Carmona Retusa (Tsaang gubat) Roots and Stem Methanolic Extract: Anti-Angiogenic Effect

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Abstract - *Cancer is one of the leading causes of death worldwide. Angiogenesis is a factor for cancer progression and most researches focuses on anti-angiogenesis to inhibit the metastasis of cancer. The purpose of the study was to screen the anti-angiogenic effect of Carmona retusa roots and stem crude methanolic extract using chorioallantoic membrane assay on fertilized duck embryo. A total of 7 mL were obtained from the stem extract and 3 mL for the roots extract and were diluted with distilled water obtaining concentrations of 25 g/mL, 50 g/mL, 75 g/mL and 100 g/mL. Experimental design was used in the study. Treatments were prepared in three trials. Extracts were applied in ovo without breaking the chorioallantoic membrane (CAM) of duck embryo. Blood vessels were viewed under a stereomicroscope and photomicrographs were taken. Branching points were counted through NIH Image J software and results were submitted to a Two-way analysis of variance. Study showed that C. retusa roots and stem extract do not have significant difference F(1,21)=.016=p=.960 in anti-angiogenic effect. Administration of C. retusa roots and stem in different concentration showed significantly F(3,21) =6.029=p=.004 anti-angiogenic effect on the branching points of the blood vessels. A higher concentration of the extract (roots and stem) would have a greater effect in lessening the blood vessel on the CAM of fertilized duck embryo. C. retusa roots and stem methanolic extract demonstrates to have an anti-angiogenic potential.*

Keywords: *Angiogenesis, Chorioallantoic membrane assay, NIH Image J, methanolic extract*

INTRODUCTION

Many people fear cancer compared to other diseases due to its increasing number of deaths worldwide. Cancer is the second leading cause of death globally and was responsible for 8.8 million deaths in 2015 [1]. Nearly 1 in 6 deaths is due to cancer. Experts are becoming more determined to find treatments and other risk managements to suppress the disease and its factors. Ribatti [2] mentioned that cancer is capable of spreading to nearby organs which makes it life threatening. Growth of the vascular network is important so that metastasis will take place. The process by which development of new blood vessels occur is angiogenesis [3], [4]. There are studies showing that angiogenesis itself and some activators make it a risk factor for cancer progression [5] - [8].

Angiogenesis means development of a new blood vessel from a pre-existing one [9]. It is a normal process that takes place inside in the body that plays an important role in organogenesis and it helps to improve embryonic and fetal progression [4]. Angiogenesis has a big part in cancer because cancers require the formation of new blood vessels in order for the cancer cells to grow and metastasize [10]. Cancer secretes a substance that helps stimulate angiogenesis where the growth of cancer begins. As the cancer grows, the oxygen level is adequate that causes the secretion of angiogenic growth factor molecules that will attach to the receptors of an endothelial cell nearby a blood vessel that initiates a tumor angiogenesis [11]. Vascular endothelial growth factor (VEGF) is the most significant angiogenic growth factor that is secreted by tumour cells [12], [13], [14]. Nowadays, anti-angiogenic therapy is considered as a cancer treatment besides surgery, chemotherapy and radiotherapy [15].

Anti-angiogenesis can be an alternative pathway to suppress or to treat cancer because it blocks the growth of blood vessels that support tumour growth rather than blocking the growth of tumour cells themselves [16], [17]. In some cancers, anti-angiogenesis appears to be most effective when combined with additional therapies [18]. Because angiogenesis inhibitors work by slowing or stopping tumor growth without killing cancer cells, they are given over a long period [19].

Certain plants have chemicals that can affect angiogenesis. *Carmona retusa* commonly known as *Tsaang gubat* is approved by DOH as stomachic. It is a plant that has many useful properties such as anti-microbial, anti-inflammatory and anti-mutagenic. Retusa [20] mentioned the phytochemical analysis of Tsaang gubat indicates that it has alkaloids, carbohydrates, tannins, phenols, alcohol and sterols that can contribute to an anti-angiogenic effect. Specifically it also has squalene that is triterpene in nature which gives different activities such as antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemopreventive, lipoxygenase inhibitor andanti HIV [21]. The implication of the study was to assess and evaluate the angiogenic activity of *C. retusa* on chorioallantoic membrane of duck embryo. This will help as an alternative way to decelerate the spread and growth of tumour or cancer cells with minimal side effects in the body. Furthermore to avoid the painful aftermath of chemotherapy and other radiation therapies involved in treating cancer related diseases.

