Original Article

Screening of Antiangiogenic Potential of Fresh *Moringa oleifera* Leaves Extract Using Chick Chorioallantoic Membrane Assay

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Abstract

Background: Angiogenesis is the formation of new blood vessels that supports the progression of cancer. Angiogenesis inhibition will inhibit metastasis and growth of tumour cells. *Moringa oleifera* leaves extracts have been reported to possess therapeutic effects like anticancer, antimicrobial, anti-inflammatory and hypotensive effects. Chick chorioallantoic membrane is an extraembryonic membrane which consists of high density of capillary networks. This enables it to be used in this research to screen the antiangiogenic potential of fresh *Moringa oleifera* leaves extracts. **Materials and Methods:** Preliminary phytochemical screenings were carried out on extracts. Fresh fertile chicken eggs were divided into different treatment groups. Sample treatment was given to the eggs on day six of incubation. Pre- and post- treatment images of the chorioallantoic membrane were taken using stereomicroscope. Percentage of blood vessels increased/ reduced after treatment were determined at the end of 24 and 48 hours of treatment. **Results:** Preliminary phytochemical screening of methanolic extract of *Moringa oleifera* leaves showed the presence of alkaloid, flavonoid, tannins, and steroid. The screening also revealed that aqueous *Moringa oleifera* leaves extracts contained alkaloid, flavonoid, saponins, tannins and steroid. Statistical analysis revealed that the antiangiogenic effect was increased with increased methanolic leaves extracts concentration ($p<0.05$). The analysis also found

that the antiangiogenic effect was not significantly increased with the increased in aqueous leaves extracts concentration ($p>0.05$). **Conclusion:** Methanolic and aqueous extracts of *Moringa oleifera* contain various phytochemicals that inhibits angiogenesis.

Keywords: Antiangiogenesis, CAM assay, *Moringa oleifera*.

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Introduction

Cancer cell undergoes rapid and uncontrolled cell division that has the ability to metastasise to different parts of the body. Tumour cells stimulate angiogenesis by releasing angiogenic growth factors like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). These factors induce endothelial sprouting and formation of new blood vessels. In few conditions, an imbalance of angiogenic and anti-angiogenic factors, results in uncontrolled growth of blood vessels that lead to development of cancer. [1-3]

Currently, patients show more interest towards natural medicine for prevention and treatment of ailments. [1,4] Exploration of natural reserves for their therapeutic potential might lead to more effective, safe and affordable treatment when compared with conventional therapy. Extensive research on medicinal plants is being conducted all over the world to meet the increasing demand for safe therapy. [5]

Moringa oleifera is a tree of Moringaceae plant family and rich in phytochemicals [6] with less reported toxicity.[7] Anti-cancer properties of *M. oleifera* leaves have been established by few research studies. However, anti-angiogenic potential of *M.*

oleifera is not reported till date. Hence, this research study is focussed on determining the angiogenesis inhibition property of the *M. oleifera* fresh leaves.

Chick choriallantoic membrane (CAM) is an extraembryonic membrane formed between day 4 and 5 of embryonic development of chick. The membrane is rich in vascular network of blood vessels. [8] During the embryonic development stage of days 11 and 12, the vascular system of CAM becomes highly angiogenic and new blood vessels are continuously formed. During this period CAM is highly responsive to proangiogenic and antiangiogenic stimuli. [2] CAM contains different extracellular matrix proteins which include type 1 collagen, fibronectin and laminin. [2,10] The presence of these proteins will create and mimic cancer cell environments. Also, CAM is an immunocompromised vascular tissue that is suitable for screening proangiogenic and antiangiogenic properties of compounds. Hence, in this research study, CAM assay was adapted to explore the anti-angiogenic potential of *M. oleifera* [9].

Materials and Methods

Plant extraction: Fresh *M. oleifera* leaves were collected and dried under shade. Dried leaves were coarsely powdered and approximately 30 g of the powder was boiled with methanol and distilled water separately for 6 hours in water baths. The filtrates were then concentrated and dried to get the final extracts.

