EVALUATION OF ANTI-ANGIOGENIC POTENTIAL OF MENTHA ARvensis LINN. LEAF EXTRACTS USING CHORIOALLANTOIC MEMBRANE ASSAY

Hemlata Sonawane¹, Ashwini Shinde² and Jaywant Jadhav³*

¹,²Dept. of Microbiology, Annasaheb Magar Mahavidyalaya, Hadapsar, Pune, India.
³*Cell Biology Section, Dept. of Zoology, Shivaji University, Kolhapur, 416 004, India.

ABSTRACT
Numerous plant extracts prevent cancer through angiogenesis suppression and are widely used in clinical practice. Mentha arvensis Linn is a popular vegetable; known to possess various medicinal properties including anti-inflammatory and anticancer effects. In the present study anti-angiogenic activity of methanol, ethanol and ethyl acetate extracts of M. arvensis leaves was evaluated using chick chorioallantoic membrane assay in vivo. The CAM treated with ethanol and methanol extracts of M. arvensis leaves with increasing concentrations showed significantly reduced total number of blood vessels with associated area. Treatment of ethyl acetate extracts of M. arvensis leaves also affected new blood vessel formation; although means are non-significantly different from normal and control CAMs. Ethanol extracts showed highest anti-angiogenic activity; followed by methanol and ethyl acetate extracts. Our results suggested that M. arvensis plant extracts effectively prevent CAM angiogenesis and could be potentially be useful in protection of cancer or other pathological conditions related to angiogenesis.

KEYWORDS: Mentha arvensis, Anti-angiogenic activity, chorioallantoic membrane, blood vessels.

INTRODUCTION
Angiogenesis is the formation of new blood vessels from an existing ones. It is controlled by the balance of many stimulating and inhibiting factors.¹ Any imbalance in the control of this complex system may promote numerous angiogenesis dependant diseases. It has fundamental
importance in several patho-physiological conditions including atherosclerosis, diabetic retinopathy, tumor growth and metastasis.\[^2\]

Various natural products derived from plants constitute a promising approach in drug discovery and development especially in cancer prevention. Numerous plant extracts exhibited angiogenic and angiostatic influence in chick CAM.\[^3,4\] *Mentha arvensis* L. belonging to Lamiaceae is cultivated throughout world for use as a vegetable. It is reported to have several pharmacological properties including anti-microbial, anti-allergic, anti-inflammatory and anticancer effects.\[^5-8\] Essential oils from some Mentha species showed cytotoxic effects in cell line.\[^9\]

The anticancer and anti-inflammatory activities of leaves of *M. arvensis* could be partly attributable to its anti-angiogenic activity, and that *M. arvensis* could be a promising candidate drug for the treatment of diseases with impaired angiogenesis. In the present study, we determined effect of methanol, ethanol and ethyl acetate extracts of *M. arvensis* leaves on angiogenesis by chick chorioallantoic membrane (CAM) model *in vivo*.

**MATERIALS AND METHODS**

**Plant material and preparation of extracts**

*M. arvensis* plant was collected from rural parts of Pune, MH, India. The leaves of *M. arvensis* were shed dried at room temperature and crushed in a blender and crude powder was extracted with methanol, ethanol and ethyl acetate for 48 hrs and filtered through Whatman filter paper (No.1). The extracts were evaporated at 37ºC. Finally extracts were dissolved in phosphate buffer saline (PBS) to make a stock solution (1mg/ml). The extracts were subjected to the preliminary phytochemical investigation.

**Experimental Protocol: Chick Chorioallantoic Membrane (CAM) Assay**

Anti-angiogenic activity of methanol, ethanol and ethyl acetate extracts of leaves of *M. arvensis* was determined using *in vivo* chick chorioallantoic membrane assay. In brief, fertilized eggs of *Gallus gallus* chick embryo (0 hrs) were obtained from local source and were disinfected with 70% alcohol and incubated at 37ºC in 65% relative humidity. On day 2 of incubation 0.5 to 1ml of albumen was aspirated from eggs with a small hypodermic syringe through a small hole, allowing the developing CAM and yolk sac to drop away from the shell membrane; the space was sealed with adhesive tape. On 96 hrs of development, a small rectangular window was made in the blunt end of eggshell. The vascular zone was easily
identified on the CAM at 96 hrs of development (Figure 1). Two drops of saline was added to moisten the inner shell membrane adjacent to the CAM so that the membrane was easy to be separated from CAM.

A 5 x 5 mm sterilized filter paper discs were used to confine the test material to a defined area of the CAM. The discs were impregnated with different concentrations viz; 50, 100, 300 and 500µg/discs of methanol, ethanol and ethyl acetate extracts of *M. arvensis* leaves were directly applied on the vascular zone of the CAM away from the central blood vessels. Following disc application, the window in the eggshell was sealed with sterile adhesive tape and eggs were placed back into the incubator for further incubation. At least 5 eggs were used for each sample dose.

