Research Article

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Anti-Angiogenesis Screening of *Moringa oleifera* Pod Extracts by *In-Ovo* Chorioallantoic Membrane (CAM) Assay

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ABSTRACT

Moringa oleifera has many therapeutic benefits one out of it is anti-cancer property. Therefore, many researchers have been screening the therapeutic potential of *Moringa oleifera*. The main objective of this study was to screen and explore the angiogenesis inhibition potential of *Moringa oleifera* pod extracts. With the aim of screening anti-angiogenic potential, extracts of *Moringa oleifera* pods were prepared by decoction method. The extracts were subjected to preliminary phytochemical screening to identify the nature of phytochemicals present in the pods. *In-ovo* chorioallantoic membrane assay was chosen to achieve the objective of the study. Water-soluble extractive value (15.00% w/w) was higher than that of alcohol-soluble extractive value (3.89% w/w), indicating that the *Moringa oleifera* pods have more water-soluble constituents. Qualitative phytochemical screening revealed presence of flavonoids. Angiogenesis inhibition effect was studied and compared with sunitinib. Statistical analysis revealed highest anti-angiogenesis activity in 100% methanolic extract. Least effect was observed in 50% aqueous extract. Anti-angiogenic potential of 100% methanolic extract was statistically significant when compared with other study groups. It is concluded that *Moringa oleifera* pods exert anti-angiogenic potential and more intensified and diversified studies are needed to enable a thorough investigation of this plant components in anti-cancer treatment.

Keywords: anti-angiogenesis screening; *in-ovo* chorioallantoic membrane assay; *Moringa oleifera*; phytochemical screening; sunitinib.

1. Introduction

Cancer research in the United Kingdom and the United States of America reported that the number of new cancer cases and cancer-related death cases has increased in 2018 and 2019 [1,2]. Angiogenesis plays a significant role in the growth and metastasis of solid tumours. Tumour cells when they grow and increase in mass suffer from deficiency of essential nutrients. To counteract this deficiency the cancerous cells release growth factors like VEGF (Vascular Endothelial Growth Factor) [3,4]. Various anti-cancer drugs derived from natural compounds are being widely studied to inhibit angiogenesis that can prevent tumour growth and metastasis.

A large number of research studies have reported that *Moringa oleifera*, of Moringaceae plant family, possess therapeutic benefits like antioxidant, anti-cancer, anti-inflammatory, neuroprotective, gastroprotective, hepatoprotective, analgesic and hypolipidaemic activity [5-7]. The beneficial effects of *Moringa oleifera* is due to the presence of phytochemicals like flavonoids, thiocarbamates, and isothiocyanates [8,9].

Anti-cancer therapy derived from natural compounds has lower toxicity and fewer adverse effects [5,10]. Conventional chemotherapy approaches have a well-established downsides like cost of treatments, adverse effects, etc. These downsides can be balanced by developing more holistic, multidimensional approaches in the treatment, palliation and survivorship. One of the strategies to develop balanced approach is integrating traditional herbs and medicines into mainstream oncology therapy. A recent review has reported evidence that diet-related and physical activity-related interventions for the primary prevention of breast cancer are cost-effective [11,12]. Targeting angiogenesis using natural products can be an effective approach in controlling the growth and metastasis of tumour. In this study the anti-angiogenic effects of pods of *Moringa oleifera* were studied by *in-ovo* chorioallantoic membrane (CAM) assay.

2. Materials and Methods

2.1. Sample preparation and chicken eggs

Fresh pods of *Moringa oleifera* were collected from trees. Pods were collected between the months of May and July in the Bangalore region of south India. The pods were authentified by a renowned botanist and a voucher specimen (TJCP/PCOG/120) is retained in Pharmacognosy department of T. John Group of Institutions. All the collected pods were washed in the running tap water and towel dried to remove wetness. Pods were cut open, the pulp and seeds from the pods were separated and shade dried in room temperature. After complete drying both the pods and seeds were grounded into coarse powder and were used for extraction. Fresh fertilised white leghorn chicken eggs were collected from a nearby farm (RH farms, Malaysia). The eggs were cleaned using distilled water to remove the residual matter on the eggshell and kept ready for incubation.

2.2. Chemicals and reagents

Methanol (99.8%) and distilled water were used for extraction of phytochemicals present in the pods. Sunitinib of 0.01 μM concentration served as positive control for chorioallantoic membrane assay.

