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Anti-Angiogenic Screening of *Moringa oleifera* Leaves Extract Using Chorioallantonic Membrane Assay

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Abstract

Angiogenesis is defined as the physiological process resulting in formation of new blood vessels from pre-existing vessels. However, angiogenesis in cancer will lead to tumor growth and metastasis. Therefore, anti-angiogenesis is one of the ways to slow down growth and spreading of tumor. *Moringa oleifera* is also known as a "Miracle tree" which has high nutritive value and various therapeutics effects in different parts of the plant. This study aims to determine the anti-angiogenic property of *Moringa oleifera* leaves extract by using chick chorioallantoic membrane (CAM) assay.

The extracts were prepared by decoction method using methanol (99.8%) and water. The qualitative phytochemical screening was carried out for both methanol and aqueous extracts. The fertilised chicken eggs were divided into six groups which include negative control group (phosphate-buffer saline with pH 7.4), positive control group (sunitinib), 50% and 100% methanol extract, 50% and 100% aqueous extract. The anti-angiogenic effect of *Moringa oleifera* leaves extract was determined by calculating the number and percentage decrease in blood vessels in post-24 and post-48 hours of treatment.

Statistical analysis by one-way ANOVA has shown significant ($p<0.05$) percentage reduction in the blood vessels between each treatment group after 48 hours of treatment. Among all the extracts, 100% aqueous extract of *Moringa oleifera* was found to have highest anti-angiogenic effect with the greater percentage decrease in blood vessels (81.33%) in post-48 hours of treatment. Furthermore, the anti-angiogenic effect of *Moringa oleifera* leaves was found to be increased when the concentration of the *Moringa oleifera* extract was increased.

Moringa oleifera leaves with various phytochemicals was found to possess anti-angiogenic potential.

Keywords: Anti-angiogenic screening, *Moringa oleifera* leaves, Chick Chorioallantoic Membrane (CAM) assay.

Introduction

Angiogenesis is defined as the formation of new blood vessels to allow nutrient supply, gaseous exchange and removal of waste products to a localized tissue. It plays an important role in the process of wound healing, tissue repair, tissue growth, foetal development and cancer [(1)]. Angiogenesis is regulated by various mediators, where the most common type of mediators is growth factors and cytokines. The examples of growth factors include vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and angiopoietin [(2)]. However, the mediators can be divided into stimulator or activator and inhibitor, hence the balance between stimulatory and inhibitory mediators are extremely important in the process of angiogenesis. Normally, when the formation of new blood vessels is required, an "angiogenic switch" will be turned on by increasing the activator growth factor [(3)].

In recent years, there are many studies regarding angiogenesis inhibition. The mechanism of inhibition of angiogenesis is by blocking the formation of new blood vessels which further slows down the cancer progression and reduces release of metastatic cells into the circulation [(4)]. Hence, angiogenesis inhibition is one of the mechanisms in anticancer therapy. Moreover, angiogenesis inhibition is not restricted to any specific types of tumor cell because all of the solid tumors are based on angiogenesis [(5)]. Therefore, inhibition of angiogenesis has become a new target for anticancer action. Angiogenesis inhibitor consists of different benefits such as fewer side effects, less resistance compare to anti-cancer medication and accessibility of endothelial cell in blood vessels are better than tumor cell [(6)].

Plant extracts are rich in bioactive compounds that can decrease the growth rate of cancer cell and induce cancer cell apoptosis by inhibiting angiogenesis. This can lead to suppression or eradication of cancer. Phytochemicals play an important role in preventing development and growth of tumour. However, further studies are still required to explore and identify more therapeutic plants with antiangiogenic effects. Furthermore, the side effect and toxicity of plant-based anti-cancer drug was found to be fewer compared to conventional chemotherapeutic agents [(7)].

