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Anti-angiogenic Potential of *Mentha longifolia* (Horse Mint): Chlorioallantoic Membrane Assay

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Authors' contributions

This work was carried out in collaboration between all authors. Author NT performed the experiment. Author US wrote the article. Author BA did the final proof reading. All authors read and approved the final manuscript.

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ABSTRACT

Inhibition of angiogenesis is one of the mechanisms of action of anticancer drugs. The current study was designed to probe into anti-angiogenic effect of *Mentha longifolia* by employing Chorioallantoic membrane assay. White leghorn fertilized eggs were used in the study (n=10). On 6th day of incubation, aqueous solution of methanol extract (300-1000 µg/mL) of *Mentha longifolia* was applied to chicken embryos via CAM route. After 24 hours incubation, all eggs were opened carefully and CAM images were taken with camera. These images were loaded to SPIP software for quantification and assessment of structural changes in CAM. Blood vessels (primary, secondary and tertiary) diameters and CAM areas decreased in concentration dependent fashion in all treated groups as compared to values of control group. All embryos died at highest used concentration. It is concluded that methanol extract of *Mentha longifolia* possessed concentration dependent anti-angiogenic effect but toxic at 1000 µg/mL.

Keywords: Anti-angiogenesis; Mentha longifolia; CAM assay.

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1. INTRODUCTION

Angiogenesis has key role in cancer growth and metastasis. Judah Folkman was the first scientist who gave the idea of inhibition of angiogenesis as a site of action for chemotherapeutic drugs and it also help preventing metastasis [1]. A number of *in vitro* methods such as endothelial cell migration assay, endothelial cell proliferation assay, aortic ring assay and tube formation assay and *in vivo* methods like CAM assay, matrigel plug assay and corneal angiogenesis assay are available to assess angiogenic and/or anti-angiogenic activity of new compounds either synthetic or obtained from plant source [2].

Mentha longifolia is a perennial herb of Family: *Lamiaceae* [3]. *Mentha longifolia* is commonly called horse mint or wild mint [4]. Folklore use of *Mentha longifolia* is in secondary amenorrhea and oligomenorrhea in the form of syrup [5]. The number of pharmacological activities such as antioxitant [6,7], anticancer [7,8], antimicrobial [9], hepatoprotective [10], anti-inflammatory and antispasmodic [11] expose the medicinal significance of the plant.

Keeping in view the medicinal importance of *Mentha longifolia*, the current study was designed to evaluate anti-angiogenic effect of different concentrations of this herb by using *in vivo* Chorioallantoic membrane assay.

2. MATERIALS AND METHODS

2.1 Chemicals

Methanol, ethanol, formaldehyde (BDH), Buffered solution of 0.9% sodium hydroxide 0.9% (Merck) were purchased from local market.

2.2 Preparation of CAM

Fifty fertilized white leghorn chicken eggs purchased from a local hatchery (Big Bird-Lahore, Pakistan) were incubated in humidified incubator (humidity: 55–60%) at 37.5ºC, this incubator was rotated upto 60º angle at hourly basis. Ethanol (70%) was used to sterilize the egg surface. On day five of incubation, a two cm wide window was cut at blunt end of eggs and in order to separate the CAM. Albumin almost 4 mL was aspirated from each egg with sterile syringe (21 gauge). This step is important for better quantification of vasculature of Chorioallantoic membrane. Eggs were incubated for 24 h after sealing with sterile parafilm tape.

2.3 Plant Material

The aerial parts of *Mentha longifolia* L. were collected from suburbs of Jauhirabad-Pakistan. The plant was identified and authenticated (voucher no. 1786) by a Taxonomist of Botany Department, Govt. Postgraduate College Jauhirabad. The plant was dried under shade and ground to fine powder after drying.

2.4 Preparation of Extract

Maceration process was used to prepare methanolic extract of *Mentha longifolia.* The pulverized plant material (3kg) was soaked in seven liters of 70% methanol for three days with occasional shaking at room temperature. The extract was filtered with muslin cloth and solvent was removed with rotary evaporator [12,13].

2.5 Experimental Design

Fifty eggs were divided into five groups (n=10). Group I served as control, group II-V were administered hundred fifty microliter of sample solution at concentration 300 µg/mL, 500 µg/mL, 700 µg/mL and 1000 µg/mL respectively via CAM route on 6th day of their incubation. All the eggs were again incubated for next 24 h.

