International Journal of Biomolecules and Biomedicine (IJBB)
ISSN: 2221-1063 (Print), 2222-503X (Online)
http://www.innspub.net
Vol. 13, No. 1, p. 1-8, 2021

RESEARCH PAPER OPEN ACCESS

Antiangiogenic activity of *Coccinia grandis* ethanolic leaf extract using the chorioallantoic membrane assay in *anas platyrhynchos* embryos

Vivian C. Peligro*

Agusan del Sur State College of Agriculture and Technology, Bunawan, Agusan del Sur, Caraga, Philippines

Key words: Antiangiogenic, Chorioallantoic membrane, Cancer, *Coccinia grandis*, Duck embryo

Article Published: 30 July 2021

Abstract

Vascular growth is vital for every aspect of tumor growth. *Coccinia grandis* is one of the medicinal plants in many countries because of its diverse ethnomedicinal uses. Hence, this study aimed to evaluate the antiangiogenic activity of *C. grandis* ethanolic leaf extract using the Chorioallantoic Membrane Assay (CAM) in *Anas platyrhynchos*. Thirty pieces of six-day-old duck eggs were used in the study with triplicates in each treatment namely: positive control (vitamin A), negative control (ethanol), and *C. grandis* ethanolic extracts in 1µg/mL, 3µg/mL, and 5µg/mL. These were then applied to six-day old duck embryos and were incubated at 37°C with 65.5% humidity. The eggs were then harvested on the tenth day of incubation. The antiangiogenic effect of *C. grandis* ethanolic leaf extract was evaluated by taking the average number of branch points using the chorioallantoic membrane. Results revealed that in negative control angiogenesis was induced while in the different treatments of the ethanolic leaf extracts, inhibition reduced significantly. Statistical analysis supports that there was a significant difference in the antiangiogenic effect of *C. grandis* ethanolic leaf extract using the CAM assay on the vascularization of duck embryos. It showed that the higher the dosage, the lesser the branch points and the smaller the ducks. Further, *C. grandis* ethanolic leaf extract contains alkaloids and tannins which are responsible for the anti-angiogenic property and antiproliferative effects of the extract. Thus, it indicated that *C. grandis* ethanolic leaf extract may have a potential and promising source of chemotherapeutic agent against tumors.

***Corresponding Author:** Prof. Vivian C. Peligro ✉ vpeligro@asscat.edu.ph

Introduction

Coccinia grandis (L.) J. Voigt, commonly known as ivy gourd and the scarlet gourd is native in North-Central East Africa. It has been introduced and naturalized in different parts of tropical Asia, Pacific, and Americas (Chun 2001). It also occurs as a naturalized weed in the degraded land, fields and roadsides of Fiji (Smith, 1981). In addition, agricultural and residential areas were being covered by this plant (Chun 2001).

Many traditional medicinal practitioners from many countries considered *C. grandis* a medicinal plant because of its diverse ethnomedicinal uses. *C. grandis* is well-known for its several medicinal uses namely anti-diabetic, anti-obesity, antimicrobial, antifungal, antileishmanic, antioxidant, antihypertensive, antitussive, antiulcer, analgesic, antipyretic, antianaphylactic, and anti-cancer properties (Kirtikar and Basu, 1994; Yadav *et al*., 2010; Tamilselvan *et al*., 2011; Pekamwar *et al*., 2013; Gill *et al*., 2014; Nagare *et al*., 2015; Sakharkar *et al*., 2017). In addition, *C. grandis* ethanol leaf extract produced significant in vivo anticancer activity (Bhattacharya *et al*., 2011). This was validated by numerous scientific reports on phytochemical constituents and pharmacological activities of the plants (Monalisa *et al*., 2014). Its leaf extract was found to have alkaloids, flavonoids, glycosides, saponins, sterol and tannins (Alamgir *et al*., 2014) which suggests that the plant can be a candidate for further studies towards isolation of efficacious therapeutic agents (Monalisa *et al*., 2014).

