

Ethnomedicines' effects on the process of vascularisation in embryonated chicken eggs

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Angiogenesis (vascularisation) is a vivacious progression during embryonic development, which is required for the growth, development, wound healing and maintenance. However, the process may turn into pathological conditions; excessive angiogenesis occurs in diseases such as cancer, diabetic blindness, age related macular degeneration, rheumatoid arthritis, etc., whereas insufficient angiogenesis occurs in diseases like coronary artery disease and chronic wounds. Since ancient times, the human population has been dependent on plants for deriving medicines to cure diseases. In the present study, aqueous extract of 04 ethnomedicinal plants parts, *Butea monosperma* (Kamarkas), *Dioscorea hispida* (Beychandi), *Myristica fragrans* (Nutmeg) and *Mesua ferrea* (Nagkesar), were evaluated for modulation of angiogenesis in chorioallantoic membrane (CAM) of embryonated chicken eggs. Out of these 04, *B. monosperma* revealed mild inhibitory effects on angiogenesis. *M. fragrans* and *D. hispida* inhibited angiogenesis, but also showed detrimental effect on growth of normal CAM with irregular outgrowths in form of projections and uneven thickness. The *M. ferrea* revealed its potential as antiangiogenic material by inhibiting the vascularisation by reducing the number of blood vessels without effecting normal morphology of CAM tissue. The study paves way to screen ethnomedicinal wealth for screening of plant produce for therapeutic purposes in ameliorating angiogenic disorders.

Keywords: Anti-angiogenesis, Chorioallantoic membrane (CAM) assay, Digimizer, Ethnomedicines, Vascularisation

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Angiogenesis is a multistep process of sprouting, splitting and remodelling of new blood vessels from the pre-existing ones¹. The process of angiogenesis is obligatory for many important physiological procedures like growth, maintenance and development of body. Both, the insufficient or excessive angiogenesis are causative of pathological conditions². Excessive angiogenesis causes disorders like diabetic retinopathy, cancer, arthritis, age related macular degeneration etc.; whereas insufficient blood supply due to poor angiogenesis increases the risk of tissue death in several diseases such as ischemic chronic wound and coronary artery diseases. This process is also essential for bone repair³.

Since ancient times, plants have been utilized in treating several disorders in various traditional medicine systems throughout the world. The information regarding ethnobotany and traditional medicine practices plays a significant role in scientific research. Systemic screening and characterization of

folk plants and medicines could be an enriched source of new and yet undiscovered biologically active molecules^{4,5}. Plants and plant produce are known to contain several phytochemicals such as flavonoids, tannins, phlobatannins, steroids, saponins, alkaloids, terpenoids, quinones, cardiac glycosides and coumarin. These have potential to cure various diseases including those related to pathological angiogenesis⁶. A broad range of plants' phytochemicals/ secondary metabolites such as tannins⁷, flavonoids⁸, saponins⁹ etc. possess anti-angiogenic or pro-angiogenic properties.

India has rich ethnobotanical heritage and its ancient system of medical treatment 'Ayurveda' is famous throughout the globe. There are some ethnomedicinal plants, which have been evaluated for angiogenesis modulation such as *Argyrea elliptica*, *Daucus carota*, *Ipomoea fistulosa*, *Lea indica*, *Papaver rhoeas*, *Scandix pecten-veneris*, *Nasturtium officinale*, and *Cynara cardunculus* extracts; and are proven to diminish endothelial cells differentiation and capillary morphogenesis⁶⁻¹². In the present study,

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we investigated the effects of the different plant products of four ethnomedicinal plants, i.e., *Butea monosperma* (Kamarkas), *Myristica fragrans* (Nutmeg), *Dioscorea hispida* (Beychandi) and *Mesua ferrea* (Nagkesar); which are commonly used in India to maintain and improve the health.

