometer (20). Figure 5 shows the cytotoxic effect of *Z. japonica* extracts expressed as mean percent inhibition. Among the three extracts, *Z. japonic*a hexane extract in 100 µg/mL exhibited the highest inhibition with 98.41% followed by ethyl acetate extract in 100 µg/mL with 96.62%. In HepG2, the extracts exhibited a dose-dependent activity and ethyl acetate extract showed the highest inhibition with 77.75% much higher than the positive control and the two other extracts (Figure 6). And lastly, in HUVEC (normal cells), n-butanol extract exhibited the highest inhibition of 108.08% among the three extracts (Figure 7).



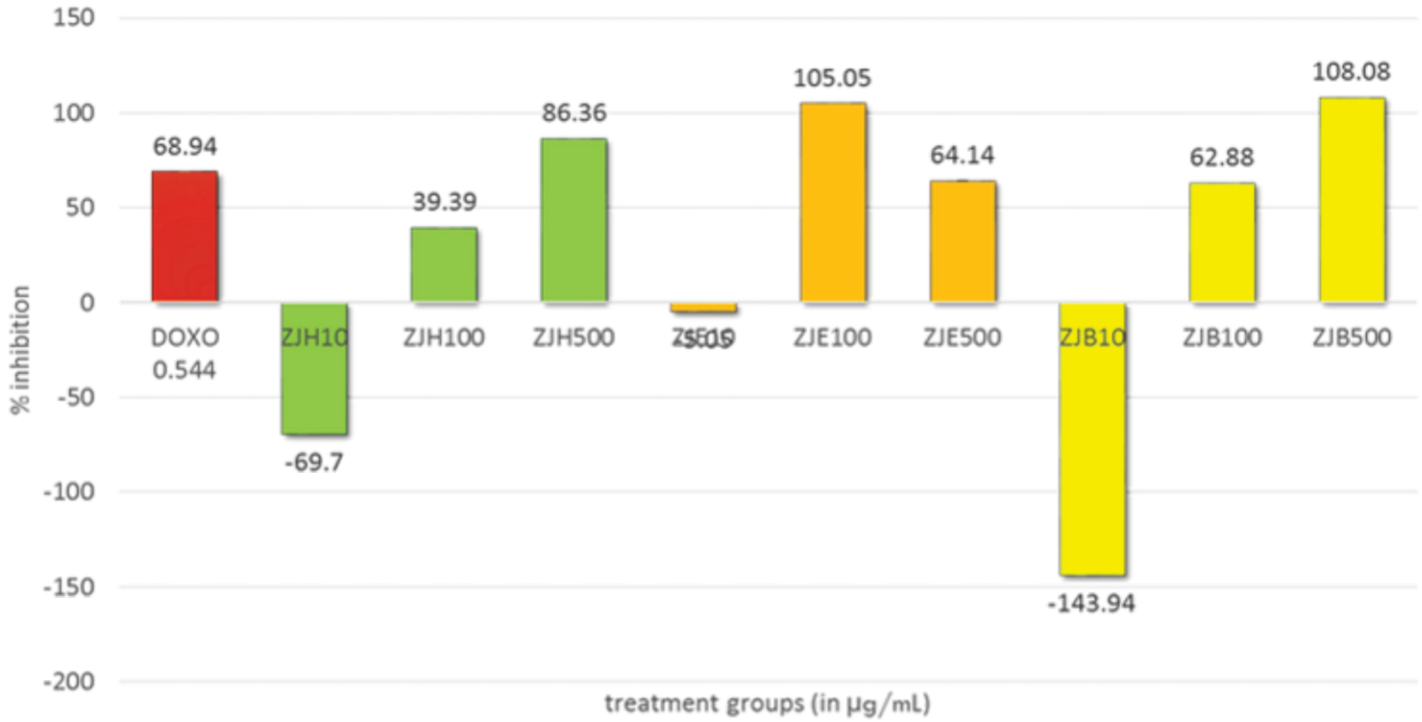
Legend: DOXO - Doxorubicin; ZJH - *Z. japonica* Hexane Extract; ZJE - *Z. japonica* Ethyl acetate Extract; ZJB - *Z. japonica* Butanol Extract

Figure 5. *Cytotoxic potential of Z. japonica crude leaf extracts against U20s cells expressed as Mean percent inhibition.*



Legend: DOXO - Doxorubicin; ZJH - *Z. japonica* Hexane Extract; ZJE - *Z. japonica* Ethyl acetate Extract; ZJB - *Z. japonica* Butanol Extract

Figure 6. *Cytotoxic potential of Z. japonica crude leaf extracts against HepG2 cells expressed as Mean percent inhibition.*



Legend: DOXO - Doxorubicin; ZJH - *Z. japonica* Hexane Extract; ZJE - *Z. japonica* Ethyl acetate Extract; ZJB - *Z. japonica* Butanol Extract

Figure 7. *Cytotoxic potential of Z. japonica crude leaf extracts against HUVECs cells expressed as Mean percent inhibition.*