Qualitative phytochemical screening : Preliminary screening for phytochemicals was carried out based on the standard procedure. [11,12] Extracts were dissolved in 5 mL of solvents and the solution was used for qualitative analysis of alkaloids, flavonoids, tannins, saponins and steroids.

CAM assay: Fresh fertile chicken eggs were collected from a nearby hatchery and the eggs were cleaned to remove the dirt sticking to the shell. They were divided into six groups for treatment with phosphate buffer saline (PBS), standard and

different concentrations of extracts. Eggs were incubated for 5 days at 37 - 37.5^{o}C and 55 - 60% humidity. On day 5, candling of eggs was done to check on viability and embryo growth. Eggs that showed development of embryo were taken to the process of window making on the same day. A window of 2 x 2 cm was made by removing the shell on each egg after displacing the CAM layer carefully. The eggs were sealed with airtight paraffin and were further incubated for another 24 hours.

On day six whatman filter paper discs soaked in sample solutions were placed onto the CAM according to the treatment groups. The eggs were further incubated for 24 and 48 hours after the treatment. Pre- and post-treatment CAM were observed under stereomicroscope and the images were taken and analysed manually by counting the number of blood vessels. Blood vessels that branched out from the main branch were considered for the analysis. Percentage reduction/ increase in blood vessels was calculated using the formula:

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

Statistical analysis : Statistical analysis was carried out by using IBM SPSS v.26 software. One-way Anova was chosen to determine the statistical significance between different treatment groups. Tukey post-hoc test was carried out to determine the significant difference between each treatment group.

Results

Fresh leaves of *M. oleifera* were dried in shade for 1 week, extracted and their yield and physicochemical characteristics are reported in Table 1.

Table 1: Yield and Physical characteristics of *M. oleifera* leaves extracts

	Methanolic extract	**Aqueous extract**

Yield (%)	2.52	19.60
Colour	Greenish dark	Brown
Consistency	Sticky and oily	Powder
Odour	Characteristic odour	Characteristic odour

Aqueous and methanol extracts were screened for phytochemicals and are reported in Table 2. The screening was done to identify the groups of phytochemicals present in the extracts.

Table 2: Qualitative Phytochemical Screening of *M. oleifera* leaves extracts

Phytochemials (Tests)	**Methanolic**	**Aqueous**
Alkaloid (Dragendorff's Test)	+	+
Flavonoid (Sodium Hydroxide Test)	+	+
Saponins (Foam Test)	-	+
Tannins (Ferric Chloride Test)	+	+
Steroids (Salkowski Test)	+	+

+ = Presence - = Absence

Photographic images of CAM layer of each sample before and after application of the study compound were captured. The region was assessed again after 24 and 48 hours of sample application. The change in the number of blood vessels is reported in Table 3.

Table 3: Percentage change in blood vessels on the CAM region of interest

Groups	% change in blood vessels (Mean ± SD)	
	Post-24 hours	Post-48 hours
Negative control	42.48 ± 4.74*	65.85 ± 7.04*
Positive control	42.39 ± 5.43#	57.19 ± 5.29#
50% methanolic extract	21.73 ± 4.45#	52.94 ± 6.58#
100% methanolic extract	64.20 ± 6.25#	89.07 ± 6.66#
50% aqueous extract	25.57 ± 5.39#	52.70 ± 7.19#
100% aqueous extract	38.22 ± 4.77#	66.64 ± 6.49#

* Percentage increase in blood vessels.

Percentage decrease in blood vessels.

Statistical analysis between treatment groups and their significance values are presented as pictographic presentation in Figure 1 and 2 below.