Choriollantoic membrane (CAM) model is widely used research tool in studying the morpho-functional aspects of angiogenesis in vivo and investigating the activity of pro-angiogenic and anti-angiogenic molecules due to its extensive vascularization (Ribatti, 2012). It is also suitable for tumor engraftment to study various aspects of the angiogenic and metastatic potential of human malignancies (Ribatti, 20012; Lokman *et al*., 2012) such as glioma (Westhoff *et al*., 2013; Warnock *et al*., 2013; Miranda *et al*., 2013), colorectal cancer (Ankri *et al*., 2013; Subauste *et al*., 2009), leukemia (Taizi *et al*., 2006), ovarian cancer ((Lokman *et al*., 2012), prostate cancer (Wittig *et al*., 2011; Conn *et al*., 2009), and osteosarcoma (Balke *et al*., 2010) because of its fully developed immune system in the chick embryo (Ribatti, 2012; Palmer *et al*., 2011).

Moreover, angiogenesis is the development of new capillary blood vessels in the body (Vinoth Prabhu *et al*., 2011) that sprouts from endothelium, where there is endothelial cell migration, tube formation and proliferation (Lokman *et al*., 2012). Seventy-two to ninety-six hours (72-96 hr), the reaction was observed after stimulation of the increasing vessel density around the implant, with the blood vessels radially joining onto the center like spokes in a wheel (Tufan, Satiroglu-Tufan, 2005). Hence, there is a flareup in the field of research for potential anti-angiogeniic agents because of the success of anti-angiogenic therapy for treating cancer (Lokman *et al*., 2012). Diseases such as cancer is an example of wherein extreme angiogenesis occurs. Thus, natural products have been discovered as angiogenesis inhibitors to treat cancer (VinothPrabhu, 2011). Hence, this study aimed to evaluate the anti-angiogenic property of *C. grandis* ethanolic leaf extract using duck embryo CAM assay.

Materials and method

Plant Extraction

The leaves of *C. grandis* were collected in San Teodoro, Bunawan, Agusan del Sur. The collected leaves were then air-dried for one (1) week. It was then powdered mechanically by the use of sterile blender and then stored in an air-tight container. At room temperature, the one hundred grams (100g) of the fine, dried powdered leaves was macerated in 1000mL of aqueous ethanol for forty-eight (48) hours with intermittent shaking. The extract was then filtered using an ordinary filter paper. It was then concentrated by the used of rotary evaporator at 60^{o}C and were placed in a water bath until crude extract is produced (Muslim *et al*., 2012).

Phytochemical Screening

The crude leaf extract of *C. grandis* was brought to the Department of Science and Technology (DOST) Laboratory, Butuan City.

Standard procedures and protocols were followed in screening and identifying the phytochemical constituents qualitatively (Fajardo *et al.*, 2015).

Preparation of Duck Eggs

Six-day-old *Anas platyrhynchos* eggs were used for the experiment. The eggs were cleaned using the 70% ethanol. This was done to ensure that the eggs were free from any dirt that could infect it when opened. The eggs were then incubated at 37°C and about 65.5% humidity. Six (6) fertile 6-day-old duck eggs were used for each treatment namely negative control, positive control, 1µg/mL, 3µg/mL, and 5µg/mL.

To check the viability of the eggs, candling experiment was done before the treatment. The eggs with underdeveloped and even dead embryos were discarded. There are three (3) treatments of the concentrated extracts that were assigned as experimental group such as: 1, 3, and 5µg/mL. For the negative and positive control, ethanol and retin as a source of retinoic acid, Vitamin A, were used respectively. To expose the CAM, about 1x1cm window in the shell was made to direct access for experimental manipulation. A parafilm was used to seal the treated eggs. The eggs were then incubated at 37°C with a humidity of 65.5% for four (4) days. The CAMs were harvested on the 10th day of incubation by removing the hard shell withdrawing intact the soft membrane covering the embryo (Tantiado and Tan, 2012).

At the site of application, the chorioallantoic membranes were examined. Two (2) days after implantation, quantitation was performed and the counting of the number of CAM vessels in the area was done under the stereomicroscope. In response to the proangiogenic stimuli, the newly formed blood vessels come out converging toward the disk in a wheel-spoke pattern. The lack of new blood vessel formation and sometimes in the disappearance of pre-existing vessel networks is the result of inhibition of angiogenesis by antiangiogenic compounds (Tantiado and Tan, 2012). Four (4) quadrants were made in the area of the CAM and these were then photographed for the measurement of branching frequency, (Norby, 1998).