OBJECTIVES OF THE STUDY

The study identified which part of *C. retusa* will have a significant reduction on the growth of blood vessels formation in the CAM of fertilized duck embryo. It determined the concentration of the plant roots and stem crude methanolic extract that will exhibit a significant reduction in blood vessel formation and also determined the average number of branching points of blood vessel after inoculation of the roots and stem crude methanolic extract.

MATERIALS AND METHODS

Experimental method was used in the study. One hundred (100) fertilized duck eggs purchased from Calamba City, Laguna were utilized and treated with *C. retusa* roots and stem methanolic extract. The plant was authenticated from the Bureau of Plant Industry. The point of branching of the blood vessel formation in sodium dodecyl sulphate served as the negative control compared with 25, 50, 75 and 100 g/mL concentrations of the roots and stem extract.

Procedure

A1. Preparation of *C. retusa* stems methanolic extract (Department of Science and Technology - National Capital Region; Appendix)

Fresh mature 465.0 g of *C. retusa* stems were garbled, washed, air-dried and cut into small pieces. Dried stems were pulverized using a Wiley Mill and soaked in 4.0 L of methyl alcohol for 48 hours. The mixture was filtered and the filtrate obtained was concentrated using rotary evaporator at 60ºC under vacuum for 2 hours. The concentrated extract was further evaporated using water bath at 60ºC to obtain a semi solid extract. Crude extraction of dried 465.0 g *C. retusa* stems produced 2.5 L methanolic extract. Concentration of the filtrate yielded 7.0 g of semisolid extract with a percentage yield 1.5%. Extract from *C. retusa* stems were prepared to the desired treatment dilution. Using distilled water as the diluent; the collected crude methanolic stem extract had a total volume of 10mL and was used to prepare the different concentrations (25, 50, 75 and 100 g/mL).

A2. Preparation of *C. retusa* roots methanolic extract (Department of Science and Technology – National Capital Region; Appendix)

Fresh mature 190.0 grams of *C. retusa* roots were garbled, washed, air-dried and cut into small pieces. Dried stems were pulverized using a Wiley Mill and soaked in 2.0 liter of methyl alcohol for 48 hours. The mixture was filtered and the filtrate obtained was concentrated using rotary evaporator at 60ºC under vacuum for 2 hours. The concentrated extract was further evaporated using water bath at 60ºC to obtain a semi solid extract. Crude extraction of dried 190.0 grams *Tsaang gubat* roots produced 1.6 L crude methanolic extract. Concentration of the filtrate yielded 3.2 grams of semisolid extract with a percentage yield 1.7%. Extract from *Tsaang gubat* stems were prepared to the desired treatment dilution. Using distilled water as the diluent; the collected crude methanolic roots extract had a total volume of 10ml and was used to prepare the different concentrations (25, 50, 75 and 100 g/mL).

B. Preparation of Sodium dodecyl as Negative control

Sodium dodecyl sulfate was prepared in 1.45 grams dissolved in 50 mL distilled water to make 0.05 mol of solution which serves as a control for the experiment.

C. Collection of Fertilized Duck eggs

One hundred (100) fertilized duck eggs known as breeders at embryonic day eight (ED 8) purchased in M.

Alcasid Farm in Calamba City, Laguna were collected using durable paper mache trays.

D. Incubation and Candling (Adopted from [22])

Before the incubation of duck eggs, they were checked individually for damage in the egg shell. Damaged eggs were not incubated to avoid contamination. The duck eggs at ED 8 were incubated at 37.8ºC in a humidified using Penguin Enterprise Automatic Egg Incubator. The temperature of the incubator was monitored every 24 hours to maintain the required temperature. Eggs were candled to determine if fertilization stage at ED 8 was developed. Candling was done in a dark room by placing the egg on top of the built-in egg candler in the incubator. Fertilized eggs were confirmed with the presence of the germ spot and small blood vessels. All fertilized eggs were placed back into the incubator until the day of inoculation of extract at ED 9 with a relative humidity of 50-55% and temperature of 37ºC. The eggs were used for the Chorioallantoic Membrane (CAM) Assay (Adopted from [23]).

