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*Full Length Article*

# Evaluation of Anti-Angiogenic Activity of Latex and Extracts of *Euphorbia helioscopia* using Chorioallontoic Membrane (CAM) Assay

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## Abstract

The aim of this study was to investigate anti-angiogenic activity of *Euphorbia helioscopia* using CAM assay. Fertilized white leghorn chicken eggs were purchased from a local hatchery and placed in humidified incubator. On 5th day of incubation, a 2 cm wide window was cut at blunt ends of eggs and 4 mL of albumin was aspired from each egg then each egg was sealed with parafilm tape and put back in incubator. On 6th day of incubation, 200 µL, containing 100 µg concentration of each sample / standard was applied over the growing embryo and again sealed and incubated. On 7th incubation day all the eggs were opened and images were taken with camera for quantification of CAM vessels using SPIP software. Sterilization was strictly maintained during the experiment. There were statistically significant variations in primary, secondary and tertiary blood vessels diameters and CAM areas of all the treated groups. Of all the extracts and latex, methanol leaves extract showed highest anti-angiogenic activity. From the data obtained in this study, it can be concluded that *Euphorbia helioscopia* possessed significant potential to cause inhibition of angiogenesis and this property could be useful in controlling oxidative stress induced angiogenesis. © 2015 Friends Science Publishers

**Keywords:** Anti-angiogenesis; *Euphorbia helioscopia* extracts; CAM assay

## Introduction

Tumor growth can be controlled by inhibiting the angiogenesis. Judah Folkman was the first scientist who proposed anti-angiogenesis as a target site for treating tumors and decreasing metastasis (Folkman, 1971). Various *in vitro* (endothelial cells proliferation assay, endothelial cells migration assay, tube formation assay and aortic ring assay) and *in vivo* (matrigel plug assay, corneal angiogenesis assay and CAM assay) methods are available to evaluate pro-angiogenic and anti-angiogenic potentials of molecules (Auerbach *et al.,* 2003).

Angiogenic factors IL 8 and vascular endothelial growth factor (VEGF) production is stimulated with generation of free radicals in the cells (Brown *et al.,* 2000). VEGF, known as vascular permeability factor, is a key mediator in angiogenesis; it enhances the expression of endothelial nitric oxide synthase (eNOS), which may play important role in VEGF-induced angiogenesis (Bouloumie *et al.,* 1999). After binding with specific receptors present on endothelium VEGF promotes cellular events such as endothelial proliferation, migration and degradation of extracellular matrix components (Ferrara and Davis-Smyth, 1997). Oxidative stress increases the secretion of matrix metalloproteinase -1 (MMP-1), a collagenase that helps in the vessel growth within tumor cells. Thus, oxidative stress can cause angiogenesis within carcinoma cells (Brown *et al*., 2000). De novo synthesis of blood vessels plays pivotal role in tumour (Pepper, 1997) and warts growth (weblink 1) and also activates embryogenesis in female reproductive system (Fraser and Lunn, 2000).

*Euphorbia helioscopia* is an annual weed belonging to medicinally rich family *Euphorbiaceae* (Nadkarni, 2002) and is extensively grows in Pakistan. In China, this is a part of traditional Chinese medicine, used to treat various diseases such as tuberculosis, ascites, dysentery, edema, cervical cancer, lung cancer and esophageal cancer (Editorial Committee of the ABTCM, 1998; Pang and Lian, 2007; Yang *et al.,* 2007). Number of research findings have confirmed the anticancer, antioxidant, antibacterial, antiviral, antifungal, vasodepressor, anti-asthmatic, phytotoxicity, and molluscicidal activities of *Euphorbia helioscopia* (Ramezani *et al.,* 2008; Uzair *et al*., 2009; Nikolova *et al*., 2011; Ben-Mohamed *et al.,* 2012; Wang *et al.,* 2012). Secondary metabolites of the plant are responsible for its every pharmacological action. Flavonoids (Kawase and Kutani, 1968; Chen *et al.,* 1979), diterpenoids (Yamamura *et al.,* 1981; Shizuri *et al.,* 1983, 1984a,b; Kosemura *et al.,* 1985; Yamamura *et al.,* 1989; Zhang and Guo, 2006; Barile *et al.,* 2008; Tao *et al.,* 2008),