In a clockwise direction, the blood vessel branch points were counted manually at each area of the different quadrants (Tantiado and Tan, 2012).

Morphometric Analysis

The weights of the embryos were measured using a digital balance. A Vernier caliper was also used to measure the morphometry of every embryo that was used for the experiment. The indices that follows were measured: (1) Crown-rump-length (CRL), describes as the distance from the crown, the skull vertex, to the midpoint between the rump of the apices of the buttocks; (2) Head beak length (HBL), it is the distance from the back of the head of the embryo to the tip of the beak; (3) Forelimb length (FL), it is the measurement between where the forelimb is connected and the tip of the forelimbs; (4) Hind limb length (HL) it is the distance between where the hind limb is connected and the tip of the recognizable hind limb; and (5) Beak length (BL).

Statistical Analysis

One-Way Analysis of Variance (ANOVA) was used to compare the two groups such as vascular densities and morphometry. Differences with $P<0.05$ between experimental groups were considered statistically significant.

Results and discussions

Phytochemical Screening

Results revealed that of *C. grandis* leaf extract has the following phytochemical properties namely secondary alkaloid, steroids, flavonoids, saponins, and tannins (table 1). Alkaloids have been reported to possess analgesic, antispasmodic and bactericidal, antimalarial and analgesic activities (Alamgir *et al.*, 2014). Steroids are substances that are good as anti-tumor, immunosuppressive, hepatoprotective, antibacterial, cytotoxic and cardiotonic activity. On the other hand, flavonoids are proven to be a good antioxidant and antibacterial (Zannah *et al.*, 2017). Flavonoids can alter enzymatic and chemical reactions, and thus it has an impact on human health positively or negatively (Beecher, 2003). While saponins are found to be good plant in fungal problems (Osbourn, 1996).

Plant tannins give the plant medical properties such as antimicrobial, anti-inflammatory, antioxidant and astringent actions (Periera, 2015). Moreover, plants usually contain phytochemicals that provide health benefits for humans. These chemical compounds will detoxify substances causing cancer. Free radicals tend to be neutralized; and enzymes that activate carcinogens will be inhibited (Saxena *et al.*, 2013).

Table 1. Phytochemical Properties of *Coccinia grandis*.

Tests				
Alkaloids	Steroids	Flavonoids	Saponins	Tannins
++	+	+	+	+

Legend: ++ secondary

+ means presence of active constituents

- means absence of active constituents

Branching Points of the CAM

Table 2 and Fig. 1 showed the comparison of the average branching points of the CAM upon application of the different treatments of *C. grandis* ethanolic leaf extract together with the positive control (Vitamin A) and negative control (ethanol). It was observed that the negative control showed the highest number of blood vessel branch points (106.33±4.55522) while 5µg/mL had the lowest (12.06±5.85947). The data further showed that the average branching points of the CAM decreased significantly as the dosage increases. This implied that the highest dosage has the greatest inhibition property. This means that it has an anti-angiogenic property (Fajardo *et al.*, 2015).

Moreover, the harvested samples treated with *C. grandis* showed many pathologies which have corresponded with the vascular densities of the chorioallantoic membrane. The growth of chorioallantoic capillaries was disrupted, and the capillaries and veins were irregularly branched and thin. Furthermore, negative control showed induced angiogenesis while there was a significant reduced in the inhibition of angiogenesis in the different treatments of *C. grandis* ethanolic leaf extracts using the CAM assay on the vascularization of duck embryos (table 1, Fig.s 1, Fig. and 2).

Other plants have many active ingredients that affect tumor angiogenesis (Keshavarz *et al.*, 2011;). *C. grandis* ethanolic leaf extract contains alkaloids and tannins which are responsible for the anti-angiogenic property and antiproliferative effects of the extract (Fajardo *et al.*, 2015; Stangl *et al.*, 2007; Karagiz *et al.*, 2007; Kampa *et al.*, 2007).