After 48 hrs of doses administration i.e. on 144 hrs of CAM development, embryos were removed from incubator and placed in glass bowls. The eggshell surrounding the window through which the filter disc was placed facing up, was carefully cut with a pair of dissecting scissors and removed to expose the underlying filter disc. CAM tissues were washed with 1ml PBS, spread on glass slide and the blood vessels were viewed in microscope and photographed. The images were transported to the computer for image analysis.

**Quantitation of angiogenesis**

Quantitation of angiogenesis was accomplished by counting the number of blood vessel branch points in the area of filter disc with the aid of stereomicroscope equipped with a digital camera. The relative number of the blood vessel branch points adjacent to the discs is indicative of the angiogenesis level (sprouts arising from pre-existing blood vessels). Vascular density was determined from each experimental condition and compared with normal and PBS treated control CAMs.

The percent of increase and inhibition was calculated using the following equation:

% *Increase*= \[\frac{(\text{Vessel Number of CAM treated by herb extract} - \text{Vessel number of CAM treated by PBS})}{\text{Vessel number of CAM treated by PBS}}\] x 100.

% *Inhibition*= \[\frac{(\text{Vessel Number of CAM treated by PBS} - \text{Vessel number of CAM treated by herb extract})}{\text{Vessel number of CAM treated by PBS}}\] x 100.
Histological preparation
For histological preparation CAM tissues were surgically removed; fixed in 10% formaldehyde for 12 hrs and was processed for light microscopy. The membrane was immersed on increasing concentrations of ethanol and infiltrated with paraffin (melting point 58-60°C). Serial sections (2µm) were cut in a plane parallel to the surface of the CAM and further processed for stained preparation of haematoxylin-eosin, which was observed under a light photomicroscope.

RESULTS
*M. arvensis* L. leaf extracts reduces vascular density in the chick CAM
To determine ant-angiogenic effect of *M. arvensis* leaves; different concentrations of methanol, ethanol and ethyl acetate extracts of *M. arvensis* leaves were administered at 96 hrs of CAM development and results were analyzed on 144 hrs by macroscopic, microscopic and quantitative evaluation that quantify vascular complexity.

![Image of CAM vessels](image.jpg)

**FIGURE 1.** The vasculature in normal and PBS treated control CAM. (A) The development of blood vessels in normal CAM at 48 hrs of incubation with embryo in inset. (B) Branch points and ends of blood vessels in normal CAM with tree like branching vessels in inset. (C) Filter discs impregnated with PBS was placed on CAM at 48 hrs of embryo development by window method. (D) Vascularized CAM in PBS treated control group. (E) Blood vessels with normal branching pattern in PBS treated control CAMs.

Macroscopic observations of normal CAM at 144 hrs of development showed normal angiogenesis with dendritic branching pattern of blood vessel formation at 144 hrs of development (**Fig. 1A&B**). Blood vessels were designated as primary, secondary and tertiary according to the branch points as explained earlier.[3] The PBS treated control CAMs showed
hardly any difference from normal CAM. It exhibited uninterrupted and clear directional
patterns of the blood vessel development. It showed numerous large and small blood vessels
with virtually no disturbance of CAM structure (Fig. 1D & E).

Treatment of ethanol extracts of *M. arvensis* exhibited progressive inhibition in blood vessel
formation and its branching pattern at all hrs of treatment studied (Fig. 2A-2H), but more
significant inhibition was noted at high doses i.e. at 300 and 500 µg dose of methanol extracts
of *M. arvensis* leaves. 500 µg dose treatment at 96 hrs of incubation showed complete
suppression of the angiogenesis, which was evidenced by a capillary less background. It
suppressed new blood vessel development not only in the filter disc area implanted but also
in the surrounding area (Fig. 2D & H). These structures were completely absent from the
normal and PBS control group and a normally developed vascular pattern was discerned.

**FIGURE 2: Effect of ethanol extracts of *M. arvensis* leaf on angiogenesis: Fig. A & E -
50µg dose of *M. arvensis* showed normal tree like branching pattern of blood vessel
development. Fig. B & F-100µg treatment reduced the branching of vessel network. Fig.
C & G-CAM treated with 300µg dose showed reduction in number of vessels with
frequent avascular zone. Fig. D & H-500µg extract treated CAM showing complete
suppression of the blood vessel development with a capillary less background.**