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triterpenoids (Nazir *et al.,* 1998), steroids and lipids (Kosemura *et al.,* 1985), polyphenols (Wei-Sheng *et al.,* 2009) have been isolated in number of studies. Quercetin, a flavonoid obtained from plant, has been quantified in the leaves of *Euphorbia helioscopia* (Liu *et al.,* 2011) and its anticancer activity was confirmed by Wang *et al.,* (2012). The antineoplastic activity of *Euphorbia helioscopia* has been analyzed and confirmed by number of researchers (Kang and Liang, 1997; Cai *et al.,* 1999 a,b; Caltagirone *et al.,* 2000).

In Pakistan, the milky juice (latex) obtained from stem of *Euphorbia helioscopia* is being used as traditional remedy for the removal of warts from the fingers and success rate of this therapy is very high but no research data are available. In case of cancer/tumor, angiogenesis plays pivotal role in metastasis of cancer cells within the body. It is hypothesized that the understudied plant possessed compounds that have anti-angiogenic effect that cure the warts and it may also be helpful as an adjunct therapy with other anticancer drugs because it will inhibit vessels growth which is necessary to supply oxygen and nutrients to cancer cells and ultimately cancer will come in remission phase from progressive phase.

The aim of study was to investigate anti-angiogenic effect of latex and different extracts of *Euphorbia helioscopia* using *in vivo* CAM assay.

## Materials and Methods

### Chemicals

Ethanol (Analytical grade; BDH Laboratory), petroleum ether (analytical grade; Sigma Aldrich), methanol and chloroform (analytical grade; Merck, Germany), quercetin [QTN] (Merck Germany), phosphate buffer saline (PBS) were procured from local market of Lahore-Pakistan.

### Plant Collection

The plant "*Euphorbia helioscopia"* was collected from suburbs of Lahore – Pakistan in the months of February and March. After identification and authentication by a Taxonomist of Botany Department, Government College University (GCU) Lahore-Pakistan, a voucher specimen (1501) was deposited to their herbarium. Leaves and stem were separated and dried under shade, then ground to fine powder separately which were later on used in extraction. The latex was collected in dried bottles by cutting the leafy part from the stem.

### Preparation of Extract

The pulverized material from both parts of the plant was extracted separately at ambient temperature by maceration in water and ethanol as solvents. Then both the materials were extracted sequentially using solvents (petroleum ether, chloroform, and methanol) in the order of increasing polarity by soxhlet apparatus. The solvents were removed from the extracts on rotary evaporator at 40$^{o}$C.

UV and FTIR fingerprints of all the extracts of leaves and stems showed overlapping behavior in our earlier research (Saleem *et al.,* 2014b), keeping in view this result, only leaves extracts were selected for the current study.

### Preparation of Chorioallantoic Membranes

Fertile white leghorn chicken eggs purchased from local hatchery (Big Bird-Lahore, Pakistan) were incubated at 37.5$^{o}$C in humidified incubator (humidity: 55–60%) that was rotated at hly cycle upto 60$^{o}$ angle. Ethanol (70%) was used to sterilize the surface of all the eggs. On 5$^{th}$ day of incubation, a window of 2 cm in diameter was cut at blunt end of eggs and 4 mL of albumin was sucked from each egg with 21 gauge sterile syringe. This step was performed in order to separate the CAM from dry white membrane lying under the egg shell to get better quantification of CAM vasculature. Eggs were sealed with sterile parafilm tape and incubated for 24 h.

### Preparation and Administration of Samples Solutions

PBS was used as vehicle in preparation of samples (leaves aqueous extract [L.AQ], leaves ethanol extract [L.ET], leaves petroleum ether extract [L.PE], leaves chloroform extract [L.CH], and leaves methanol extract [L.MT]) solutions of concentrations 10 µg/mL, 30 µg/mL, 50 µg/mL, 80 µg/mL, and 100 µg/mL. Standard (QTN) solution, 100 µg/mL, was prepared similarly. The pH of each solution was maintained between 6.5 and 7.5.