Table 2. Average Branch Points of the CAM.

Treatment	Branching Points
+C	90.83 ± 0.316228^{a}
-C	106.33 ± 4.55522^{a}
1µg/mL	76.56 ± 4.40265^{a}
3µg/mL	43.06 ± 14.8593^{a}
5µg/mL	12.06 ± 5.85947^{a}

mean±std. error of the different treatment.

Superscript *a* means significantly different from each other ($P<0.05$).

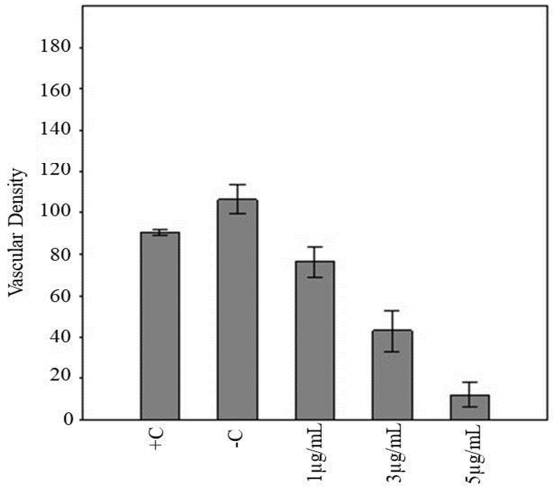

Fig. 1. Vascular Densities of different treatments at $P<0.05$. The angiogenesis was induced in the negative control while the angiogenesis inhibition had been strongly reduced upon the treatment of *C. grandis* ethanolic leaf extract. Results are presented as mean ±SEM.

Morphometry Analysis

Results revealed that there is a significant difference ($P<0.05$) on the weights and sizes of the duck embryos on the different treatments of *C. grandis* ethanolic leaf extracts (Table 3 and Fig. 3). Further, duck embryos in controlled treatments (positive and negative) were bigger compared to the duck embryos treated with *C. grandis* ethanolic leaf extracts. Generally, it is observed further that the higher the dosage, the smaller the duck embryos.

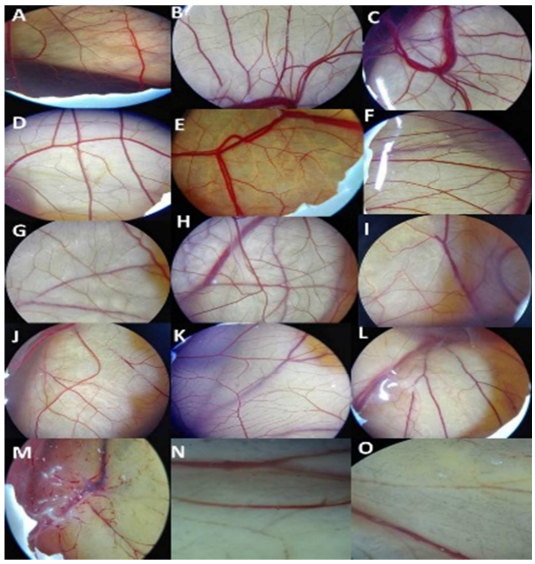

Fig. 2. Blood vessels of different treatments. A-C. Duck eggs that were treated by positive control show high branching points. D-F. Duck eggs that were treated negative control shows very high branching points. G-I. Duck eggs that were treated with 1mg/mL *C. grandis* ethanolic extracts show low branching points. J-L. Duck eggs that were treated with 3mg/mL *C. grandis* ethanolic extracts shows slightly low branching points. M-O. Duck eggs that were treated with 5mg/mL *C. grandis* ethanolic extracts shows almost no branching points

Table 3. Summary of the morphometry with its mean±std. error of the different treatment. Superscript *a* means significantly different from each other (P<0.05).