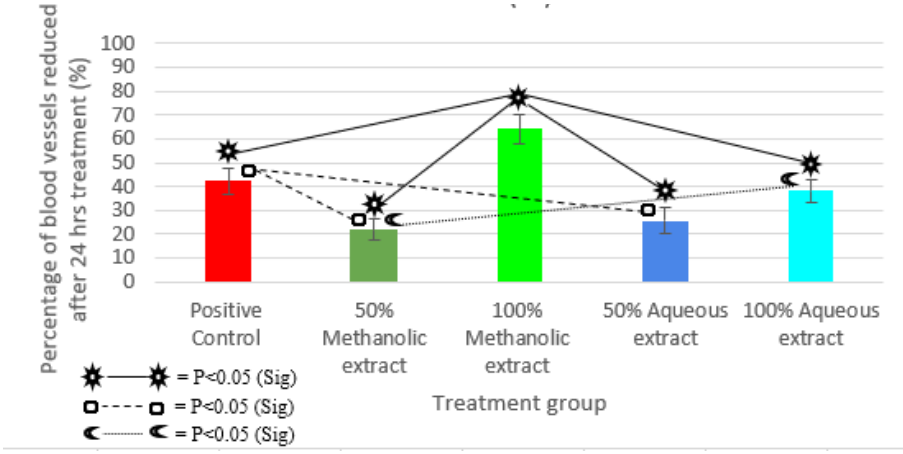

Figure 1: Comparison of percentage reduction in blood vessels after 24 hours of treatment

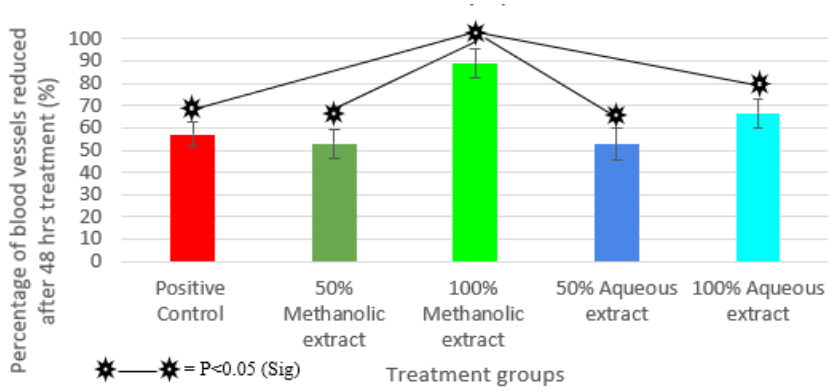

Figure 2: Comparison of percentage reduction in blood vessels after 48 hours of treatment

Discussion

Phytochemicals present in *M. oleifera* fresh leaves were extracted by decoction method of extraction using methanol and distilled water. The percentage yield and physical characteristics of the completely dried extracts are presented in Table 1. The aqueous extracts yield (19.60%) was found to be higher than methanolic extracts (2.52%) which explains that the leaves of *M. oleifera* are rich in water soluble components. Methanolic extract was sticky and oily after complete drying. This could be due to the presence of steroids or any other oily constituents that are easily soluble in methanol.

Preliminary qualitative analysis revealed the presence of various phytochemicals. Both the extracts contained alkaloids, flavonoids, tannins and steroids. Additionally, aqueous extract also has saponins which are absent in the methanolic extract. These phytochemicals have already been studied by various researchers and have proven pharmacological benefits.[12-14] The most common flavonoid found in the leaves of *M. oleifera* include kaempferol and quercetin [14]. Flavonoids have been reported to possess antiangiogenic, antioxidant and antiproliferative

properties. Angiogenesis inhibition will prevent neo angiogenic processes and cause rapidly multiplying cancer cells deficient of oxygen and nutrients which will eventually lead to death of cancer cells by inducing apoptosis.[15,16]