E. Administration of *C. retusa* roots and stems crude methanolic extract (Adopted from [24] - [26])

The test specimens were divided into five treatments: four groups for each dilutions respectively (25, 50, 75, 100 g/ml) and one group for the negative control. Each group were treated with their corresponding dilutions. After incubation, the eggs were wiped individually with 70% ethanol and were transferred to a biosafety cabinet. Marking the air cell of the egg was done then the section marked was cut with a rotary dentist saw blade. The eggs were punctured at the site above the CAM embryo to which the air sac is exposed for administration of the treatment. A Terumo brand tuberculin disposable syringe with 26 gauge was used for the treatment administration. One syringe was used per egg and was used to assess the sterility of the treatment of the egg. An LED light was used to guide the needle to prevent damaging the CAM of the egg. A volume of 0.5mL of different concentrations of *C. retusa* roots and stem crude methanolic extract was administered into the CAM. The punctured site was covered with surgical tape to prevent from rotting and contamination. Then was incubated for 3 days and monitored with 37.8℃ humidity. After incubation, branching points of blood vessels were observed under dissecting microscope and was documented using a camera.

Data gathering (Adopted from [27], [28])

Incubated fertilized duck embryos were observed at ED 9, ED 10 and ED 11 for the development of the embryo and the branching of blood vessels. Photomicrograph documentation was taken using a mobile phone camera that was attached to the stereomicroscope and were submitted to image analysis using Image J software.

Statistical Analysis

Two-way ANOVA was used to compare the significant difference between the roots and stem crude methanolic extract. Difference among various concentrations of both roots and stem were compared using the same statistical analysis. Multiple comparisons were performed and all concentration was compared with the negative control and with one another.

RESULTS AND DISCUSSION

Table 1 shows the difference in concentrations of *C. retusa* extract as compared to the negative control. Based from the four concentrations, 25% concentration showed the greatest number of branching points (M=27;SD=2.65), while 100% concentration showed the least number of branching points of blood vessel (M=17; SD=4.58).

Table 1. Mean and SD of branching point in different concentrations of crude methanolic stem extract of *C. retusa* on chorioallantoic membrane of fertilized duck embryo

Concentrations	Mean	SD
Negative control	45.33	12.34
25%	27	2.65
50%	21.33	6.66
75%	18	1.73
100%	17	4.58
Total	25.73	5.59

The negative control showed (M=45.33; SD=12.34) the highest number of branching point of blood vessel compared to the four concentrations. This means the higher the concentration of the stem extract used, the number of branching points are reduced. Reduction of branching points of blood vessels are seen after inoculation of crude methanolic stem extract of *C. retusa* in chorioallantoic membrane of fertilized duck embryo which agreed with Lontoc [24], Wang [25], Alves De Paulo [30], and Seow [31] showed a significant decrease in the number of blood vessels compared to the negative control. The angiogenic activity of the blood vessels formation maybe affected by the bioactive phenolic chemical compound of *C. retusa* because it is considered to be an antioxidant.

Table 2. Mean and SD of branching point in different concentrations of crude methanolic roots extract of *C. retusa* on chorioallantoic membrane of fertilized duck embryo

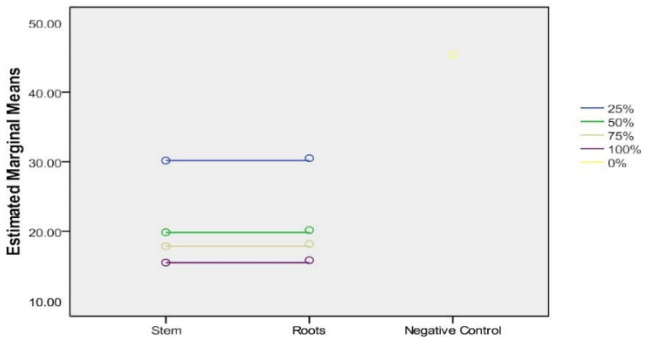
Concentrations	Mean	SD
Negative control	45.33	12.34
25%	33.67	10.50
50%	18.67	5.51
75%	18.00	3.61
100%	14.33	3.51
Total	130	35.47