B. monosperma is a common deciduous tree of Fabaceae family. In the Indian traditional system of medicine, a number of medicinal uses of its flowers are well known¹³. The gum is popularly known as kamarkas and is given post-partum to the ladies¹⁴. As an ethnomedicine, *B. monosperma* used by local inhabitants of Kathua district of Jammu and Kashmir (India) showed 100% fidelity level, indicator of high healing potential, for treating urological disorders¹⁵. Also, it is widely used for healing wounds in Ayurvedic medicine system.

Traditional uses of *D. hispida* includes its usage against arthritic and rheumatic pains¹⁶. In Odisha (India), the tubers are used for birth control as it contains dioscorine, a lactonic alkaloid, which is a component of birth control pills¹⁷. Jeypore tract people, in same state use its intoxicating effect, to get an effect similar to drinking alcohol¹⁸. In Chinese medicinal system, tuber decoction is used as post-partum remedy and roasted pounded tubers are applied in form of paste to cure wounds and injuries.

Since long, *M. fragrans* and its oil are being used in Indian traditional medicine systems for nervous and digestive systems and also as condiment¹⁹. Nag clan of the Rai Ghatual tribal community of Moulvibazar district of Bangladesh, use it for fever and to reduce menstrual pain²⁰. It is a stimulant, carminative, astringent and aphrodisiac; but when large amounts are taken, it has toxic effect and cause hallucination. Myristicin is considered to be responsible for this effect. It also has anti-depressant effects²¹. In Ayurveda, *M. ferrea* has been used for protecting and stabilizing conception, bleeding piles, gastritis, bronchitis, sexual debility etc.²² It also has anti-arthritic properties²³. It may be used as single medicine to treat vessel related disorders like excessive menstrual bleeding and bleeding piles. It

has wound healing capacity and able to contract wound and increase epithelialisation in rat wound model, possibly due to presence of tannins²⁴.

These plants have diverse uses through ancient medicine systems in curing several human ailments. In Ayurveda, *B. monosperma* (Kamarkas), *M. fragrans* (Nutmeg), and *M. ferrea* (Nagkesar) are given with other medicines in form of Battisa powder (a mixture of 32 herbal medicines), where *D. hispida* (Beychandi) is given as a part of Chinese medicinal system, to mothers post-partum. All the four ethnomedicines are described in the Ayurvedic and Chinese medicine system to have positive effects on uterine and menstrual health. In the present study, we evaluated the efficacy of these 04 medicinal plants in modulating angiogenesis (the process of blood vessel formation), to treat angiogenesis associated disorders.

Materials and methods

Plant Materials

Different plant produces, such as gum (*Butea monosperma*), tuber (*Dioscorea hispida*), seeds (*Myristica fragrans*) and dried stamens (*Mesua ferrea*) were used in the present investigations; these plant materials were procured from traditional medicine vendors (*Pansari*) from the local market of Bhopal (Madhya Pradesh).

Aqueous extract preparation

All the plant materials were thoroughly rinsed with pH balanced water, shade dried and powdered. Two-grams powder of each plant produce was heated at 70°C with 50 mL of double distilled water for 1 h and filtered.

Characterization of Plant Material

These plant materials were tested for the presence of various secondary metabolites following standard published protocols for tannins²⁵, anthocyanins²⁶, steroids²⁷, terpenoids²⁸, flavonoids²⁹, leucoanthocyanins³⁰, coumarins³¹ and saponins³². Based on the intensity of the colour produced by the test material, the quantity of metabolite was designated in range from lowest to highest (single + to +++) by three blind observations (Table 1).

Table 1 — Secondary Metabolites present among 04 ethnomedicinal plants.

Plant of ethnic importance	Anthocyanins	Tannins	Steroids	Terpenoids	Leuco-anthocyanins	Coumarins	Flavonoids	Saponins
<i>Butea monosperma</i>	+	-	-	-	+	+	+++	+
<i>Dioscorea hispida</i>	-	-	-	-	-	-	+	-
<i>Myristica fragrans</i>	-	+	+	-	-	-	-	-
<i>Mesua ferrea</i>	-	++	+	-	-	-	-	+++

Test Material

The fertile, clean embryonated chicken eggs (9-11 days of age) were selected for the experiments. These were surface sterilized with 70% ethanol. Fifty eggs were divided into 05 groups (10 eggs in each group). The eggs were candled for screening of viability. The control group was treated only with PBS containing antibiotic/antimycotic solution; other four groups were treated with aqueous extract of *B. monosperma*, *D. hispida*, *M. fragrans*, and *M. ferrea* respectively.