Treatment	BW (g)	CRL (mm)	HBL (mm)	FL (mm)	HL (mm)	BL (mm)
+C	2.65±0.949883^a	31.50 ±1.6667^a	27.17 ±2^a	11.50 ±1.09545^a	13.67 ±0.557773^a	7.67 ±0.333333^a
-C	2.21 ±0.193468^a	36.83 ±1.05409^a	28.50 ±0.703167^a	9.83 ±0.428174^a	10.83 ±0.881917^a	8.67 ±0.401386^a
1µg/mL	1.82 ± 0.199527^a	28.67 ±2.30217^a	25.33±1^a	8.83±0.57735^a	9.67 ±0.509902^a	6.17±0.374166^a
3µg/mL	1.60 ±0.066458^a	24.50±0.894427^a	23.33 ±0.957427^a	8.67±0.316228^a	8.33 ±0.25^a	5.17 ±0.547723^a
5µg/mL	1.29±0.0807465^a	20.50 ±1.18145^a	20.83 ±0.4^a	7.33 ± 0.25^a	6.83 ±2.43721^a	4.17±0.288675^a

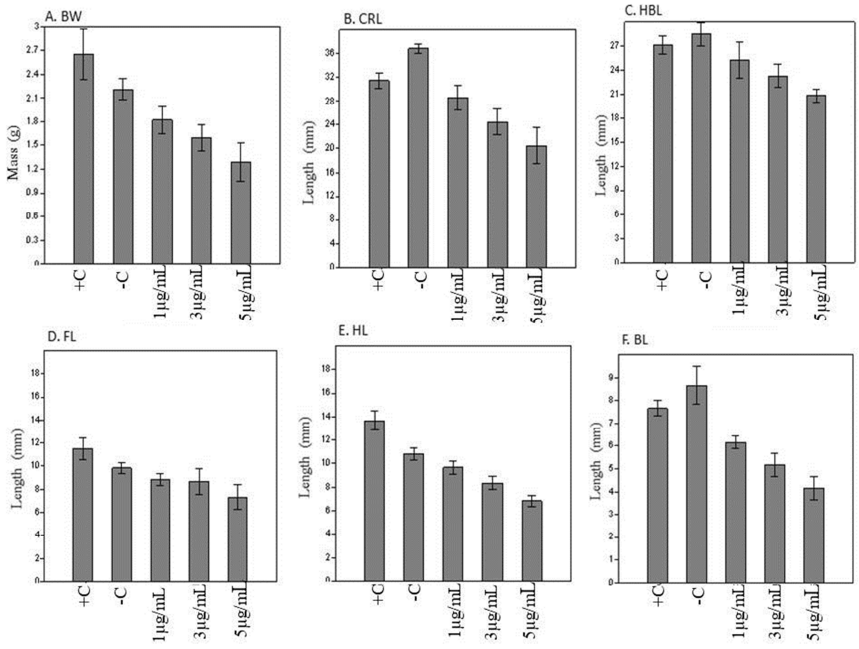

Fig. 3. Morphometry in millimeters of the embryos with the different treatments at P<0.05. A) BW- Body weight; B) CRL-Crown rump length; C) HBL-Head beak length; D) FL-forelimb length; E) HL-Hindlimb length and F) BL-Beak length. Results are presented as mean ±SEM.

Conclusion

The duck chorioallantoic membrane assay was used for examining the antiangiogenic activity of *C. grandis*. Results indicated that *C. grandis* in a dose-dependent manner inhibits angiogenesis. In this manner, the plant as a source of the new chemical substance that can inhibit angiogenesis is being actively explored. It has been observed that *C. grandis* leaf extract inhibits the development of capillary networks in CAM significantly. Moreover, duck embryos were smaller as the dosage got higher. Furthermore, *C. grandis* ethanolic leaf extract contains alkaloids and tannins which are responsible for the anti-angiogenic property and antiproliferative effects of the extract. Hence, based on the findings of the study it is indicated that *C. grandis* ethanolic leaf extract may have a potential and promising source of chemotherapeutic agent against tumors.

References

Alamgir AN, Minhajur Rahman, Ataur Rahman M. 2014. Phytochemical Characteristics, Antimitotic, Cytotoxic and Antiinflamatory Activities of *Coccinia grandis* (L.) J. Voigt. Journal of Pharmacognosy and Phytochemistry **3(1),** 222-225.