2.3. Extraction

Phytochemical extraction was done by the method of decoction [13,14]. About 50 g of *Moringa oleifera* coarse pod powder was used for extraction. The coarse powder was completely immersed in respective solvents. The mixtures were boiled separately for 2 hrs by placing over a water bath. The temperature of the water bath was set closer to the solvents boiling point. Water bath containing mixture of powder and distilled water was set to a temperature of around 80^0C and water bath containing mixture of powder and methanol was set to a temperature of around 50^0C. After 2 hrs the mixtures were filtered separately using whatman filter paper and the extracts were concentrated. The concentrated extracts were dried by placing in an oven and to get a solid dry powder. The solidified extracts were stored at 4°C until use [13,14]. The extractive values were calculated by using the formula below [15].

$$\text{Extractive value (\% w/w)} = \frac{\text{Amount of dried extract after extraction (g)}}{\text{Amount of pod powders used for extraction (g)}} \times 100$$

2.4. Preliminary phytochemical screening

Dried aqueous and methanolic extracts of *Moringa oleifera* pods were dissolved in respective solvents to make into liquid extract solutions. The liquid extracts were then screened for identifying various phytochemical components like alkaloids, flavonoids, saponins, tannins and steroids present in the pods extracts [1,9,10].

2.4.1. Test for alkaloids

Dragendorff test: About 1 mL of extract solution was treated with 0.5 mL of 2% hydrochloric acid (HCl) and then heated by placing in a water bath for 2 min. To the mixture 3 drops of Dragendorff reagent was added after filtration. Appearance of orange-brown precipitate was considered as positive result for presence of nitrogen containing compounds.

Wagner's test: About 2 mL of Wagner's reagent was added to 1 mL of the extract solution. Formation of reddish-brown precipitate was deliberated as presence of alkaloids.

2.4.2. Test for flavonoids

Alkaline reagent test: About 1 mL of the extract solution was made alkaline by adding 0.5 mL of 10% sodium hydroxide solution. Development of yellow colour in the alkalinized extract solution indicates presence of flavonoids.

2.4.3. Test for saponins

Foam test: About 1 mL of the extract solution was mixed with 0.5 mL of distilled water in a stoppered test tube. Solution was shaken vigorously for 10 min. Formation of foam which is stable for 10 min was considered as a positive result for the presence of saponins.

2.4.4. Test for tannins

Ferric chloride test: About 1 mL of the extract solution was heated for 2 min by placing inside a water bath. The mixture was cooled and three drops of 10% ferric chloride solution was added. Formation of dark green colour in the mixture confirms presence of tannins.

2.4.5. Test for steroids

Salkowski test: About 0.5 mL of chloroform solution was added to 1 mL of the extract solution. To this mixture 0.5 mL of concentrated sulfuric acid was added in drops in the sides of the test tube. Development of red or brown colour ring in the bottom region of chloroform layer indicates presence of steroids.

2.5. Preparation of sample extracts and filter paper discs

Both methanolic and aqueous solid extracts were dissolved in phosphate buffer saline. After dissolving the concentration of the extracts were made to remain in the concentration of 100% and 50% using phosphate-buffer saline (PBS). The pH of the phosphate-buffer saline was adjusted, using sodium hydroxide solution or hydrochloric acid, to 7.4 before its use. Whatman filter paper discs of 5 mm diameter were prepared using paper puncher and all the paper discs were sterilized suitably before proceeding to CAM assay to remove any infecting material if present [16].

2.6. In-ovo CAM assay

CAM assay was performed in 6 groups including negative control (PBS), positive control, 50% methanolic extract, 100% methanolic extract, 50% aqueous extract and 100% aqueous extract. Positive control group was treated with sunitinib 0.01 µg/mL which is prepared using phosphate-buffer solution.

Fertilised white-leghorn chicken eggs were selected for performing CAM assay. Fresh fertilised chicken eggs were collected from a local hatchery farm at Klang. The eggs were subjected to superficial cleaning process using water to remove dust and dirt adhering to the surface of the shells.

2.6.1. Incubation of eggs

Prior to incubation the incubator was sterilised using 70% alcohol. Also the incubator was pre-tested for 3 days to ensure the temperature and humidity control falls in the range of 36.5 to 37.5^{0}C and 55 to 60% respectively. Seven-days old fertile eggs were incubated for 5 days for development of embryos [17]. During the incubation period all the eggs were rotated horizontally 3 times every day. The humidity and temperature of incubator was maintained between the range of 55% to 60% and 37 ± 0.5°C respectively to avoid dehydration, cracking and damage to the eggshells [18].