Chorioallantoic membrane (CAM) assay

The modulating angiogenic effects of different ethnomedicines were evaluated on chorioallantoic membrane (CAM) of embryonated chicken eggs. A small window of 1.0 cm² was made in the egg shell. A sterile Whatman paper disk, impregnated with 0.2 mL of aqueous extract, antibiotics (ampicillin 50 µg/mL, Streptomycin 10 µg/mL) and antimycotic solution (amphotericin 10 µg/mL) was placed over the surface CAM. The window was sealed with the parafilm and incubated for 72 h in humid chamber at 37°C.

After incubation, the egg shells were opened and observed for vascularisation. Gross examination was recorded with Nikon D5100 camera. Small pieces of CAM were excised (10 per egg); placed onto a glass coverslip and examined with a drop of xylene, under 5x microscopic field. The harvested CAM tissue was fixed in neutral buffered formalin (10%) and subjected to histopathological studies following

standard haematoxylin and eosin staining method. Histopathological slides were observed under 20x and 200x magnifications.

Software applied

The number of branch points of blood vessels were counted using Digimizer image analysis software (<https://www.digimizer.com/>). Using this software, the width of CAM tissue was measured at 8 different points and means were calculated. Also, the area occupied by blood vessels with perimeter and width was also calculated. All the calculations were done by measuring pixels (px) as unit of measurement.

Statistical analysis

One-way analysis of variance (ANOVA) for the variance between the group and pairwise comparison of all means was done using Tukey's test. Mann-Whitney U test was also applied along with ANOVA for further validation.

Results

The results of phytochemical analysis have been depicted in Table 1. *B. monosperma* was found to be rich in flavonoids, whereas *M. ferrea* was rich in tannins and saponins. Mild flavonoids were revealed in *D. hispida* and in *M. fragrans*, mild tannins and steroids were observed (Table 1).

The shell of treated eggs was opened, examined and compared with the control (Fig. 1a). In comparison to control, out of the four treated