Two hundred microliter of each sample was applied over the growing embryos of all groups on 6$^{th}$ day of their incubation and again incubated for next 24 h.

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

Chick embryos of all the treated, control and standard groups (n=10 per each group), aged seven days and staged 31-32 along with CAM were collected in separate petri dishes and images were recorded with COOLPIX (Nikon-china): wide 10X zoom, Lense-shift VR 16.0 megapixels camera. All images were cropped in Adobe Photoshop 6.0 to highlight the vessels on every image and then imported to SPIP version 6.2.5, an image-processing program that works on specific algorithm for automatic measurement of 3D surface roughness and related parameters to evaluate the anti-angiogenic response. For precise quantification, the x, y and z dimensions of each image were loaded to software.

### Statistical Analysis

SPIP software version 6.2.5 was used for quantification of angiogenesis. Results were expressed as mean ± SD. One sample t-test was applied using SPSS version 12.

Graphs were drawn with graphpad prism version 4.00. Significance value was set at $P < 0.05$.

## Results

Blood vessels growth pattern, diameter, 3D surface roughness, CAM areas of control, standard, L.AQ, L.ET, L.PE, L.CH, L.MT, and latex were observed and quantified. All the samples showed prominent results at 100 µg/mL, so remaining concentrations were excluded from the study in subsequent experimentation.

On macroscopic examination of CAMs, normal vascular architecture i.e. main "Y" branch (primary blood vessel) which further divided into secondary and tertiary blood vessels was found in control group. Quercetin (QTN) is a known anti-angiogenic compound, used as standard in the study. There was decrease in the secondary and tertiary blood vessels branching along with reduction in their thickness in all the extracts-treated groups. The branching pattern of blood vessels of latex, L.MT, L.CH, L.AQ and L.ET treated groups showed similarity with that of QTN treated group. L.PE treated group exhibited gross changes in vascular architecture (Fig. 1).

CAM areas of control and standard groups were 1372 $mm^2$ and 1228 $mm^2$ respectively. There was significant decrease ($P < 0.05$) in CAM areas of extracts-treated groups as compared to control (Fig. 2). The diameters of blood vessels growing on CAMs were also significantly reduced ($P < 0.05$) with respect to control group blood vessels (Fig. 3). It strengthens our hypothesis that *Euphorbia helioscopia* possesses anti-angiogenic activity.

Neovascularization on CAMs was quantified by evaluating 3D surface roughness parameters (Table 1). Average surface roughness values of control and standard were 7.534 ± 5.8 mm and 6.150 ± 3.5 mm respectively. There was decrease in average surface roughness of all the test groups, confirming anti-angiogenic effect of *E. helioscopia* (Table 1).

Anti-angiogenic activity of samples was calculated with following formula:

$$\%\ Inhibtion\ of\ angiogenesis = \frac{CAM\ area\ of\ control - CAM\ area\ of\ standard\ or\ sample}{CAM\ area\ of\ control} \times 100$$

QTN exhibited 10.50% anti-angiogenic effect. L.MT showed 11.64% inhibition of angiogenesis. The effect of atex was same as that of standard. L.AQ and L.PE showed minimum anti-angiogenic effect i.e. 5.39% among all the samples while L.CH and L.ET had 10.20% and 7.94% respectively (Fig. 4). There was non-significant difference in anti-angiogenic property of latex and L.CH extract as compared to standard while anti-angiogenic potential of L.MT extract was significantly greater than that of standard. L.AQ, L.ET and L.PE extracts showed significantly lower anti-angiogenic power with respect to that of standard (Fig. 4).