Studies have proven that *M. oleifera* possess anticancer properties against cell lines of colorectal cancer and breast adenocarcinoma.[17,18] However, the antiangiogenic effect of the leaves has not been proven yet. Hence in this study CAM assay was carried out using chicken eggs to screen for antiangiogenic *M. oleifera* leaves extracts. Fertilised and incubated chicken eggs were divided into 6 groups of negative control, positive control, 50% methanolic extract, 100% methanolic extract, 50% aqueous extract and 100% aqueous extract. PBS was used as solvent to dissolve the standard drug and extract samples. PBS helps to moisturise cells, balance the pH and prevents shrivelling and rupturing of cells as it is non-toxic and isotonic to the cells. [19] Hence, negative control group was treated with PBS of pH 7.4 to nullify the solvent effect in the study. Sunitinib, tyrosine-kinase inhibitor is widely used clinically to treat various gastrointestinal tumours. It specifically inhibits VEGF receptor, PDGF receptor and proto-oncogenes [20] with the IC_{50} of 0.01 µM.[21] Hence, sunitinib was used as a standard drug to compare the antiangiogenic effect of extracts. Samples of negative control group showed an increase in blood vessels of about 42.48% after 24-hours of treatment and 65.85% after 48 hours of treatment (Table 3). This confirms that PBS did not show any effect on the process of angiogenesis, and the embryo development was not altered by PBS. However, 33% of negative control samples faced a survivability problem after making a window on the shells which may be due to exposure to the dust during the process of window making or contamination.

The antiangiogenic effects of the treatment groups were determined and percentage reduction in number of blood vessels after 24 and 48 hours of treatments were calculated and are tabulated in Table 3.

Sunitinib showed an average reduction of 42.39% and 57.19% after 24 hours and 48 hours of treatment, respectively. Out of four sample treatment groups the highest percentage reduction of blood vessels was observed in 100% methanolic leaf extract after 24- and 48-hours of treatment. An average of about 64.20% and 89.07% reduction of blood vessels was seen in 100% methanolic extract post- 24 and 48 hours of treatment. Whereas 50% methanolic extract showed less inhibition than 50% aqueous extract after 24 hours. After 48 hours of treatment, both 50% aqueous and methanolic extract groups showed similar inhibition of blood vessels. Aqueous extract of 100% concentration exerted weaker antiangiogenesis after 24-hours, whereas, after 48 hours it showed stronger antiangiogenic potential when compared with the standard sunitinib.

Based on this research findings antiangiogenic potential of *M. oleifera* fresh leaves extract can be sequenced as 100% methanolic extract > sunitinib > 100% aqueous extract > 50% aqueous extract > 50% methanolic extract post-24 hours treatment. Antiangiogenic potential post 48 hours treatment can be sequenced as 100% methanolic extract > 100% aqueous extract > sunitinib > 50% methanolic extract group > 50% aqueous extract.

Statistical analysis by one-way ANOVA showed that the treatment groups post-24 and post-48 hours of treatment displayed significant ($p<0.05$) data. Post-hoc test was also carried out to determine the significance between treatment study groups. Significance data is presented in Figure 1 and Figure 2 which shows that the study groups possess a comparable antiangiogenic effect with the standard agent. [22,23]

The objectives of this research were successfully achieved as various phytochemicals had been extracted out from the fresh *M. oleifera* leaves using methanol and distilled water. Studies have shown that the anticancer effect of *M. oleifera* leaves could mostly be due to the presence of phytochemicals like isothiocyanate, niazimicin and quercetin.[24, 25] Through this research, it is shown that *M. oleifera* fresh leaves extracts possess

antiangiogenic properties and an attempt to fill the research gap by starting another path in understanding the anticancer mechanism has been initiated.

Conclusion

This study is preliminary research that proves antiangiogenic potential of fresh *M. oleifera* leaves extracts. Methanolic and aqueous extracts contain various phytochemicals that need to be isolated and elucidated for its structure. It is concluded that methanolic leaves extract shows higher antiangiogenic effect than aqueous extract. However, more studies are needed to further evaluate the phytochemicals and antiangiogenic effects of *Moringa oleifera* leaves by overcoming the limitation of this study.

Limitations: Separation, isolation and quantification of phytochemicals was not done in the study. Another limitation is, in the CAM assay possibility for elimination of inflammatory triggers was not carried out. Hence, these inflammatory triggers could have influenced the process of angiogenesis. Advanced software can be used to quantify the antiangiogenic effects in the CAM like MountainsSPIP 8 software and Wimasis Image Analysis.

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