When above mentioned concentrations of methanol extracts of *M. arvensis* leaves were
administered at 96 hrs of embryonic development, it exhibited marked inhibition of new
small blood vessels inducing strong avascular zone more specifically at 500-µg doses (Fig. 3
D & H). Treatment of 50 µg and 100µg dose produced fewer branches and abnormal
branching patterns and appeared to decrease vessel diameter and vessel area. The vessels
were thinner and showed less branching (Fig. 3A, B, E & F); which was strongly noted at
500µg dose of treatment, where large avascular zone with free of vessels surrounding
implanted filter disc area was observed.
FIGURE 3: Effect of methanol extracts of *M. arvensis* leaf on angiogenesis: Fig. A & E - 50µg dose of *M. arvensis* showed inhibition of blood vessel numbers and branching pattern. Fig. B & F-100µg treatment showed inhibition of the formation of new blood vessels. Fig. C & G-CAM treated with 300µg dose exhibited distorted vascularization as well as perturbation on existing vasculature. Fig. D & H-500µg extract treated CAM showing complete suppression of the vessel formation.

Administration of 50µg dose of ethyl acetate extracts of *M. arvensis* did not alter the normal vasculature of CAM (Fig. 4A & E). Treatment of 100 and 300µg dose of ethyl acetate extracts of *M. arvensis* exhibited marginal suppression in capillaries (Fig. 4B, C, F & G). The pattern of vasculature showed pre-existing, medium and large sized vessels were unaffected. But treatment of 500µg dose of ethyl acetate extracts of *M. arvensis* caused marked inhibition of neovascularization (Fig. 4D&H).

FIGURE 4: Effect of ethyl acetate extracts of *M. arvensis* leaf on CAM angiogenesis. Angiogenesis was examined on 144 hrs of CAM development: Fig. A & E-50µg dose *M. arvensis* showed normal pattern of blood vessel development. Fig. B & F-100µg treatment showed similar observations with normal angiogenesis. Fig. C & G-Treatment of 300µg dose showed less branching with disruption of blood vessel development. Fig. D & H-Treatment of 500µg dose of *M. arvensis* leaf showed thin and less branching of blood vessels.
The results from quantitative analysis showed that PBS treatment promoted blood vessel formation which was remain unaffected by the treatment of 50µg dose of *M. arvensis* leaves extracts. But it was significantly reduced with increasing concentrations of ethanol and methanol extracts. This inhibitory response was also noted with ethyl acetate extract; but was non-significantly different from normal and PBS treated control CAMs.

The effect of mentha leaves extracts on formation of micro-vessels *in vivo* was also examined histologically. Transversal section of normal CAM at 144 hrs of development exhibited a chorionic epithelium with small blood vessels; a mesenchyme with medium sized blood vessels and allantoic epithelium (Fig. 5A & B). Three layered morphology was also observed in tissues treated with PBS as a control; where numerous capillaries were observed on the mesoderm close to the chorionic epithelium; which tend to extend towards mesoderm resulting increased vascular area in mesoderm (Fig. 5C). Tissue sections treated with methanol and ethanol extracts of mentha leaves at 50µg dose each showed a normal architecture of CAM vasculature without apparent alterations (Fig. 6A & C). However treatments of 300 and 500µg doses exhibited progressive reduction in CAM vascularization with reduced thickness of the CAM (Fig. 6B & E). At 500µg dose treatment significantly decreased and abnormally formed blood vessel formation were observed. Treatment of 50µg and 300µg doses of ethyl acetate extracts did not alter normal CAM vasculature considerably (Fig. 6 G & H); but 500µg dose induced alterations caused inhibition of neovascularization (Fig. 6I).
FIGURE 5. Histological structure of normal and control CAM: Fig. A & B. Normal CAM at 144 hrs of development showing three-layered morphology (1. ectoderm, 2. mesoderm and 3. endoderm) with inner shell membrane (ISM). Numerous blood vessels noted in mesoderm and beneath the ectoderm. Fig. C- PBS treated control CAM showing well-vascularized tissue with mesodermal vessels filled with RBCs.

FIGURE 6. Histological alterations in CAM angiogenesis by *M. arvensis* leaf extracts: CAMs treated with 50µg methanol extract (Fig. A), 300µg methanol extract (Fig. B), 500µg methanol extract (Fig. C), 50µg ethanol extract (Fig. D), 300µg ethanol extract (Fig. E), 500µg ethanol extract (Fig. F), 50µg ethyl acetate extract (Fig. G), 300µg ethyl acetate extract (Fig. H) & 500µg ethyl acetate extract (Fig. I).

Phytochemical screening

Table 1. Preliminary phytochemical screening of *M. arvensis* leaf extracts

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Preliminary phytochemical investigation of *M. arvensis* leaves revealed the presence of flavonoids, tannins, steroids, phenolic compounds, glycosides and sugars in methanol extract. Flavonoids, alkaloids, glycosides, sugars and tannins in ethanolic extract; while flavonoids, alkaloids, glycosides and sugars in ethyl acetate extract.