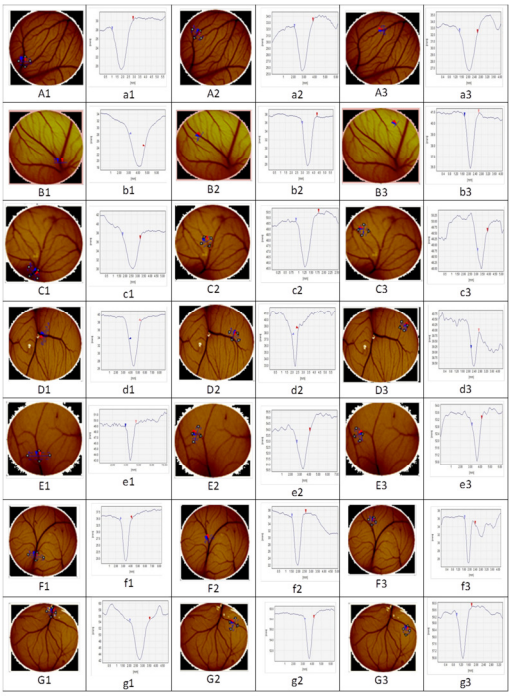


**Fig 1:** SPIP generated branching pattern and quantification of primary, secondary and tertiary blood vessels

1=Primary blood vessel, 2= Secondary blood vessel, 3= Tertiary blood vessel; A = Control, B = Standard (QTN), C = Methanol extract, D = Petroleum ether extract, E = Chloroform extract, F = Aqueous extract, G = Ethanol extract, H = Latex, a – h = quantification of blood vessels in term of diameter of above mentioned control, standard and test samples

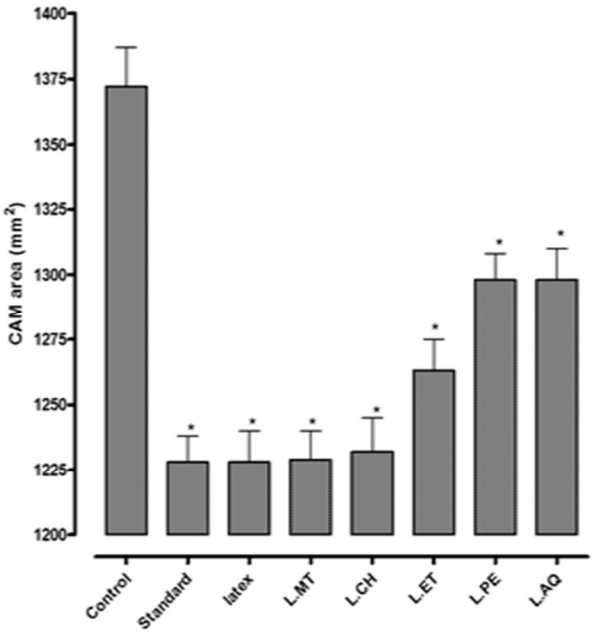


**Fig 2:** Effect of different extracts and latex of *Euphorbia helioscopia* on CAM areas. *$P < 0.05$ as compared to control

## Discussion

The CAM is a thin and extensively vascularized membrane. Due to its easy use and simplicity, the CAM assay is most commonly used to study normal angiogenesis and screen anti-angiogenic activity of new compounds (Patel *et al*., 1994; Cao *et al.,* 1995; Friedlander *et al.,* 1995; Maragoudakis *et al.,* 1995; Hatjikondi *et al.,* 1996). The results of *in vitro* techniques, based on visual scoring to evaluate angiogenesis, are valid only when there is marked change in vascular area while minor changes due to physiological processes need accurate quantitative technique (Defouw *et al.,* 1989; Strick *et al.,* 1991; Danesi *et al.,* 1993; Nguyen *et al.,* 1994). Quantitative assessment of angiogenesis requires selection of appropriate parameters (Ejaz *et al.,* 2004). Thus, CAM areas, blood vessels diameter, surface roughness parameters were quantified with SPIP software to assess even minute anti-angiogenic effect.