2.6 Preparation and Administration of Sample

Sample solutions of 300 µg/mL, 500 µg/mL, 700 µg/mL and 1000 µg/mL concentrations were prepared in distilled water. The pH of each solution was adjusted at 6.5 - 7.5. Hundred fifty microliter of each sample was applied over the growing embryos of all groups on 6th day of their incubation and again incubated for next 24 h.

2.7 Image Acquisition and Image Probing System (IPS) for the Quantification of Blood Vessels Growth over CAM

On 7^{th} day of incubation, chick embryos (n=10 per each group) of all the treated and control groups were collected in separate petri dishes along with their CAM and images were recorded with COOLPIX (Nikonchina): Lense-shift VR 16.0 megapixels and wide 10X zoom camera. All images were cropped in Adobe Photoshop 6.0 was used to crop images in order to make blood vessels prominent on each image. This cropped image was loaded to SPIP version 6.2.5 to measure 3D surface roughness parameter along with all related parameters to evaluate the anti-

angiogenic potential of extract. The x, y and z dimensions of each image were loaded to software for accurate quantification.

2.7 Statistical Analysis

Results were expressed as mean ± SD. For quantification of angiogenesis, SPIP software version 6.2.5 was used. ANOVA was applied using SPSS version 12.P < 0.05 was considered statistically significant.

3. RESULTS

Blood vessels diameter, CAM area, and anti-angiogenic activity were quantified at concentration ranging 300 µg/mL – 1000 µg/mL. The highest concentration (1000 µg/mL) was found embryo-toxic, it caused death of treated embryos. Thus, all the parameters were quantified at concentrations i.e. 300 µg/mL to 700 µg/mL.

Fig. 1 showing the concentration dependent decrease in diameters of as compared to that of control group.CAM area of control group was 1380 mm^2. There was significant decrease ($P < 0.05$) in CAM areas of treated groups as compared to CAM area of control (Fig. 1). The diameter of primary, secondary and tertiary blood vessels growing on the CAM was also reduced significantly ($P < 0.05$) with respect to diameter of blood vessels of control group (Fig. 2). This is indicative of anti-angiogenic potential of the plant.

3D surface roughness parameters quantify neovascularization on CAM. Average surface roughness of control was 81.1±1.0. There was reduction in average surface roughness of all the treated groups which confirm the anti-angiogenic power of the understudied plant (Table 1).

Anti-angiogenic activity of samples was determined by applying following formula:

$$Anti - angiogenic\ activity\ (\%) = \frac{CAM\ area\ of\ control - CAM\ area\ of\ sample}{CAM\ area\ of\ control}\ X\ 100$$

Methanol extract of *Mentha longifolia* at 300 µg/mL exhibited 5.07% anti-angiogenic activity. Whereas, 500 µg/mL and 700 µg/mL concentrations of *Mentha longifolia* displayed 9.42% and 11.01% anti-angiogenic activity (Fig. 3).

4. DISCUSSION

The CAM is very thin and highly rich vascular membrane. This is commonly used to probe into angiogenic and anti-angiogenic potential of plant extracts and new compounds because of its simplicity and easy handling [14-16]. This is simple *in-vivo* method which quantifies minor changes in CAM area and diameter of blood vessels; hence, the results of *in-vitro* techniques show visual score of angiogenesis [17,18]. Selection of appropriate parameters is required for quantitative evaluation of angiogenesis.