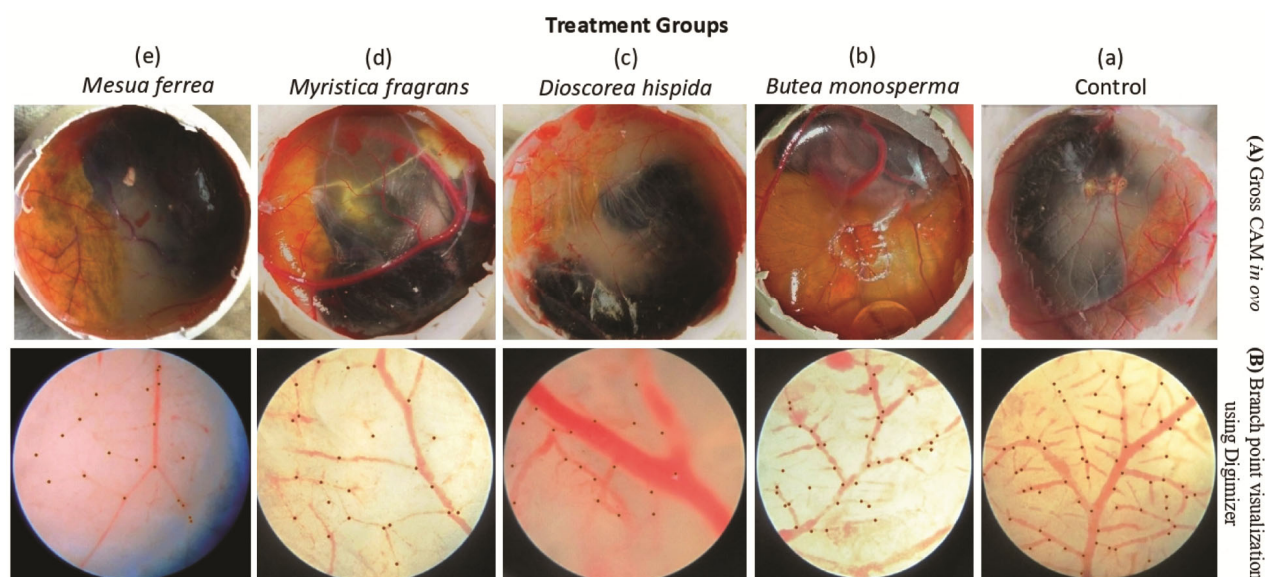


Fig. 1 — (A) *In ovo* CAM of control (a) and treated eggs (b-e) (B) visualization and analysis of branch points using Digimizer showing well arborized vascular system in control group; *Butea monosperma* treated group (b) exhibited very thin blood vessels; however, reduction in the secondary and tertiary blood vessels observed in CAMs treated with *Dioscorea hispida* (c), *Myristica fragrans* (d) and *Mesua ferrea* (e).

groups, *B. monosperma* treated group exhibited very thin blood vessels; however, the number of blood vessels was not remarkably reduced (Fig. 1b). Reduction in the number of blood vessels was observed in CAMs treated with *D. hispida* (Fig. 1c), *M. fragrans* (Fig. 1d) and *M. ferrea* (Fig. 1e) in comparison to control (Fig. 1a).

The CAMs were observed under microscope (20x) and analysed through Digimizer; an average of 66 branch points have been observed for control; whereas 40, 24, 20, 17 branch points were observed in *B. monosperma*, *M. fragrans*, *M. ferrea* and *D. hispida* respectively (Fig. 1). The comparison of the width of CAM, area and perimeter of the blood vessels among control and treated groups were analysed (Fig. 2); which reveals that the width of CAM of *D. hispida* treated group was significantly higher than other treated groups including the control CAM ($p < 0.01$). The minimum value for the width of CAM was observed in *B. monosperma* treated group (Fig. 2A). The mean area of a single vessel was recorded maximum in the group treated with *M. fragrans*, followed by control and lowest in *B. monosperma* treated group (Fig. 2B). The mean perimeter of blood vessel was maximum among *M. fragrans* treated group ($p < 0.01$) and lowest in *Mesua ferrea*. The mean perimeter of the vessels of *B. monosperma* and *M. ferrea* treated groups were lesser than the control (Fig. 2C). In comparison to controls as well among all the treated groups, number of blood vessels was lowest and tiniest in *M. ferrea* treated group. The results of Mann-Whitney U test have been depicted in Table 2, indicating that *D. hispida* had significant impact on the width of CAM, whilst other treatment groups did not significantly alter the width of the CAM. However, both *B. monosperma* and *M. ferrea*, reduced the area of vessels significantly, only *M. ferrea* reduced the perimeter of vessel (Table 2).

The CAM treated with *B. monosperma* extract exhibited regular thickness along with normal vasculature (Fig. 3B); whereas the *D. hispida* treated CAM showed the presence of abnormal growth and projections in the CAM tissue (Fig. 3C). The CAM

was abruptly irregular in thickness at different places with sparsely distributed blood vessels; indicating detrimental effects of *D. hispida* extract on CAM tissue growth as well as angiogenesis (Fig. 3C). The *M. fragrans* also exhibited abnormal projections and irregular thickening of CAM tissue (Fig. 3D). Contrary to *D. hispida* and *M. fragrans*; *M. ferrea* treated group had no detrimental effect on CAM tissue growth, and still the number of blood vessel branch points per magnification field was less (Fig. 3E).