2.6.2. Candling and marking of fertilised eggs

Candling of eggs was done on sixth day of incubation using torch light in a dark room. This process was done to ensure and track the development of embryo and identify the vasculature. The eggs were transilluminated in order to check for the viability and embryonic phase. The eggs with poor vasculature, non-fertile eggs, or eggs presented with blood rings were excluded. The egg was held gently with naturally occurring air sac toward the light source. The

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region of eggshells receiving openings was marked with a pencil. The attachment of the developing embryo to the CAM was identified first and marked to prevent any interventions in this region. Secondly, a well vascularised area was chosen as the operating window area and a circle was drawn on it. The operating window area should be drawn at least 2 cm away from the embryo attachment. In the operating window area, a mark was made in a less vascularised area to allow the air flowing into the egg. Third, the air sac region was marked with a cross [19,20].

2.6.3. Egg shell grafting and creating working window

Two holes were made at the region marked with cross and air displacement was done. The egg was placed in the light source to visualize the air sac. A gentle suction was applied using a rubber bulb by placing on the small hole made in the air sac area. The pressure was released in the bulb until the two membranes were separated in the operating window area. This step was repeated as many times as needed in order to achieve complete separation of the membranes in the operating window area. A small window of 2 cm x 2 cm was made on the eggshells using a sterilised pen knife. The egg surface was cleaned by gently sticking an adhesive tape to remove all loose particles. With blunt forceps, the eggshell was removed at the operating window area. The inner eggshell membrane was carefully removed, to avoid contamination of CAM layer with shell dust. The window was covered with parafilm and incubated for another day [18].

2.6.4. Application of samples, visual assessment and photography on CAM

The sterilised filter paper discs were loaded with respective study sample by soaking them in the extract solution, PBS for negative control and sunitinib solution for positive control group. After soaking the filter paper discs were dried to remove the extra solvent adhering to the paper. Dry filter paper discs loaded with samples were applied to the CAM through a small window made on the shell. After application of the disc the window was sealed using parafilm and incubated further for two more days. The CAM layer was studied using stereomicroscope and images of CAM layers were captured as images for analyzing the changes in the blood vessels. Images were captured before placing the disc, immediately after placing the disc (post-0 hours), 24 hours after placing the disc (post-24 hours) and 48 hours after placing the disc (post-48 hours). Inhibition of blood vessels or reduction in the number of blood vessels surrounding the discs indicated the anti-angiogenic potential of the sample. In this method reduction in the number of blood vessels was calculated from the images captured. The procedure of counting blood vessels was done in triplicate by three different samples to minimize random error. The percentage inhibition of blood vessels was calculated using the formula given below [21].

$$\text{Percentage inhibition} = \frac{\text{No of blood vessels in CAM of (negative control - sample)} \quad \text{x 100}}{\text{No of bloood vessels in CAM of negative control}}$$

2.7. Statistical analysis

Mean of groups were compared by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The probability, $p < 0.05$ was indicated as statistically significant. Data analysis and graph plotting were done using International Business Machine Statistical Package for the Social Sciences (IBM SPSS) statistics version 26.0 software.

3. Results and Discussion

3.1. Extractive parameters of Moringa oleifera pod extracts

The organoleptic characteristics of dried solid extracts are presented in Table 1. The extractive values are significant for evaluating quality and purity of the plant sample [22]. Yield and extractive values are affected by various experimental, environmental and plant related factors [23]. High aqueous extractive value indicates that sample contained more polar constituents which are soluble in water [24,25].

3.2. Preliminary phytochemical screening of Moringa oleifera pod extracts

Alkaloids, flavonoids and saponins were present in both methanolic and aqueous extract. Steroids were present in methanolic extract while absent in aqueous extract. Tannins were not found in methanolic and aqueous extract (Table 2.). The results of the phytochemical test were different for each study because chemical constituents of natural products differ according to the cultivation parameters [26].

Table 1. Organoleptic properties, yield and extractive value of *Moringa oleifera* pod extracts

Extract	Appearance	Yield (g)	Extractive value (% w/w)
Methanol	Colour:Greenish-black Consistency: Sticky and oily	1.97	3.89
Aqueous	Colour: Brown Consistency: Fine Powder	7.55	15.00

Table 2. Phytochemical constituents of *Moringa oleifera* pod extracts.

Phytochemical constituents	Methanolic extract	Aqueous extract
Alkaloids (Dragendorff's test)	+	+
Flavonoids (Sodium hydroxide test)	+	+
Saponins (Froth test)	+	+
Tannins (Ferric chloride test)	-	-
Steroids (Salkowski Test)	+	-

Note: (+) indicates presence of phytochemicals
(-) indicates absence of phytochemicals

Among the phytochemical constituents, flavonoids are groups of polyphenols responsible for anti-angiogenesis activity in plant samples.