Based on the statistical treatment, the mean percentage of the three crude extracts exhibited no significant difference (p-values <0.05) as compared with the positive control, Doxorubicin. However, comparison of the three extracts with their mean percentage in different concentrations significantly differ. For U2Os cells, the mean percentage of 2 µg/mL is significantly less (p-value <0.011) than 20 µg/mL, and that the mean percentage of the 100 µg/mL is significantly higher (p-value <0.001) than 20 µg/mL. For HepG2 cells, it was evident that the mean percentage of the three extracts significantly differ (p-value 0.037). The mean percentage inhibition of Doxorubicin, n-butanol and ethyl acetate extracts did not differ (p-value 0.430), and that the mean percentage inhibition of hexane is significantly the least (p-value <0.05). Additionally, for hexane, ethyl acetate and n-butanol extracts, the mean percentage of the different concentrations significantly differ (p-value <0.001), indicating that the mean percentage of 10 µg/mL is significantly less (p-value 0.011) than the 100 µg/mL, and that the mean percentage of the 500 µg/mL concentration is significantly higher (p-value <0.001) than the 100 µg/mL. Finally, for the HUVEC, there are no significant differences in the mean percentage inhibition of the three extracts and Doxorubicin. Yet, the mean percentage of the concentrations of the three extracts significantly differ (p-value <0.001), indicating that the mean percentage of 10 µg/mL is significantly less (p-value <0.001) than the 100 µg/mL and 500 µg/mL. The mean percentage inhibition of 100 µg/mL and 500 µg/mL did not differ (p-value 0.730).

The IC_{50} values of ZjH, ZjEA and ZjB for U2Os cells were 19.65 µg/mL, 9.89 µg/mL and 31.04 µg/mL respectively. Similarly, the $IC5_0$ values of ZjH, ZjEA and ZjB for HepG2 cells were 770.90 µg/mL, 130.10 µg/mL and 231.60 µg/mL respectively. Lastly, the IC_{50} values of ZjH, ZjEA and ZjB for HUVEC were 69.46 µg/mL, 42.23 µg/mL and 63.44 µg/mL respectively (Table 4).

Table 4. *In vitro cytotoxicity effects of Zehneria japonica extracts against U2Os, HepG2 and HUVEC.*

Test Extracts	ZJH			ZJE			ZJB		
Cells	U2Os	HepG2	HUVEC	U2Os	HepG2	HUVEC	U2Os	HepG2	HUVEC
IC_{50} (µg/mL)	19.65	770.90	69.46	9.89	130.10	42.23	31.04	231.60	63.44
R^2	0.813	0.867	0.560	0.825	0.886	0.441	0.653	0.884	0.731

In summary, the plant *Zehneria japonica* also possesses an anti-angiogenic activity that may be related to other studies of its related plant species under Cucurbitaceae [21,22]. The anti-angiogenic effect of *Z. japonica* may be attributed to phytochemical constituents that are prominent in the three extracts such as sterols, triterpenes, anthrones, flavonoids, and alkaloids.

The polyphenols exhibit anti-angiogenic effect and metastasis through the regulation of multiple signaling pathways. It may also influence some steps in tumor angiogenesis. Similarly, presence of triterpenoids and sterols have strong anticancer activity [23]. Among the three extracts, n-butanol extract exhibited the highest potency and inhibition in CAM assay comparable to the positive control, Celecoxib.

Cytotoxic compounds directly targeted cells and are utilized in chemotherapy in cancer patients. According to the United States National Cancer Institute, a sample or compound is considered cytotoxic if its IC_{50} value is ≤ 20 µg/mL. Based on the results, *Z. japonica* extracts exhibited strong cytotoxicity against U2Os cells with an IC_{50} values within 20 µg/mL except for n-butanol extract with 31.04 µg/mL, and no cytotoxic effect against HepG2 cells with an IC50 values of >100 µg/mL. The extracts exhibited mild cytotoxicity against HUVEC with IC_{50} values of 40-60 µg/mL. Among the three extracts, ethyl acetate extract has the highest IC_{50} values based on the three cell lines and is considered the most potent among the extracts. Since it exhibits activity against the cancer and normal cells, n-butanol extract is much safer to use than ethyl acetate because its IC_{50} values revealed to be in the middle when compared to hexane and ethyl acetate extracts.

Presence of terpenoids and flavonoids are attributed to the cytotoxic activity of *Z. japonica*. Flavonoids, specifically, are reported to have cytotoxic activity due to presence of phenolic groups while terpenoids, prevent the cancer cell growth and size reduction [24,25].

Conclusion

The present results suggest that *Z. japonica* leaf extracts have a cytotoxic and angiosuppressive effect. Phytochemical screening through TLC revealed that the extracts contain alkaloids, anthrones, flavonoids, and sterols. Further studies should evaluate the in vitro and in vivo benefits of *Z. japonica* extracts.

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