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Moringa oleifera is rich in different types of phytochemicals which makes it as a good source of anti-cancer agents. Furthermore, *Moringa oleifera* leaves are proved to possess various medicinal properties, such as analgesic activity, antimicrobial activity, antidiabetic activity, anti-inflammatory activity and antihypertensive activity [8–13] . The anti-cancer effect of *Moringa oleifera* leaves was well-studied and established by certain researchers but studies regarding antiangiogenic effect of *Moringa oleifera* are least [14,15]. Hence, this research was conducted to study the antiangiogenic effect of *Moringa oleifera* leaves extract by in-ovo chick chorioallantoic membrane (CAM) assay.

Materials and Methods

Sample preparation and extraction

Moringa oleifera leaves powder with product name LOHAS® was purchased from a shop named Kait Lifestyle store located at Desa Parkcity, Kuala Lumpur. Approximately 25 g of *Moringa oleifera* leaves powder was dispersed in methanol and distilled water separately. The mixtures were boiled by placing over a water bath for two hours setting the temperature of the water bath closer to the solvent boiling point. Aqueous extraction was done by placing the water bath at 80℃ and methanol extraction was done at 50℃. The mixtures were then filtered and the filtrate was concentrated by placing in an oven at 60℃ till complete solvent evaporation and dried extracts are obtained. The percentage yield was calculated and the dry extracts were stored separately with appropriate label in the refrigerator at 4 °C.

Preliminary qualitative analysis of phytochemicals

Preliminary phytochemical screening was done for both extracts by the following procedure [16,17]

1. Alkaloid (Dragendroff's and Wagner's tests)

Approximately 1 mL of plant extract was added to 0.5 mL of 2% hydrochloric acid (HCl) then heated in a water bath for two minutes. The mixture was filtered and treated with three to four drops of Dragendroff's reagent. Formation of orange-brown precipitate indicates presence of nitrogen containing compounds.

About 1 ml of plant extract was treated with 2 ml of Wagner's reagent. Reddish-brown precipitate was obtained indicating the presence of alkaloids.

2. Flavonoids (Alkaline reagent test)

Approximately 1 mL of plant extract was mixed with 0.5 mL of distilled water. The mixture was treated with 0.5 mL of 10% sodium hydroxide (NaOH) solution. Formation of yellow colour solution indicates presence of flavonoid.

3. Saponins (Foam test)

Approximately 1 mL of plant extract was added with 0.5 mL of distilled water in a test tube then closed with test tube stopper. The mixture was shaken vigorously for 5 minutes. Formation of foam indicates presence of saponin.

4. Tannins (Ferric chloride test)

Approximately 1 mL of plant extract was heated on a water bath for two minutes then cooled. Three drops of 10% ferric chloride ($FeCl_3$) solution were added into the extract. Formation of dark green colour indicates presence of tannin.

5. Steroids (Salkowski test)

Approximately 1 mL of plant extract was added with 0.5 mL of chloroform. Then 0.5 mL of concentrated sulfuric acid (H_2SO_4) was added drop by drop on the sides of the test tube containing the mixture. Formation of red or brown colour ring at the lower part of the chloroform layer indicates presence of steroid.

Preparation of treatment solutions

The study was conducted among six treatment groups which included positive control (sunitinib), negative control (phosphate buffer saline), aqueous *Moringa oleifera* extract (50% and 100%) and methanolic *Moringa oleifera* extract (50% and 100%).

Phosphate buffer saline (PBS) was prepared according to the formula from the United States Food and Drug Administration (FDA) [18]. The pH of PBS solution was measured using Eutech Instruments CyberScan pH 510 and adjusted to pH 7.4 by adding a few drops of NaOH or HCl. The PBS solution was sterilised by autoclave at 121 °C for 15 minutes. Sunitinib 0.01 µg/mL solution was prepared by serial dilution from the stock solution (100 mg/mL) using PBS.

The different concentrations of extracts namely, 50% methanol extract, 100% methanol extract, 50% aqueous extract and 100% aqueous extract were prepared by dissolving concentrated extract in PBS. The required amount of concentrated extract was weighed using an analytical balance and the concentrated extract was dissolved in PBS. The mixture was stirred until the concentrated extract has fully dissolved and stored in the refrigerator at 4 °C.