Discussion

Since long, plants have been the source of bioactive compounds; several such compounds, also

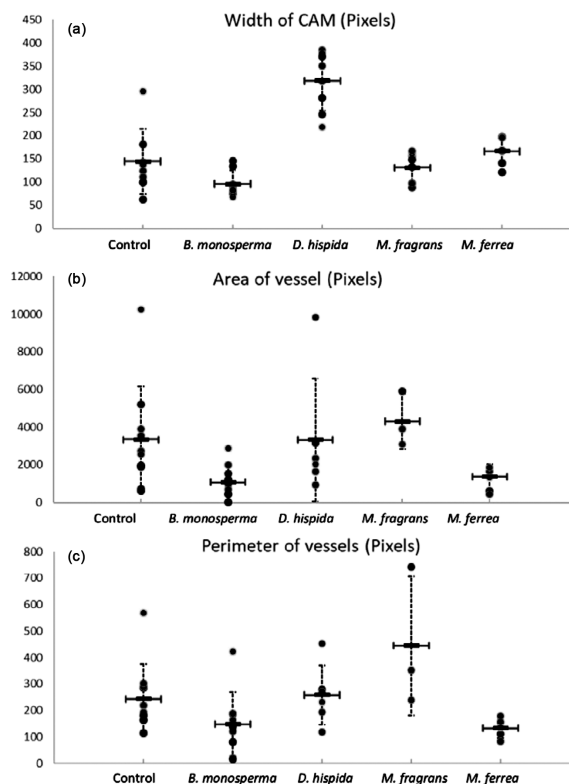


Fig. 2 — The CAMs were observed under microscope (20x) and analysed through Digimizer, the overall comparison of (A) the width of CAM, (B) area and (C) perimeter of blood vessels among the control and *Butea monosperma*, *Dioscorea hispida*, *Myristica fragrans* and *Mesua ferrea* treated group.

Table 2 — Mann-Whitney U Test to determine the anti-angiogenic effect.

	P-value			
	<i>Butea monosperma</i>	<i>Dioscorea hispida</i>	<i>Myristica fragrans</i>	<i>Mesua ferrea</i>
Width of CAM	0.1031	0.0028**	0.9601	0.1556
Area of vessel	0.0244*	0.8807	0.0735	0.0232*
Perimeter of vessel	0.0784	0.7872	0.0989	0.0323*