Table 2 shows the difference in concentrations of *C. retusa* roots extract as compared to the negative control. Based from the four concentrations, 25% concentration had the greatest number of branching points (M=33.67; SD=10.50), while 100% concentration showed the least number of branching points of blood vessel (M=14.33; SD=3.51). The negative control showed (M=45.33; SD=12.34) the highest number of branching point of blood vessel compared to the four concentrations. This means the higher the concentration of the stem extract used, the number of branching points is reduced. Reduction of branching points of blood vessel are seen after inoculation of crude methanolic roots extract of *C. retusa* in chorioallantoic membrane of fertilized duck embryo which agreed with Lontoc [24], Wang [25], Kuete [32], and Mahapatra [33] showed a significant decrease in the number of blood vessels compared to the negative control. The angiogenic activity of the blood vessels formation might be affected by the bioactive phenolic chemical compound of *C. retusa* that is considered to be an antioxidant.

Table 3. Two-way ANOVA of the reduction of blood vessels with the different centration of roots and stem of Carmona retusa

Source	Df	F	Sig.
Roots of *C. retusa*	1	.016	.901
Stem of *C. retusa*	1	.016	.901
Concentration	3	6.029	.004
Error	21		

Table 3 shows the two-way ANOVA on the reduction of blood vessels in chorioallantoic membrane of fertilized duck embryo. Parts of *C. retusa* include two parts (roots and stem) and concentration consisted of five levels (25%, 50%, 75%, 100% and 0% as negative control). All effects were significant at the .05 significance level except for the parts of *C. retusa* factor. The main effect for concentration (25%, 50%, 75%, 100% and 0% as negative control) yielded an F ratio of F(3,21) =6.029=p=.004 indicating a significant difference between the different concentrations. The main effect for parts (roots and stem) of *C. retusa* yielded an F ratio of (1,21)=.016=p=.960 indicating that the effect for parts of *C. retusa* was not significant. Therefore, any part of the plant used will have the same reduction on the blood vessels. The reduction results differ when a higher concentration is used. 100% concentration had the highest anti-angiogenic effect. This is in conformity with the study of Lontoc [24] using other plant extract on chorioallantoic membrane of chick embryo. They revealed that the reduction of branching points of blood vessels decreases as the concentration of extract increases.



Non-estimable means are not plotted

Fig. 1. Comparison of roots and stem of *C. retusa* by the average number of branching points of blood vessels in CAM of fertilized duck embryo.

The first line in figure 1 represents 25% concentration that yields less than 30 branching points of blood vessels. At 50%, 75% and 100% there are less than 20 branching points of blood vessels counted. When a higher concentration is used, the greater effect it has in lessening the number of branching points of blood vessels. This is in conformity with the study of Lontoc [24], Alves De Paulo [30], Germanò [34], and Mathur [35] using other plant extract on chorioallantoic membrane of chick embryo. They revealed the reduction of branching points of blood vessels decreases as the concentration of extract increases. The roots and stems are not different in the reduction of the branching point of blood vessel; it had the same anti-angiogenic effect. The different concentrations showed a significant difference with each other and the best concentration in the reduction of angiogenesis was the 100% concentration which showed the least number of branching points counted. This concurs with the phytochemical analysis of the study of screening for the anti-angiogenic activity of selected Philippine medicinal plants using chorioallantoic membrane assay. Camposano [29] mentioned that the

use of *C. retusa* yielded a higher reduction of blood vessel in CAM for the presence of phenolic compounds which exhibits inhibition of angiogenesis cascade.

CONCLUSION AND RECOMMENDATION

Findings showed that when parts of *C. retusa* crude methanolic extracts were compared to each other, it has the same effect on the chorioallantoic membrane of fertilized duck embryo. Multiple comparisons of various concentrations of both roots and stem extract were done. When the concentration of both roots and stem extract of *C. retusa* were compared based on their anti-angiogenic effect on the CAM of fertilzed duck embryo, all concentrations exhibited anti-angiogenic effect. 100% concentration both roots and stem had the highest anti-angiogenic effect among other concentrations. When a higher concentration of the extract (roots and stem) was used, it had a greater effect in lessening the blood vessel on the CAM of fertilized duck embryo. Therefore, *C. retusa* roots and stem methanolic extract demonstrates to have an anti-angiogenic effect on the chorioallantoic membrane of fertilized duck embryo.