The biochemical compounds, kaempferol and quercetin have effects on biochemical pathways [27]. These effects include cell proliferation inhibition, detoxification of mutagenic metabolites, anti-angiogenic activity and apoptosis. Besides, flavonoids are water-soluble antioxidants, because they protect the body from the influence of ROS. Free radicals become inactive due to the high reactivity of the hydroxyl group in flavonoids. Thus, cell damage can be prevented due to the presence of flavonoids in both extracts of *Moringa oleifera* pods [28]. However, a thorough understanding of their phytochemical compounds is important because these compounds may be the bio-active constituents that are responsible for the efficacy of the whole pods of *Moringa oleifera* studied. Therefore, it is inferred that the *Moringa oleifera* pod extracts could be the rich source of phytoconstituents, which can be used for synthesis of drugs and for treating diseases.

3.3. Anti-angiogenic activity by in-ovo CAM assay

Negative control group was administered with PBS because it is an isotonic buffer, which is frequently used for diluting, washing cells and transporting tissues in biological research. In addition, PBS mimics the osmolarity, pH and ion concentration of the human body. Therefore, compared to distilled water, PBS can prevent the cells from rupturing due to osmosis. Compared to dimethyl-sulfoxide (DMSO), PBS shows no toxicity to cells [29]. Increase in the number of blood vessels i.e angiogenesis was observed in negative control group indicating that PBS did not show any interference with the samples.

Anti-angiogenic potential of two concentrations of methanolic and aqueous extract of pods of *Moringa oleifera* was compared with sunitinib (0.01 µg/µM). Positive control group showed anti-angiogenesis effect. This is because sunitinib is a vascular endothelial growth factor-tyrosine kinase inhibitor (VEGF-TKI). It inhibits many tyrosine kinase receptors, some of which are related to tumour growth, pathogenic angiogenesis and metastatic progression of cancer. It competitively inhibits the binding of ATP to the tyrosine kinase domain on the vascular endothelial growth factor receptors (VEGFRs) [30]. Changes in the blood vessels after application of samples and the percentage changes in blood vessels are presented in Table 3 and Table 4 respectively.

Percentage reduction of blood vessels given in Table 4 indicates that the pod extracts of *Moringa oleifera* have the potential to inhibit angiogenesis. The an-

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giogenesis inhibition effect on CAM was exhibited in a dose-dependent manner for *Moringa oleifera* pod extracts. The results of methanolic and aqueous extract demonstrated that the greater the concentration of the applied samples, the fewer the blood vessels were observed, and the higher the percentage of blood vessels reduced after 24 hours and 48 hours of treatment. It was observed that the two extracts with a concentration of 100% showed a higher percentage reduction of blood vessels compared with extracts with a concentration of 50% [3,31]. Methanolic extract of 100% concentration showed highest anti-angiogenic activity compared with other samples.

Statistically significant reduction in percentage of blood vessels was observed between the groups as determined by one-way ANOVA where the $p<0.05$ for 24 hours and 48 hours post-treatment. From the results of Tukey post-hoc test, it showed that the anti-angiogenesis groups were different from each other. The test also revealed that there was a statistically significant difference in percentage reduction of blood vessels after 24 hours and 48 hours of treatment between 100% methanolic extract and four other groups ($p<0.05$). For percentage reduction of blood vessels after 24 hours of treatment, there was a statistically significant difference between 50% aqueous extract and 100% aqueous extract ($p<0.05$).

The preliminary phytochemical study revealed the presence of flavonoids in methanolic and aqueous extracts of pods of *Moringa oleifera*. It is reported that many natural compounds containing flavonoids possess cytotoxic and anti-cancer potentials [28,32,33]. Studies have also shown that flavonoids can inhibit angiogenesis and proliferation of tumour cells and endothelial cells *in-vitro*, and inhibit the cell proliferation and migration in human umbilical vein endothelial cells (HUVECs) induced by vascular endothelial-growth factor (VEGF) [34]. This

Table 3. Blood vessel changes in the CAM layer.