** $p < 0.01$; * $p < 0.05$

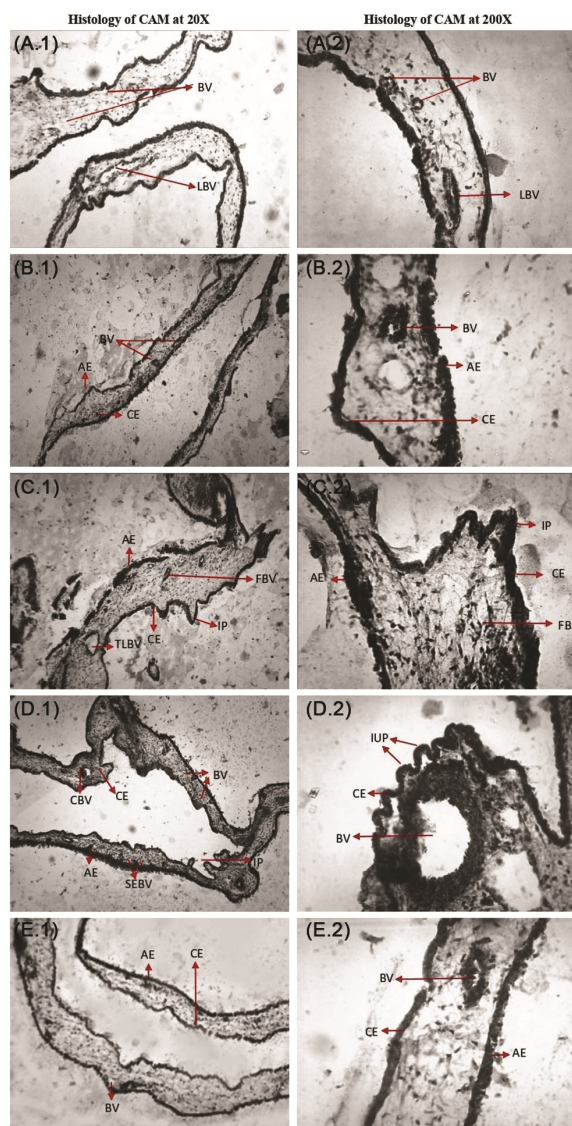


Fig. 3 — (A) Sections of CAM of the control (A.1 at 20x and A.2 at 200x) appeared to have thin chorionic (CE) and allantoic epithelium (AE) layer due to minimal accumulation of fibroblast cells (FB) with the presence of uniformly dispersed large blood vessels (LBV) and vein (V). (B) *Butea monosperma* treated group showed similar histology with presence of uniformly scattered blood vessels and veins. (C) *Dioscorea hispida* treated CAM revealed abnormal and irregular thickness with increased width of allantoic and chorionic epithelium. Chorionic epithelium showed irregular projections (IP) of uneven size. Instead of circular or oval, triangular large blood vessels (TLBV) and flattened blood vessels (FBV) were visible. (D) *Myristica fragrans* treated CAM abnormal projections were present at site of centrally located circular blood vessel with thickened AE and CE. Presence of numerous sub epithelial blood vessels (SEBV) beneath the AE was peculiar feature. Irregular unguilate projections (IUP) were visible at 200X. Also, abnormally large sized veins (LV) were visible. (E) *Mesua ferrea* treated group scanty blood vessels were visible.

referred as nutraceuticals, are being tested for their potential clinical applications. Though, their

effectiveness still needs to be validated in scientific trials³³. The present study is an attempt to evaluate the effects of the 04 common ethnomedicines, i.e., *B. monosperma* gum, *D. hispida* tuber, *M. fragrans* seed and *M. ferrea* dried stamens on the modulation of angiogenesis. All these medicines are given to mothers post-partum to retain good uterine and menstrual health in traditional medicinal systems. In humans, physiological formation of new blood vessels takes place only during wound healing or during menstruation, otherwise it happens only in case of pathology like cancer, diabetic blindness, age related macular degeneration, rheumatoid arthritis or coronary artery diseases.

Artemisia annua, *Viscum album*, *Curcuma longa*, *Scutellaria baicalensis*, *Camellia sinensis*, *Magnolia officinalis*, *Ginkgo biloba*, *Zingiber officinale*, *Rabdosia rubescens* hora, *Panax ginseng* etc. are the natural medicines which revealed anti-angiogenic properties through modulating gene expression, signal processing, and enzyme activities³⁴. Research on angiogenesis relies on diverse assessments including in vitro, in vivo and in ovo models. In vitro experiments include Boyden chamber assay for endothelial migration^{35,36}, scratch wound assay for endothelial migration^{37,38}, microtissue assay^{39,40}, cell migration and capillary-like Formation⁴¹, cell sheet assay^{42,43} etc.; in vivo model includes corneal assay⁴⁴, zebrafish assay⁴⁵, and aortic ring assay⁴⁶. In ovo model is also very popular among researchers due to ease in handling, possibilities to manipulate during experimentation, less requirement of technical expertise and low cost and ethical permissions are not required. The chorioallantoic membrane (CAM), of embryonated egg is an extraembryonic membrane, serves as a gaseous exchange surface. Due to extensive vascularisation, CAM has been used to study morpho-functional aspects of the angiogenesis process to study the efficacy and mechanism of action of pro- and anti-angiogenic molecules⁴⁷.

The flower extract of *B. monosperma* is known to heal wounds, which is a pro-angiogenic activity⁴⁸, however in our experiment, little reduction in the number of vessels has been observed (40 branch points) with small area and diameter as evidenced by data obtained through Digimizer software. Due to higher number of branch points, despite having vessels with less area and perimeter and having no detrimental effect of CAM, *B. monosperma* can't be used as anti-angiogenic treatment. A species of

Dioscorea found in China, i.e., *D. hypoglauca*, in combination with other herbs has been reported to possess anti-angiogenic effect in collagen-induced arthritis in rats⁴⁹; our results also augment the result of Li *et al.*⁴⁹, since we also observed that *D. hispida* had anti-angiogenic effects. Abnormally triangular and flattened blood vessels in the mesoderm of CAM tissue exhibited angiogenesis inhibition⁵⁰. Though the extract showed anti-angiogenic property by reducing the number of blood vessel with area of perimeter of blood vessels similar to that of control vessels, it also had detrimental effect on normal physiology of CAM tissue. The CAM tissue revealed varied thickness at varied places and accumulation of fibroblast. The mean width of CAM tissue was highest among the treated group. The abnormal irregular projections seen in chorionic ectoderm tissue indicated detrimental effect of extract on normal CAM tissue growth, so the extract can't be used in treating angiogenesis related ailments.