Application of a positive control on the methodology is recommended to check if the plant causes the same anti-angiogenic effect against the chorioallantoic membrane (CAM). Isolation and identification of the specific compound of *C. retusa* for angiogenesis in ovo experimentation is recommended and also the determination of the lethal dose concentration of the plant to ensure the best survival rate of the embryo.

REFERENCES

[1] World Health Organization (2018). Cancer. Retrieved from https://www.who.int/news-room/fact-sheets/detail/cancer

[2] Ribatti, D., Nico, B., Crivellato, E., Roccaro, A. M., & Vacca, A. (2007). The history of the angiogenic switch concept. Leukemia, 21(1), 44.

[3] Adair, T. H., & Montani, J. P. (2010, October). Angiogenesis. In Colloquium Series on Integrated Systems Physiology: From Molecule to Function (Vol. 2, No. 1, pp. 1-84). Morgan & Claypool Life Sciences.

[4] Mentzer, S. J., & Konerding, M. A. (2014). Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. Angiogenesis, 17(3), 499-509.

[5] Stephenson, J. A., Goddard, J. C., Al-Taan, O., Dennison, A. R., & Morgan, B. (2013). Tumour angiogenesis: a growth area—from John Hunter to Judah Folkman and beyond. Journal of Cancer Research, 2013.

[6] Staton, C. A., Reed, M. W., & Brown, N. J. (2009). A critical analysis of current in vitro and in vivo angiogenesis assays. International journal of experimental pathology, 90(3), 195-221.

[7] Nyberg, P., Xie, L., & Kalluri, R. (2005). Endogenous inhibitors of angiogenesis. Cancer research, 65(10), 3967-3979.

[8] Noy, P. J., Lodhia, P., Khan, K., Zhuang, X., Ward, D. G., Verissimo, A. R., ... & Bicknell, R. (2015). Blocking CLEC14A-MMRN2 binding inhibits sprouting angiogenesis and tumour growth. Oncogene, 34(47), 5821.

[9] Rusnati, M., Urbinati, C., Bonifacio, S., Presta, M., & Taraboletti, G. (2010). Thrombospondin-1 as a paradigm for the development of antiangiogenic agents endowed with multiple mechanisms of action. Pharmaceuticals, 3(4), 1241-1278.

[10] Folkman, J. (2006). Angiogenesis. Annu. Rev. Med., 57, 1-18.

[11] Bergers, G., & Benjamin, L. E. (2003). Angiogenesis: tumorigenesis and the angiogenic switch. Nature reviews cancer, 3(6), 401.

[12] Schlieve, C. R., Mojica, S. G., Holoyda, K. A., Hou, X., Fowler, K. L., & Grikscheit, T. C. (2016). Vascular endothelial growth factor (VEGF) bioavailability regulates angiogenesis and intestinal stem and progenitor cell proliferation during postnatal small intestinal development. PloS one, 11(3), e0151396.

[13] Winkler, F., Kozin, S. V., Tong, R. T., Chae, S. S., Booth, M. F., Garkavtsev, I., ... & Munn, L. L. (2004). Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer cell, 6(6), 553-563.

[14] O'Donnell, R. K., Falcon, B., Hanson, J., Goldstein, W. E., Perruzzi, C., Rafii, S., ... & Benjamin, L. E. (2016). VEGF-A/VEGFR inhibition restores hematopoietic homeostasis in the bone marrow and attenuates tumor growth. Cancer research, 76(3), 517-524.

[15] Boosani, C. S., & Sudhakar, Y. A. (2011). Proteolytically derived endogenous angioinhibitors originating from the extracellular matrix. Pharmaceuticals, 4(12), 1551-1577.

[16] Butler, J. M., Kobayashi, H., & Rafii, S. (2010). Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nature Reviews Cancer, 10(2), 138.

[17] Yang, W. H., Xu, J., Mu, J. B., & Xie, J. (2017). Revision of the concept of anti-angiogenesis and its applications in tumor treatment. Chronic diseases and translational medicine, 3(1), 33-40.