Preparation of CAM membrane for antiangiogenic screening

Fertilised chicken eggs were used in CAM assay. The fertilised eggs were purchased from a local hatchery farm at Klang. The eggs were cleaned by using wet sponge and tissues papers to remove any dirt, stain and marks on the surface of the shell. The incubator was disinfected with 70% alcohol to remove if any microbes present. Incubator was pre-treated for 3 days to ensure the temperature maintained inside falls within the range of 37.0 to 37.5 °C and humidity within the range of 55-60 %. The temperature and humidity of incubator were set at 37.5 °C and 55-60 % respectively [19].

Eggs were turned manually in horizontal direction three to four times daily for five days throughout the incubation period. This was done to

increase the viability rate of the embryo and prevent the sticking of CAM with the shell [20]. Egg candling (Figure 1) was performed on the fourth day (Day-4) of incubation by using a torchlight in a dark room to provide a clear visualization to identify the development of embryo.

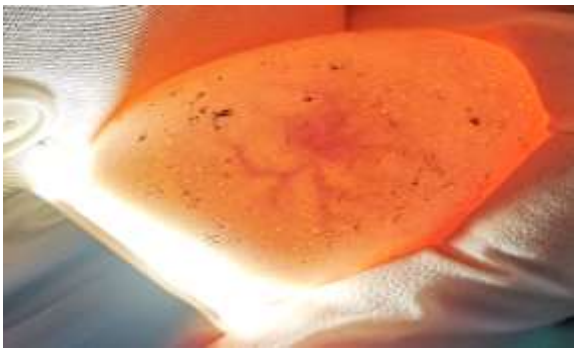


Figure 1. Egg candling

Air sac displacement and window grafting was done on the fifth day (Day-5) of incubation (Figure 2). To displace air sac, one small hole was made on the air sac area which is located at the blunt end of the egg and another small hole was made on the avascular area using a needle. Then, air in the air sac area was sucked out through the small hole of blunt end by using a rubber pipette pump.

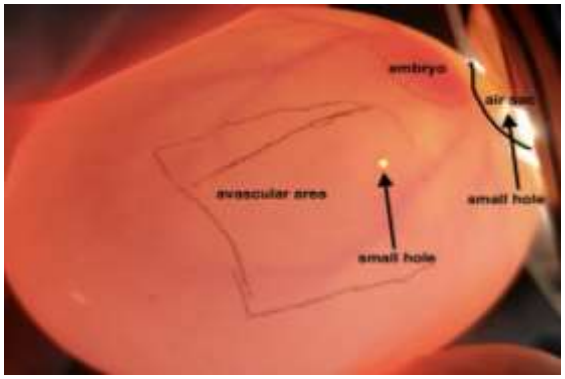


Figure 2. Regions identified during embryo development.

After successful air sac displacement, 1 cm x 1 cm square was drawn on the eggshell and cut with a penknife. The outer membrane located under the eggshell was gently ripped off with a forceps to expose CAM layer (Figure 3). Then, the window was sealed with paraffin tape and returned to the incubator [21,22].

Figure 3. Window for accessibility to CAM.

CAM assay

The eggs were divided into six groups and appropriate treatment solutions were applied on sixth day (Day-6) of incubation. It was carried out in Biosafety cabinet and all the apparatus required in the process were disinfected with 70% alcohol to minimize contamination. The treatment solutions were taken out from the refrigerator to reach room temperature before applying on the CAM.

The filter paper discs of 5 mm diameter were made using whatman filter paper. The discs were soaked into the treatment solutions and air-dried to remove the excess solution. Soaked and dried discs were applied onto the CAM membrane labelled appropriately (Figure 4). .

Figure 4. Application of sample treated filter paper disc on CAM.

After applying sample discs, the CAM was observed under a stereomicroscope and images were captured (Figure 5). The windows were sealed with paraffin tape and returned to incubator for further incubation and changes of blood vessels in CAM. The CAM layer was further observed under stereomicroscope after 24-hours and 48-hours of sample application which was noted as post-24 hours and post-48 hours changes respectively. Images of CAM after 24 and 48 hours were captured for further analysis.