Ankri R, Peretz D, Motiei M, Sella-Tavor O, Popovtzer R. 2013. New optical method for enhanced detection of colon cancer by capsule endoscopy. Nanoscale **5,** 9806-11.

Balke M, Neumann A, Kersting C 2010. Morphologic characterization of osteosarcoma growth on the chick chorioallantoic membrane. BMC research notes **3,** 58.

Beecher GR. 2003. Overview of Dietary Flavonoids: Nomenclature, Occurrence and intake. The Journal of Nutrition **133(10),** p. 3248-3254.

Bhattacharya B, Pal P, Lalee A, Mal DK, Samanta A. 2011. In vitro anticancer activity of Coccinia grandis (L.) Voigt. (Family: Cucurbitaceae) on Swiss albino mice. J Pharm Res **4,** 567-569.

Chun ME. 2001. Biology and host specificity of Melittia oedipus (Lepidoptera: Sesiidae), a biological control agent of Coccinia grandis (Cucurbitaceae). Proceedings of the Hawaiian Entomological Society **35,** 85-93.

Conn EM, Botkjaer KA, Kupriyanova TA, Andreasen PA, Deryugina EI, Quigley JP. 2009. Comparative analysis of metastasis variants derived from human prostate carcinoma cells: roles in intravasation of VEGF-mediated angiogenesis and uPA-mediated invasion. The American journal of pathology **175,** 1638-52.

Fajardo WT, Cancino LT, Dudang EB, Suratos ECM, Macayana FB. 2015. Phytochemical Analysis and Antiangiogenic Potential of Gmelina Arborea Roxb. (Paper Tree) Fruit Exocarp Using Duck Chorioallantoic Membrane (Cam) Assay. Asia Pacific Journal of Multidisciplinary Research, **Vol. 3, No. 5,** P-ISSN 2350-7756 | E-ISSN 2350-8442 | www.apjmr.com

Gill NS, Kaur P, Arora R. 2014. Review on Coccinia cordifolia auct. Non (L) Cogn. Int J Adv Pharmaceutical **5,** 234-241.

Kampa M, Nifli A-P, Notas G, Castanas E. 2007. Polyphenols and cancer cell growth. PubMed [17] Bravo L. Polyphenols: Chemistry, dietary source.

Karagiz AN, Turgut-kara O, Cakir R, Demirgan S. 2007. Ari. Cytotoxic activity of crude extracts from Astragalus chrysochlorus (Leguminosae). Biotechnol and Biotechnol **21(2),** 220-222.

Keshavarz M, Bidmeshkipour A, Mostafaie K, M-Motiagh HR. 2011. Anti-tumor activity of Salvia officinalis is due to its angiogenic, antimigratory and antiproliferative effects. Cell Journal, **vol. 12 no. 4,** winter pp. 477.

Lokman NA, Elder AS, Ricciardelli C, Oehler MK. 2012. Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis. International journal of molecular sciences **13,** 9959-70.

Miranda-Goncalves V, Honavar M, Pinheiro C. 2013. Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets. Neuro-oncology **15,** 172-88.

Monalisa MN, Al-Nahain A, Rahmatullah M. 2014. *Coccinia grandis*: a plant with multiple ethnomedicinal uses. World journal of pharmacy and pharmaceutical sciences. **Volume 3, Issue 9,** 1382-1394.

Muslim NS, Nassar ZD, Aisha A, Shafei A, Idris N, Majid AM. 2012. Antiangiogenesis and antioxidant activity of ethanol extracts of *Pithecellobium jiringa*. BMC Complementary and Alternative Medicine **12,** 210.

Nagare S, Deokar GS, Nagare R, Phad N. 2015. Review on *Coccinia grandis* (L.) voigt (Ivy Gourd). World J Pharm Res **4910,** 728-743.

Osbourn A. 1996. Preformed Antimicrobial Compounds and Plant Defense Against Fungal Attack. Retrieved on November 16, 2018 fromdwww.cell.com/trends/plant-science/abstract/S1360-1385 (96)80016-1

Palmer TD, Lewis J, Zijlstra A. 2011. Quantitative analysis of cancer metastasis using an avian embryo model. Journal of visualized experiments: JoVE.