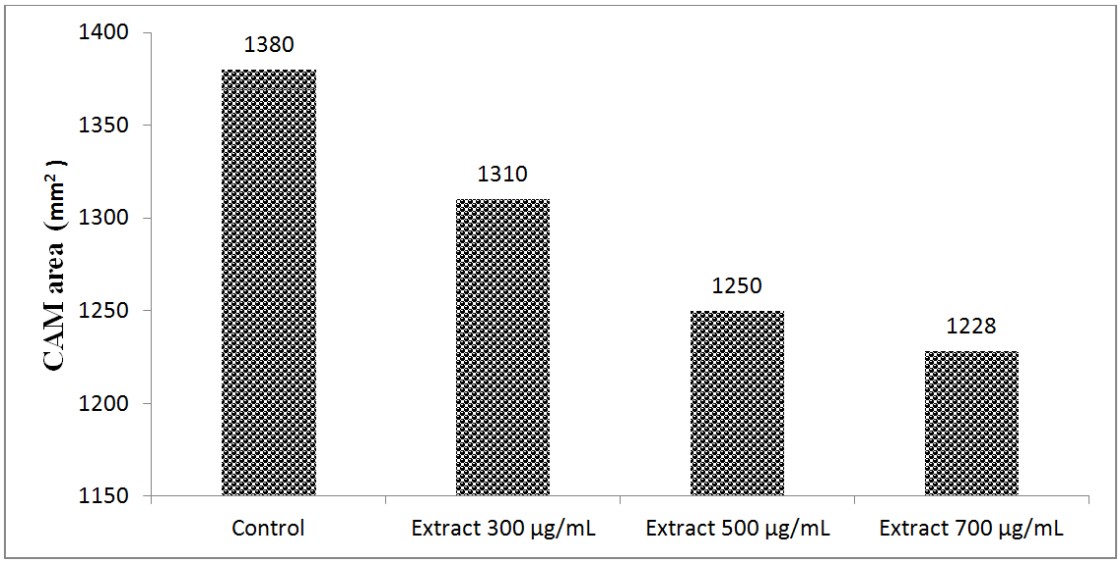

Fig. 1. Effect of different concentrations of methanol extract of *Mentha longifolia* on CAM areas

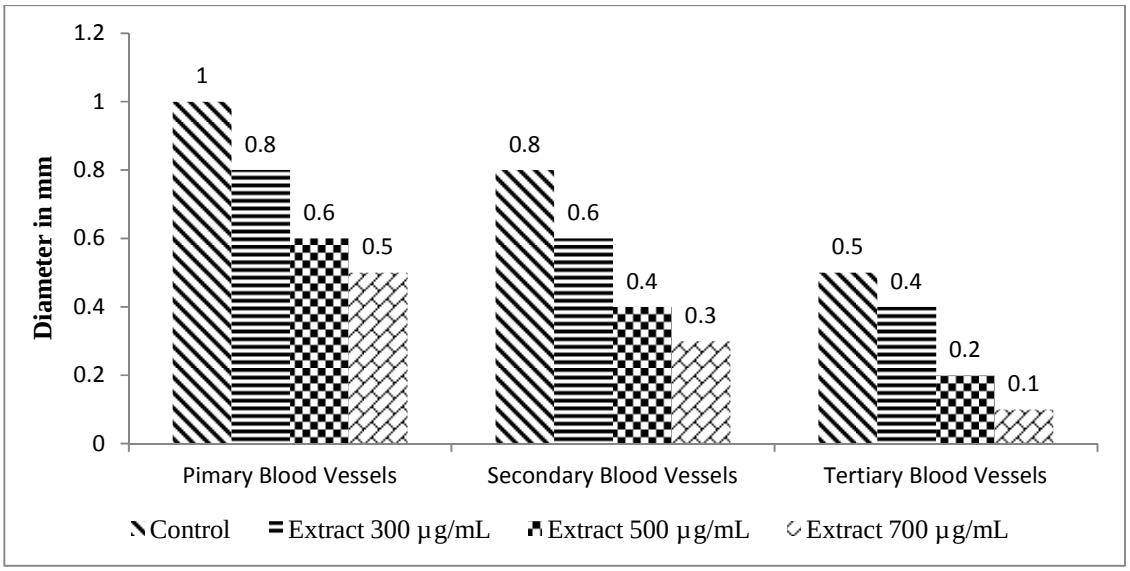

Fig. 2. Effect of different concentrations of methanol extract of *Mentha longifolia* on diameter of primary, secondary and tertiary blood vessels