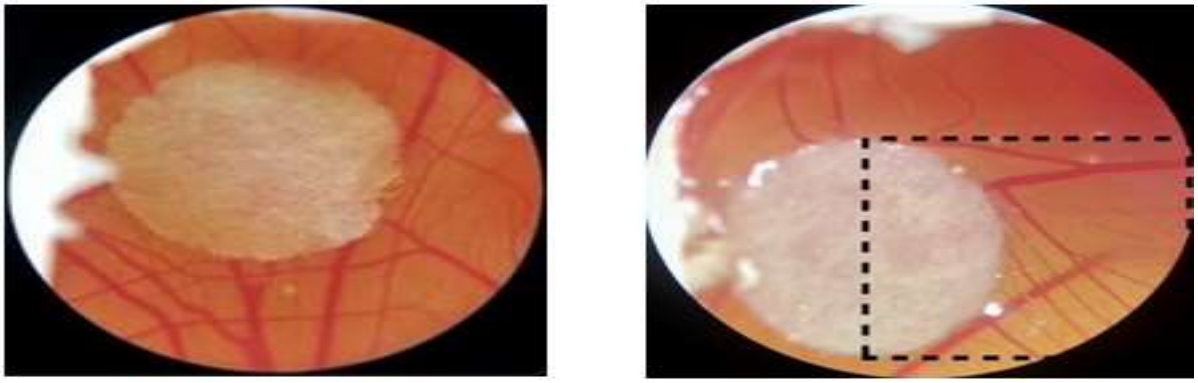


Figure 5. Observation of CAM under stereomicroscope.

The percentage increase/ decrease in blood vessels for post-24 and post-48 hours of treatment were calculated using the formula[23].

$$\text{Percentage increase/decrease in blood vessels} = \frac{\text{Difference in number of blood vessels after treatment}}{\text{Number of blood vessels before treatment}} \times 100\%$$

One-way ANOVA and post-hoc test analysis of data was done using statistical software SPSS version 26.

Results

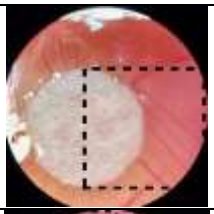
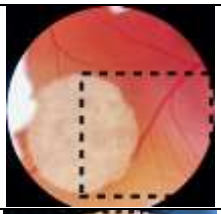
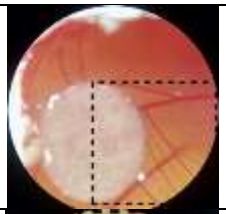
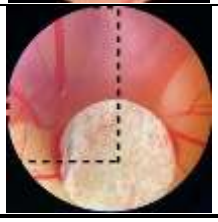
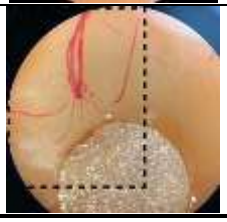
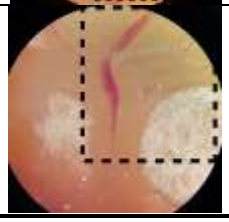
Pharmacognostical parameters

Table 1. Percentage yield, Physical characteristics and Phytochemical screening of *Moringa oleifera* leaves extracts.