Pekamwar SS, Kalyankar TM, Kokate SS. 2013. Pharmacological activities of Coccinia grandis - a review. J App Pharm Sci **3,** 114-119.

Periera A, Vieira G, Mateus Santana M, Biondoro Gois and D.m. Goncales Sant'Ana. 2015. Tannins obtained from medicinal plants extracts against pathogens: antimicrobial potential. The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs (A. Méndez-Vilas, Ed.) p. 228-235.

Ribatti D. 2012. Chicken chorioallantoic membrane angiogenesis model. Methods in molecular biology **843,** 47-57.

Sakharkar P, Chauhan B. 2017. Antibacterial, antioxidant and cell proliferative properties of Coccinia grandis fruits. Avicenna J Phytomed, Epub ahead of print.

Saxena J, Rajeev N, Dharmendra S, Abhishek Gupta. 2013. Phytochemistry of Medicinal Plants. Journal of Pharmacognosy and Phytochemistry **1(6),**168.

Smith AC. 1981. Flora Vitiensis Nova: A New Flora of Fiji. **Vol. 2.** Lawai, Kauai, HI: National Tropical Botanical Garden, 810 pp.

Stangl V, Dreger H, Stangl K, Lorenz M. 2007. Molecular targets of tea polyphenols in the cardiovascular system. Cardiovasc. Res **73(2),** 348- 358.

Subaustemc, Kupriyanova TA, Conn EM, Ardi VC, Quigley JP, Deryugina EI. 2009. Evaluation of metastatic and angiogenic potentials of human colon carcinoma cells in chick embryo model systems. Clinical & experimental metastasis **26,**1033-47.

Taizi M, Deutsch VR, Leitner A, Ohana A, Goldstein RS. 2006. A novel and rapid in vivo system for testing therapeutics on human leukemias. Experimental hematology **34,** 1698-708.

Tamilselvan N, Thirumalai T, Elumalai EK, Balaji R, David E. 2011. Pharmacognosy of Coccinia grandis - a review. Asian Pac J Trop Biomed **1,** S299-S302.

Tantiado RG, Tan VP. 2012. Evaluation of the Angiosuppresive Activity of *Tinospora rumphii* Boerl. Stem Extract Using the Chorioallantoic Membrane Assay in *Anas platyrhynchos* Embryos. International Journal of Bio- Science and Bio-Technology **4(2),** 93-102.

Tufan AC, Satiroglu-Tufan NL. 2005. The chick embryo chorioallantoic membrane as a model system for the study of tumor angiogenesis, invasion and development of anti-angiogenic agents. Current Cancer Drug Targets **5,** 249-2664.

Vinoth Prabhu V, Chidambaranathan N, Gopal G. 2011. A Historical Review on Current Medication and Therapies for Inducing and Inhibiting Angiogenesis. J. Chem. Pharm. Res **3(2),** 526-533.

Warnock G, Turtoi A, Blomme A. 2013. In vivo PET/CT in a human glioblastoma chicken chorioallantoic membrane model: a new tool for oncology and radiotracer development. Journal of nuclear medicine: official publication, Society of Nuclear Medicine **54,** 1782-8.

Westhoff MA, Zhou S, Nonnenmacher L. 2013. Inhibition of NF-kappa B signaling ablates the invasive phenotype of glioblastoma. Molecular cancer research: mcR **11,**1611-23.

Whistler WA. 1995. Wayside Plants of the Islands. Hong Kong: Everbest Printing Company.

Wittig-Blaich SM, Kacprzyk LA, Eismann T. 2011. Matrix-dependent regulation of AKT in Hepsin-overexpressing PC3 prostate cancer cells. Neoplasia **13,** 579-89.

Yadav G, Mishra A, Tiwari A. 2010. Medical properties of Ivy Gourd (Cephalandra indica) – a review. Int J Pharm Res Dev **2,** 92-98.

Zannah F, Amin M, Suwono H, Lukiati B. 2017. Phytochemical screening Diplazium esculentum as medical plant from Central Kalimantan, Indonesia. In AIP Conference Proceedings (**Vol. 1844, No. 1,** p. 050001). AIP Publishing.