**DISCUSSION**

In the present study, a new pharmacological properties of *M. arvensis* have been investigated by evaluating inhibition of angiogenesis by *in vivo* chick CAM assay. From quantitative, macroscopic and microscopic analysis it is observed that *M. arvensis* leaves extracts has significant anti-angiogenic activity especially ethanol and methanol extracts at all studied concentrations and ethyl acetate extracts at high dose. All parameters evaluated (CAM structure, total branch points (vessels) and blood vessel pattern formation) are altered to varied degrees in a dose dependent manner. Out of three solvents used for extraction; ethanol extracts showed highest inhibitory activity in angiogenic response; followed by methanol and ethyl acetate extracts.

Macroscopically it is observed that normal CAM at 144 hrs of development showed normal pattern of blood vessel formation. PBS treatment did not affect vessel area when compared to normal CAM. Administration of ethanol and methanol extracts of *M. arvensis* through filter discs with increasing concentrations significantly reduced the total number of blood vessels. In both cases there was a dose dependent inhibitory effect on blood vessel formation without affecting embryo growth. Treatment of ethyl acetate extracts of *M. arvensis* leaves also affected blood vessel formation area; although means are non-significantly different from normal and control CAMs. Small tortuous blood vessels, typical of neovascularization are visible in a PBS control CAMs. These vessels are absent or substantially less visible in ethanol and ethyl extracts treated CAMs. More areas where plexus should form, appeared abnormal and normal dendritic branching is lost. This pattern of alterations is also observed in histological preparation; where newly formed large and small vessels in ectoderm and mesoderm region suppressed with strongly reduced CAM thickness by the administration of
300µg and 500µg doses of ethanol and methanol extracts. It is also evident in 500µg dose of ethyl acetate.

Various plant extracts and components are reported to inhibit angiogenesis and induce apoptosis in many tumor cell types.\textsuperscript{[10-13]} Here we investigated that leaves extracts of \textit{M. arvensis} inhibit angiogenesis in the CAM \textit{in vivo} in a dose dependent manner. The anti-angiogenic efficacy of \textit{M. arvensis} may be attributed to the phytoconstituents present in the plant. It could be function of either the individual or the additive effects of phytoconstituents. Different species of mentha having various biological properties indicating supportive action for anti-angiogenenic response. It has been suggested that essential oils prepared from \textit{M. arvensis} plant has good antimicrobial and cytotoxic potential.\textsuperscript{[14]} It has been also recently suggested that methanolic extracts of \textit{Mentha longifolia} have the potency to act as powerful antioxidants and protect against DNA damage and have cytotoxic activity against MCF-7 cell line. Hexane extract obtained from \textit{M. spicata} flower exhibited anti-neoplastic activity against KB and MCF-7, although an antiproliferative effect at a high concentration of the extract was observed toward NIH 3T3.\textsuperscript{[15]} Cytotoxicity of essential oils of \textit{M. spicata} on some cancer cells has also been reported\textsuperscript{[16]}.

Many polyphenols, terpenoids, triterpenoids and flavonoids have shown to inhibit proliferation and angiogenesis \textit{in vivo} and \textit{in vitro}.\textsuperscript{[17-21]} Moreover, several alkaloids are considered to be anti-angiogenic natural products.\textsuperscript{[22,23]} Flavonoids, triterpenoids, steroids, alkaloids and the related compounds are widely distributed in whole plant of \textit{M. arvensis}. It has been recently reported that reactive oxygen species (ROS) induce migration and proliferation of endothelial cells.\textsuperscript{[24]} The essential oils of some Mentha species including \textit{M. arvensis}, \textit{M. piperita}, \textit{M. longifolia} and \textit{M. spicata} are potential candidates for exhibiting antimicrobial, antioxidant, radical-scavenging and cytotoxic activities.\textsuperscript{[25-28]} Antioxidants serve as a potent inhibitor of angiogenesis.\textsuperscript{[29]} Such multiple biological activities of Mentha essential oils might be ascribed to the presence of some chemical components, such as menthone, piperitone oxide, camphor and linalool.\textsuperscript{[30-32]} The anti-inflammatory property of \textit{M. arvensis} which has been reported by Verma \textit{et al.}\textsuperscript{[5]}, Malik \textit{et al.}\textsuperscript{[6]} and Biswas \textit{et al.}\textsuperscript{[33]} might have played a synergic role in the inhibition of CAM angiogenesis as observed in the present investigation possibly via down-regulating the level of nitric oxide.\textsuperscript{[34]} It might have attenuate proliferation, migration and differentiation of endothelial cells.
These results suggest that *M. arvensis* leaves contain phytochemicals that inhibit angiogenesis and could be potentially be useful in the prevention of pathological conditions related to angiogenesis, however further study is needed to elucidate its anti-cancer effects and mechanisms of action under controlled clinical investigations.

**REFERENCES**