Since long time, the natural products have key place in healthcare system (Goldman, 2001). The popularity of plant based medicine has also proved statistically by the Consumer Association in the United Kingdom indicating that in 1991 one out of four individuals is consuming such remedies whereas the corresponding estimate in 1985 was one in seven (Brtitish Medical Association, 1993). Newman and Cragg (2012) reported that approximately 85% of new drugs introduced from 1981-2010 have resulted from the research on natural products. Furthermore, the global documentation of more than 85000 plant species for medical use indicates the interest of scientists in this area (Balunas and Kinghorn, 2005).

There are few examples of anticancer drugs isolated from plants such as: vincristine and vinblastine, used to treat leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma, topotecan to treat ovarian and small cell lung cancer and irrinotcan, is used in colorectal cancer (Cragg and Newman, 2005), etoposide and teniposide are used in lymphomas, bronchial and testicular cancer (Harvey, 1999; Cragg and Newman, 2005).

Anticancer activity of *Euphorbia helioscopia* has been studied on five cancer cell lines by Wang *et al.* (2012), and found promising effects on apoptosis, inhibitory effect on cell invasion and matrix metalloproteinase (MMP) 9 expression with ethyl acetate fraction (Wang *et al.,* 2012).

In the present study, different extracts of *Euphorbia helioscopia* were studied for anti-angiogenic effect. Average surface roughness and CAM areas of standard and all treated groups were lower than control group values. A direct relationship has been observed between CAM area and angiogenesis; it increases with increase in blood vessels growth and vice versa (Ejaz *et al.,* 2004).

There was statistically ($P < 0.05$) significant reduction in blood vessels diameters of extracts treated groups with respect to control value. Percent inhibition of angiogenesis was calculated and methanolic extract of leaves showed maximum anti-angiogenic effect among all the treated groups. Leaves methanol extract showed maximum *in vitro* antioxidant activity in DPPH and FRAP assay and high contents of flavonoids i.e., quercetin and kaempferol were quantified in the methanol leaves extract in our

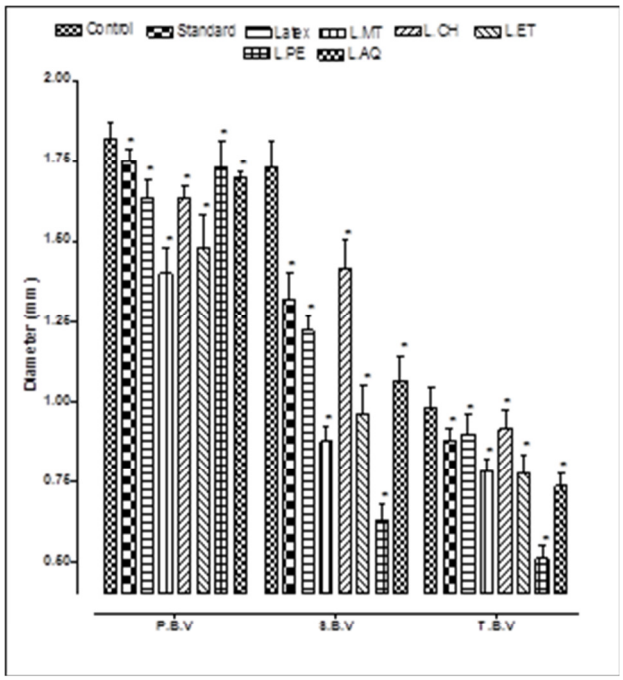


**Fig 3:** Comparison of diameters of blood vessels of CAMs treated with different extracts / latex

P.B.V = Primary blood vessels, S.B.V = Secondary blood vessels, T.B.V = Tertiary blood vessels. *P < 0.05 with respect to control.