M. fragrans contains potential antiangiogenic compounds such as myristicin, limonene, eugenol and terpinen-4-ol⁵¹. Its oil is having antioxidant property and inhibition of reactive oxygen species (ROS), (which is important angiogenesis mediator), may be important in preventing angiogenesis^{51,52}. Its methanolic extract inhibits the *in vitro* proliferation of human umbilical vein endothelial cells (HUVECs)⁵³. However, Contradictory results have been obtained by Cuong *et al.*⁵⁴, who isolated malabaricone C, a natural diarylnonanoids from *M. fragrans*, and the compound has been shown to have pro-angiogenic and cell proliferating role⁵⁵. Supercritical *M. fragrans* oil, extracted at low temperature between 40-50°C and high pressure significantly inhibited angiogenesis in a rat aorta ring assay with significant non-toxicity⁵⁶. Our results were in concordance with the previous research of Piaruet *al.*⁵¹ and revealed anti-angiogenic activity. At histological level also, anti-angiogenic properties were evidenced by presence of mesodermal blood vessels which failed to migrate to epidermis and these mesodermal blood vessels had highest perimeter (maximum in all treated CAMs). Its anti-angiogenic potential can't be explored in therapeutics, as it causes abnormal growth and irregular projection formation during the growth of CAM.

So far, *M. ferrea* has been used traditionally to treat bleeding piles and menorrhagia, possibly through its action on capillaries. Traditionally it is given to women post-partum in order to reduce heavy bleeding. However, it has not been tested

scientifically for its applicability in angiogenesis modulation. In our study, its extract caused reduction in number of branch points (20), with smallest vessels (in terms of perimeter and area); revealed its potential to be used in anti-angiogenic therapy. It allowed normal growth of CAM tissue and didn't altered the normal CAM morphology as evidenced by comparable width of *M. ferrea* treated CAM with control. It also reduced the area and perimeter of blood vessel showing reduction in angiogenesis along with unhindered normal CAM physiology. *M. ferrea* contained high amounts of saponins and the results are in concordance with results of previous studies⁵⁷⁻⁵⁹, which revealed anti-angiogenic activity of saponins from *Rumex hastatus*, *Maesa lanceolata*, *Momordica cymbalaria* and *Polygonum hydropiper*. Our results aid into the traditional knowledge, that possibly *M. ferrea* prevents excessive bleeding through inhibiting new blood vessel formation. The results obtained have also been buttressed by Mann-Whitney U test, where reduction in both the area as well as perimeter of blood vessel is seen in *M. ferrea* treated CAM, without detrimental effect on CAM in terms of width of CAM or any other abnormalities. These properties make it an ideal therapeutic ethnomedicine against angiogenesis disorders associated with excessive angiogenesis.

Conclusions

Out of the four-plant produce used in the present study, gum of *B. monosperma* revealed mild inhibitory effects on angiogenesis; where *M. fragrans* and *D. hispida* found to inhibit angiogenesis strongly but exerted detrimental effect on growth of normal CAM, which was evident with the irregular outgrowths in form of projections and uneven thickness of CAM. Out of the 04, only *M. ferrea* had no detrimental effect on normal morphology of CAM tissue (even thickness of CAM) and also reduced the number of blood vessel while reducing the area as well as the perimeter of blood vessel and inhibited the vascularisation. Hence, the *M. ferrea* can be utilized for the therapeutic purposes in ameliorating angiogenic disorders associated with excessive angiogenesis.

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