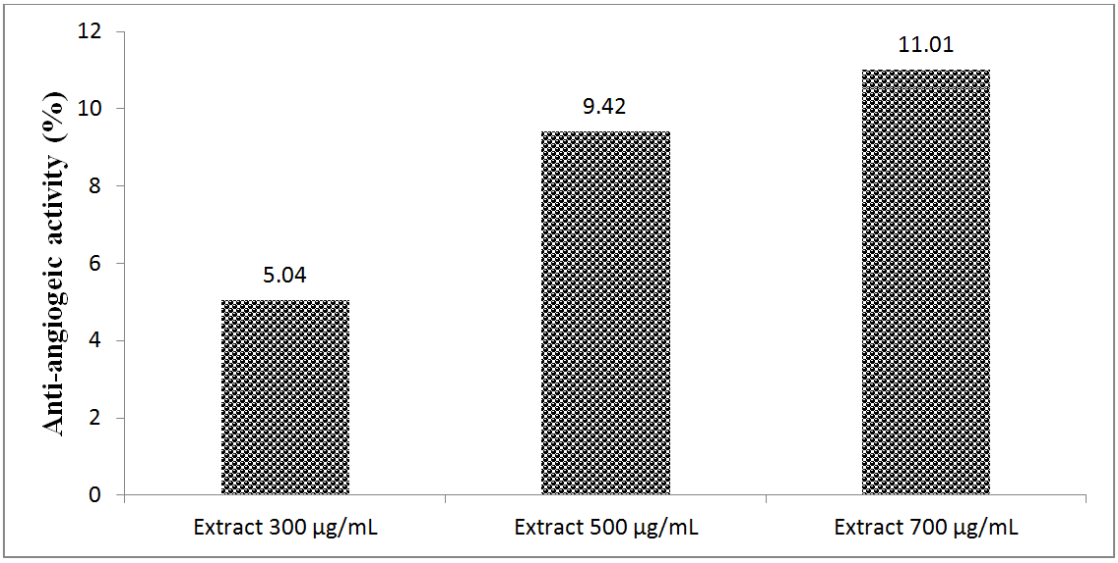

Fig. 3. Anti-angiogenic activity of methanol extract of *Mentha longifolia* at various concentrations

Table 1. 3 D surface roughness parameters of control and treated CAM

Groups	Roughness parameters				
	Sa (mm)	Sq (mm)	Sz (mm)	Sv (mm)	Sp (mm)
Control	81.1 ± 1.0	86.1 ± 3.0	282.4 ± 2.2	1.8 ± 0.1	266.1 ± 1.2
Extract 300 µg/mL	70.6 ± 1.70	76.3 ± 2.51	268.1 ± 2.2	1.4 ± 0.03	219.5 ± 5.6
Extract 500 µg/mL	69.5 ± 1.02	75.2 ± 1.89	262.2 ± 3.9	1.3 ± 0.1	237.4 ± 5.9
Extract 700 µg/mL	66.81 ± 0.81	74.4 ± 1.99	260.1 ± 3.9	1.1 ± 0.1	220.3 ± 2.3

Sa: average roughness, Sq: root mean square deviation, Sz: maximum height of the surface, Sp: reduce summit height, Sv: reduce valley depth

The use of plants in the health care system has ancient history [19]. Even in the current era, scientists are trying to discover disease remedies from natural sources; hence, 85000 plants having medicinal importance have been documented globally [20]. Vincristine, vinblastine, etoposide and teniposide are anticancer drugs which are being obtained from plant sources and are successfully used in the cancer treatment [21].

Anticancer activity of *Mentha longifolia* has been studied against HEPG2 (liver carcinoma cell), HELA (cervix cancer cell) and HTC-4 (colon carcinoma cell), A 549, HCI-H322 (Lung cancer), MCF 7 (Breast cancer), THP-1 (Leukemia), U-87MG (Glioblastoma) were the studied cell lines [7,8].

In the current study methanol extract of *Mentha longifolia* were studied for anti-angiogenic activity.CAM areas and average surface roughness of treated groups were reduced as compared to value of control group. There is inverse relationship between anti-angiogenic activity and diameter of blood vessels; it increases with decrease in blood vessels diameter [22].

Blood vessels diameter of all the treated groups was reduced significantly ($P < 0.05$) as compared to value of control group. Anti-angiogenic effect was calculated and concentration dependent anti-angiogenesis was measured in treated groups. Generally polyphenols with powerful antioxidant activity tend to possess good anti-angiogenic activity [23]. For example resveratrol [12], quercetin [24,25], genistein [26] and rosmarinic acid [27] are polyphenols isolated from plant sources showed promising anti-angiogenic effect. The data obtained in this study is consistent with these findings as *Mentha longifolia* had pronounced antioxidant power and good anti-angiogenic activity.

5. CONCLUSION

Mentha longifolia possessed concentration dependent anti-angiogenic effect at concentration range 300 µg/mL to 700 µg/mL while 1000 µg/mL concentration was embro-toxic causing death of embryos. It may be included as adjunct therapy with other anti-cancer drugs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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