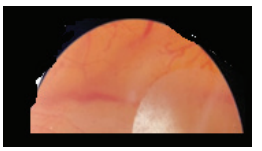
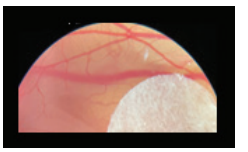
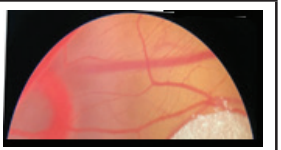
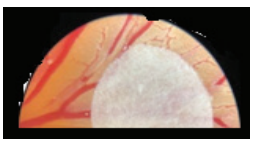
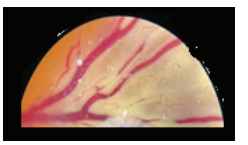
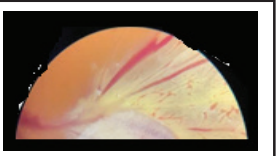
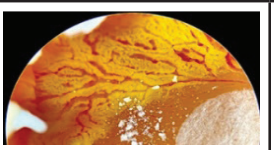
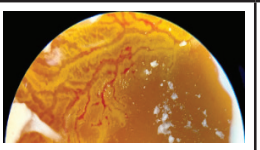
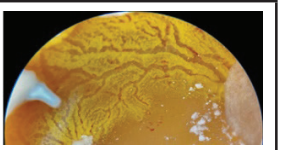
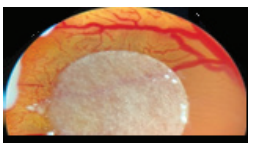
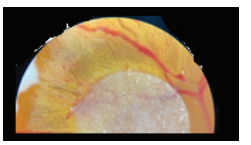
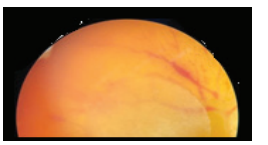
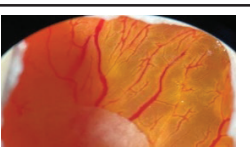
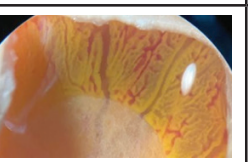
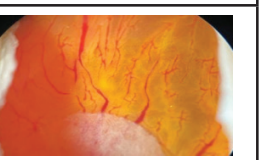
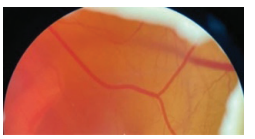
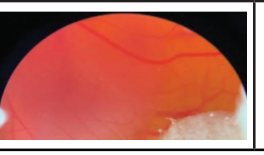
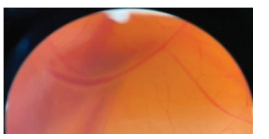
Groups	Post 0-hrs	Post 24-hrs	Post 48-hrs
Negative control (PBS)			
Positive control (Sunitinib)			
50% methanolic extract			
100% methanolic extract			
50% aqueous extract			
100% aqueous extract			

Table 4. Percentage reduction of blood vessels after 24 and 48 hours of treatment.

Samples	Post-24 hours of treatment (Mean ± SD)	Post-48 hours of treatment (Mean ± SD)
Positive control (Sunitinib)	25.61 ± 1.7563	45.51 ± 2.9431
50% methanolic extract	23.98 ± 1.8153	43.36 ± 3.3431
100% methanolic extract	50.34 ± 2.1404	64.46 ± 3.9003
50% aqueous extract	22.21 ± 2.5511	42.00 ± 3.0028
100% aqueous extract	28.31 ± 1.0710	46.52 ± 3.0834

indicates that the extracts of *Moringa oleifera* may suppress VEGF that leads to decrease in growth factor of new blood vessels. Therefore, the preliminary results obtained in this study supported further evaluation and study of potent anti-angiogenic properties of pods of *Moringa oleifera*.

4. Conclusion

This study showed that the pods of *Moringa oleifera* is a potential natural compound, which can be used to develop anti-cancer drugs by inhibiting angiogenesis. Anti-cancer potential of *Moringa oleifera* contributes lower cost and lesser side effects in cancer treatment. The results of phytochemical screening revealed presence of flavonoids, which was reported in other studies to possess anti-cancer and antioxidant activities. The results of *in-ovo* CAM assay showed that *Moringa oleifera* pod extracts have anti-angiogenic properties. 100% methanolic extract showed the highest anti-angiogenesis properties while compared to 100% aqueous extract and positive control. The anti-angiogenic potential shown by the *Moringa oleifera* pod extracts, imply a need for more intensified and diversified study to enable a thorough investigation of these plant components in anti-cancer treatment.

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Conflict of Interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

Statement of Contribution of Researchers

Concept – N.S.C., T.S.Y.; Design – N.S.C., T.S.Y.; Supervision – N.S.C.; Resources – T.S.Y.; Data Collection and/or Processing – N.S.C., T.S.Y.; Analysis and/or Interpretation – N.S.C., T.S.Y.; Literature Search – T.S.Y.; Writing – N.S.C., T.S.Y..

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