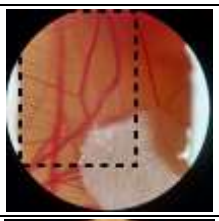
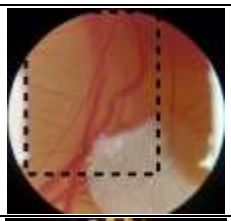
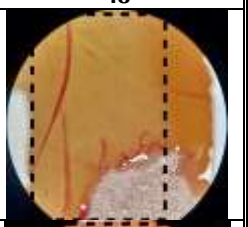
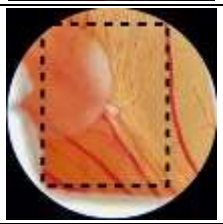
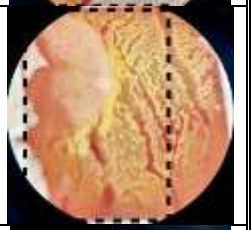
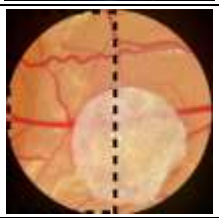
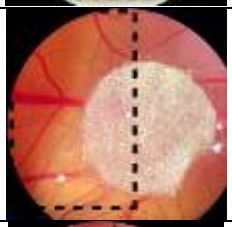
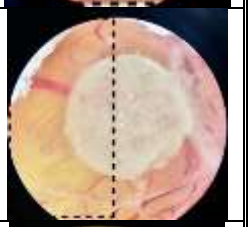
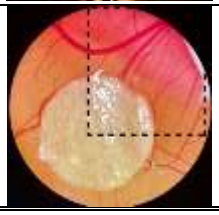
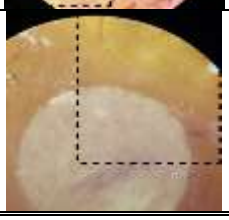
	Aqueous extract	**Methanol extract**
Extractive yield (g%)	11.27	1.68
Colour	Straw yellow	Dark green
Consistency of dried extracts	Fine powder	Coarse granules
Odour	Characteristic	Characteristic
Alkaloids (Dragendroff's test)	Present	Present
Alkaloids (Wagner's test)	Present	Present
Flavonoids (Alkaline test)	Present	Present
Saponins (Foam test)	Present	Present
Tannins (Ferric chloride test)	Present	Present
Steroids (Salkowski test)	Present	Present

CAM assay

Table 2. Changes in chorioallantoic membrane after sample treatment.

Treatment group	**Duration of treatment (hours)**		
	0	**24**	**48**
Negative Control			
Positive control			

Counited Table 2. Changes in chorioallantoic membrane after sample treatment.

Treatment group	Duration of treatment (hours)		
	0	24	48
Aqueous extract- 50%			
Aqueous extract- 100%			
Methanol extract- 50%			
Methanol extract- 100%			

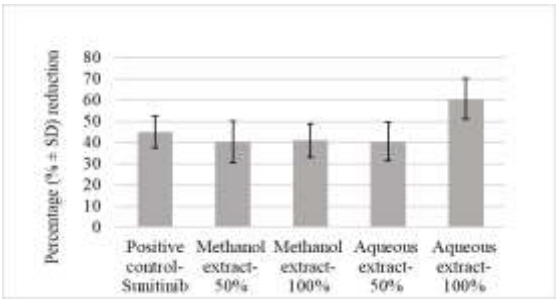


Figure 6. Percentage reduction in blood vessels post-24 hours of treatment

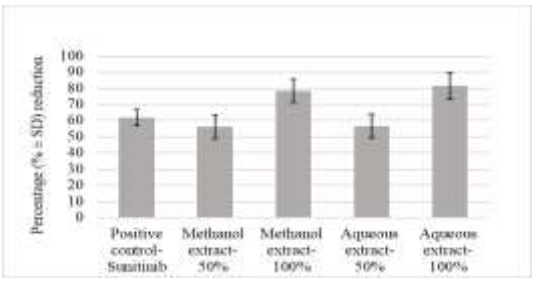


Figure 7. Percentage reduction in blood vessels post-48 hours of treatment

Discussion

Qualitative phytochemical testing revealed presence of various types of phytochemical components in both the extracts investigated (Table 1). Angiogenesis inhibition was assessed by counting the number of blood vessels. Decrease in the number of blood vessels in the area of interest reveals the inhibition angiogenesis. The negative control group showed no reduction in blood vessels, as an alternative the number of blood vessels has increased in post-24 and post-48 hours of treatment contributing to percentage increase in blood vessels of about 22.59% and 36.83% respectively. Therefore, PBS which was used as solvent for dilution of extracts does not show any effect on angiogenesis and did not interfere with the normal angiogenesis process in the embryo. Hence, it can be concluded as PBS does not cause any damage to the cell and will not affect the cell growth [24].