[18] Melkonian, G., Munoz, N., Chung, J., Tong, C., Marr, R., & Talbot, P. (2002). Capillary plexus development in the day five today six chick chorioallantoic membrane is inhibited by cytochalasin D and suramin. Journal of Experimental Zoology, 292(3), 241-254.

[19] Salas, G. M., & Totaan, E. V. (2015). Selected Philippine Herbal plant extracts as Angiogenesis

inhibitors using Chick Chorioallantoic Membrane (CAM) assay. International Research Journal of Biological Sciences, 28-32.

[20] Retusa, C. (2012). Phytochemical Screening And Antibacterial Property Of Carmona Retusa (Vahl.) Masam.

[21] Amudha, M., & Rani, S. (2016). Evaluation of in vitro antioxidant potential of Cordia retusa. Indian journal of pharmaceutical sciences, 78(1), 80.

[22] Parsons-Wingerter, P., Lwai, B., Yang, M. C., Elliott, K. E., Milaninia, A., Redlitz, A., ... & Sage, E. H. (1998). A novel assay of angiogenesis in the quail chorioallantoic membrane: stimulation by bFGF and inhibition by angiostatin according to fractal dimension and grid intersection. Microvascular research, 55(3), 201-214.

[23] Ribatti, D. (2010). The chick embryo chorioallantoic membrane as an in vivo assay to study antiangiogenesis. Pharmaceuticals, 3(3), 482-513.

[24] Lontoc, K. L. C. Angiogenic effect of Talisai (Terminalia catappa L.) crude extract on chorioallantoic membrane of chick embryo.

[25] Wang, S., Zheng, Z., Weng, Y., Yu, Y., Zhang, D., Fan, W., ... & Hu, Z. (2004). Angiogenesis and anti-angiogenesis activity of Chinese medicinal herbal extracts. Life sciences, 74(20), 2467-2478.

[26] Výboh, P., Zeman, M., Bilčík, B., Šárniková, B., & Košťál, Ľ. (2010). Angiogenic effect of leptin in the quail chorioallantoic membrane. Acta Veterinaria Brno, 79(1), 13-17.

[27] Doukas, C. N., Maglogiannis, I., & Chatziioannou, A. A. (2008). Computer-supported angiogenesis quantification using image analysis and statistical averaging. IEEE Transactions on Information Technology in Biomedicine, 12(5), 650-657.

[28] Rasband, W. S. ImageJ. US National Institutes of Health, Bethesda, Maryland, USA. 1997-2018.

[29] Camposano, J. E., Torre, G. D., Laxamana, J. G., & Larcia II, L. L. H. Screening for the anti-angiogenic activity of selected Philippine medicinal plants using chorioallantoic membrane assay. Screening, 173, 182.

[30] Alves De Paulo, S., Teruszkin Balassiano, I., Henriques Silva, N., Oliveira Castilho, R., Kaplan, C., Currie Cabral, M., & da Costa Carvalho, M. G. (2000). Chrysobalanus icaco L. extract for antiangiogenic potential observation. International journal of molecular medicine, 5(6), 667-676.

[31] Seow, L. J., Beh, H. K., Majid, A. M. S. A., Murugaiyah, V., Ismail, N., & Asmawi, M. Z. (2011). Anti-angiogenic activity of Gynura segetum leaf extracts and its fractions. Journal of ethnopharmacology, 134(2), 221-227.

[32] Kuete, V., Krusche, B., Youns, M., Voukeng, I., Fankam, A. G., Tankeo, S., ... & Efferth, T. (2011). Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts. Journal of ethnopharmacology, 134(3), 803-812.

[33] Mahapatra, S. S., Mohanta, S., & Nayak, A. K. (2011). Preliminary investigation of the angiogenic potential of Ziziphus oenoplia root ethanolic extract using the chorioallantoic membrane model. Science Asia, 37, 72-74.

[34] Germanò, M. P., Certo, G., D'Angelo, V., Sanogo, R., Malafronte, N., De Tommasi, N., & Rapisarda, A. (2015). Anti-angiogenic activity of Entada africana root. Natural product research, 29(16), 1551-1556.

[35] Mathur, R. A. J. A. N. I., Gupta, S. K., Mathur, S. R., & Velpandian, T. (2011). Calotropis procera root extracts block VEGF-induced angiogenesis: quantitative analysis. Indian J Physiol Pharmacol, 55(1), 5-12.