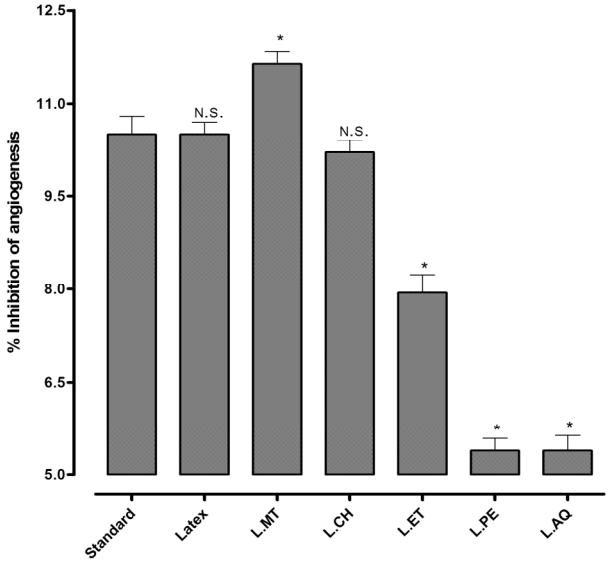


**Fig 4:** Anti-angiogenic effect of different extracts and latex of *Euphorbia helioscopia*. N.S. = non-significant, *P < 0.05 with respect to standard

**Table 1:** 3D surface roughness parameters of normal and treated CAM

| Groups | Roughness Parameters | | | | |
|---|---|---|---|---|---|
| | Sa (mm) | Sq (mm) | Sz (mm) | Sv (mm) | Sp (mm) |
| Control | 7.534 ± 5.8 | 9.772 ± 4.3 | 41.254 ± 8.3 | 21.962 ± 5.2 | 19.292 ± 4.9 |
| Standard | 6.150 ± 3.5 | 8.220 ± 6.4 | 41.300 ± 6.6 | 23.200 ± 7.6 | 18.100 ± 6.4 |
| Latex | 6.150 ± 4.3 | 7.970 ± 3.6 | 40.000 ± 9.1 | 22.500 ± 5.4 | 17.400 ± 3.9 |
| L.MT | 6.400 ± 3.3 | 8.340 ± 6.4 | 40.100 ± 4.5 | 23.100 ± 6.1 | 17.100 ± 5.3 |
| L.PE | 5.784 ±5.6 | 7.594 ± 6.8 | 39.870 ± 6.2 | 21.530 ± 4.6 | 18.340 ± 6.3 |
| L.CH | 6.890 ± 6.2 | 8.759 ± 5.2 | 40.288 ± 4.8 | 20.448 ± 4.2 | 19.840 ± 6.2 |
| L.AQ | 7.164 ± 8.1 | 9.348 ± 5.9 | 41.523 ± 5.5 | 23.027 ± 3.6 | 18.495 ± 6.8 |
| L.ET | 6.025 ± 4.8 | 7.738 ± 7.5 | 39.506 ± 5.6 | 21.826 ± 4.6 | 17.68 ± 7.4 |

Each value represents mean ± SD of 10 observations.
Sa = average roughness, Sq = root mean square deviation, Sz = maximum height of the surface, Sv = reduce valley depth, Sp = reduce summit height

earlier work (Saleem *et al.,* 2014a,b,c). This suggests that highest anti-angiogenic activity of methanol leaves extract is due to its strong antioxidant activity which can be attributed to high flavonoids contents, quercetin and kaempferol.

Approximately 5000 polyphenols had been described till 2002 and categorized into subgroups flavonoids, lignans and isoflavones (Cao and Brakenhielm, 2002). Polyphenols are one of the abundantly found secondary metabolites in plants (Mojzis *et al*., 2008; Jager *et al*., 2009). Generally polyphenols with powerful antioxidant activity tend to possess good anti-angiogenic activity (Aisha *et al*., 2010). For example quercetin (Tan *et al*., 2003; Pratheeshkumar *et al*., 2012), resveratrol (Cao *et al*., 2005), rosmarinic acid (Huang and Zheng, 2006) and genistein (Su *et al*., 2005) are polyphenols isolated from plant sources showed potent anti-angiogenic activity. These findings are in full agreement with data obtained in present study.

## Conclusion

*Euphorbia helioscopia* has significant anti-angiogenic potential due to its high flavonoid contents. The folklore use of the latex obtained from this plant seems to be justified and attributed to anti-angiogenic activity. The latex / methanol extract can be exploited as adjuvant therapy with anti-neoplastic drugs to inhibit tumor growth/metastasis.

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