Sunitinib is an angiogenesis inhibitor, approved by U.S Food and Drug Administration. It is a multitargeted receptor tyrosine kinase (RTK) inhibitor which mainly inhibits the PDGF receptor and VEGF receptor which is important in angiogenesis progression (25). IC_{50} of sunitinib for angiogenesis suppression was found to be 0.01µM

(26) and the same concentration of sunitinib was selected to compare the antiangiogenic potential of *Moringa oleifera* extracts. Hence, sunitinib served as positive control sample in this study.

Different concentrations of *Moringa oleifera* leaves extract (50% and 100%) showed reduction in the number of blood vessel on the sample treated region on CAM after 24 and 48 hours of treatment (Table 2). It was observed that aqueous extract with 100% concentration has the highest percentage reduction in blood vessels after 24 hours and 48 hours of treatment compared to other treatment groups. The treatment group with the weakest anti-angiogenesis effect was 50% methanol extract which has the lowest percentage reduction of blood vessels in post-24 hours and post-48 hours of treatment (Figure 6 and 7).

The one-way ANOVA test result and post-hoc test result for post-24 hours of treatment showed an insignificant difference in post-24 hours of treatment. But for the one-way ANOVA test result of post-48 hours there was statistically significant difference ($p<0.05$).

The anti-angiogenesis activity was found to increase with the increase of concentration of both aqueous and methanol extracts. This showed that *Moringa oleifera* leaves extracts possess concentration-dependent anti-angiogenesis effect. This research findings has encountered an agreement with the study of Pachava *et al.* [23] and Dharani *et al.* [27] that proposes the anti-angiogenesis properties of *Moringa oleifera* leaves were dose-dependent manner.

The main flavonoids found in *Moringa oleifera* leaves were myricetin, kaempferol and quercetin [28]. Kaempferol has been reported to possess anticancer activity against pancreatic cancer cells by suppressing cell proliferation and inducing cancer cell apoptosis [29]. In addition to the above mentioned mechanism, kaempferol also suppresses angiogenesis [30]. Luo et al. have reported that kaempferol causes angiogenesis inhibition in CAM. Further, he also concluded that kaempferol also responsible for inhibition of VEGF expression in ovarian cell lines [31]. Moreover another study has also reported anti-angiogenesis effect of kaempferol through regulated VEGF and FGF signalling pathway [32]. Furthermore, another flavonoid, quercetin has been reported to show anticancer activity in various types of cancer [33]. The mechanism of quercetin includes inhibition of cell proliferation, inhibition of angiogenesis, promote apoptosis in cancer cell and altering the cell cycle progression [34]. One of the study reported quercetin to have anti-angiogenesis effect by targeting VEGF-2 receptor thereby resulting in downregulation of AKT/mTOR/P70S6K signaling pathway that contributes to restrain of tumour growth [35]. In summary, both flavonoids were found to suppress VEGF receptor that leads to anti-angiogenesis activity. Qualitative analysis of the extracts in the present study also revealed presence of flavonoids which could have contributed for the anti-angiogenic property of *Moringa oleifera* leaves.

Conclusion

This study reports anti-angiogenesis effect of methanolic and aqueous extract of *Moringa oleifera* leaves screened by in-ovo CAM assay. Different phytochemicals including flavonoids, alkaloids, saponins, tannins and steroids are present in both the extracts. In present study, 100% aqueous *Moringa oleifera* leaves extract has shown significant ($p<0.05$) reduction in the number of blood vessels. Hence, it can be concluded that the antiangiogenic effect of aqueous extract is stronger compared to the standard sunitinib and methanol extract. This research finding also suggests that *Moringa oleifera* leaves extract has a potential anti-angiogenic effect which could be one of the reasons for its anticancer activities.

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