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Evaluation of the angiogenesis activity of *Crataeva magna* Lour (DC) extract using the Chorio Allantoic membrane assay in Chick Embryos

R. Meera[1]

[1]*Department of Pharmaceutical Chemistry, K.M. College of Pharmacy, Uthangudi, Madurai – 625 107. Tamilnadu, India.*

Abstract

Aim: To evaluate the antiangiogenesis activity of root bark of *Crataeva magna* Lour DC ethanolic extract. Angiogenesis plays a critical role in embryonic development and various physiological processes. However, excessive angiogenesis is associated with several pathological conditions including cancer. *Crataeva magna* Lour DC (family Capparidaceae) is a traditional medicinal plant.

Materials and methods: This in vivo antiangiogenesis effect was studied by utilizing fertilized chick embryos assay. The extract loaded on the sterile filter paper was removed and observed the changes. The most of the eggs in this extracts formed lacuna on the nerve line. In 1000 and 750 ppm there is a strong lacuna formation.

Results: The root bark of *Crataeva magna* Lour DC ethanol extract inhibit angiogenesis by blocking the VEGF expression thus inhibiting endothelial cells proliferation, migration and differentiation most likely due to presence of the antioxidant phenolic compounds in the plant. The results of preliminary phytochemical studies confirmed the presence of alkaloid, carbohydrate, glycoside, protein, tannin, flavonoid and phenol.

Conclusion: From the present study, it is evident that, the root bark of *Crataeva magna* Lour DC ethanol extract possesses significant angiogenesis effect on chick embryos and suggests that the plant may have therapeutic value in cancer and related complications.

Keywords: *Crataeva magna* Lour DC, Root extract, CAM model, Anti-angiogenesis.

INTRODUCTION

Angiogenesis, the formation of new capillaries from pre-existing vessels, is an important natural process in the body used for healing and reproduction. In early 1970s, it was noted that solid tumors appear to be highly vascularized [1, 2]. Recruitment of new blood vessels plays an important role in tumor survival and growth. In 1971, a concept of anti-tumor strategy by controlling angiogenesis was hypothesized by Folk man, as tumor growth is angiogenesis dependent and every aspect of tumor growth requires increment in vascular growth [3, 4]. Endothelial cells in tumour bed tend to be more susceptible to cytotoxic agents due to their high proliferation rate. In addition, endothelial cells, on the contrary to cancerous cells, are genetically stable as they do not undergo mutations and hence more sensitive to apoptotic effects of the cytotoxic agents. Thus, these features of endothelial cells make them a compelling target for antiangiogenesis treatment[5]. Highly vascularized chorioallantoic membrane of the chicken embryo is used as in vivo model and widely used for determination of anti-angiogenesis activity. Due to the increasing interest in anti-angiogenic therapy for cancer, several agents are investigated which act as angiogenesis inhibitors [6].It was reported that excessive angiogenesis is an important factor of the pathogenesis of many industrialized western countries [7]. Plants with anti-angiogenesis properties are therefore of considerable importance for diseases such as cancer, macular degeneration, diabetic retinopathy, and others[8–11]. Plants have been used in the treatment of cancer since ancient period. Natural products and related drugs are used to treat 87% of all human diseases including cancer. About 25% of prescribed drugs in the world are obtained from various plants. Over 3000 species of different medicinal plants have been reported to have anticancer properties[12]. Ayurvedic system of medicine reveals that there are several herbs with anticancer and anti-inflammatory activity[13]. Majority of Phytochemical are phenolic compounds. Dietary polyphenols can modulate the process of carcinogenesis by several mechanisms. Anticancer activity has been shown to be associated with a different Phytochemicals like polyphenols, flavonoid and catechins[14, 15]. Currently over 60% of used anticancer agents are derived in one or the other way from natural sources, including plants, marine organisms and microorganisms. There are worldwide efforts to discover new anticancer agents from plants[16]

Crataeva magna Lour DC (family Capparidaceae) is known as three leaved caper in English, Varuna in Sanskrit and Baruna in Hindi, a small tree with a much branched head, found to be distributed mainly in the warmer (tropical) parts of the world. In folk medicine, its stem pith in the tribal peoples of Kandhamal district of Orissa known as Eastern Ghats of India that the bark is used for lactation after child birth, treat urinary disorders, kidney bladder stones, fever, vomiting and gastric irritation[17-19]. Leaves are deciduous three foliolate; petioles 3.8–7.6 cm long; leaflets 5–15 ovate, lanceolate or obovate, acute or acuminate, attenuate at the base, entire, glabrous on both surfaces, pale beneath, and reticulately veined[20]. The traditional plant used to treat various ailments in particular to Urolithiasis [21], Hepatoprotective [22], Cardio protective [23], anti arthritic and rubifacient. [24-26] Bark juice of this plant is given orally to prevent childhood

diseases among the inhabitants of the Kanyakumari district. [27] The literature revealed that wide variety of medicinally important compounds including friedelin, diosgenin, sitosterol, butulic acid, dodecanoic anhydride, methyl pentacosanoate, kaemferol-3-O-α-D-glucoside and quercitin-3-O-α-D-glucoside have been reported from *C. magna* [28]. As there is no report on anti-angiogenic activity, extract was tested for antiangiogenic activity by CAM assay.

MATERIALS AND METHODS

Root bark of *Crataeva magna* Lour DC were collected in and around local forest area of Kanyakumari, Tamilnadu and authenticated by the Botanist Prof.Chelladurai, Department of Botany, Govt. Siddha Medical College, Tirunelveli. A voucher herbarium specimen number KMCP/CM/01/2015 was also preserved in the K.M.College of Pharmacy, Madurai.

Preparation and Extraction of Plant material

The root bark is collected were subjected to dried in shade and then coarsely powdered. The 500 gms of powdered root bark of *Crataeva magna* Lour DC were defatted with petroleum ether and extracted successively with chloroform and ethanol using soxhlet apparatus. The extraction was carried out until the extractive becomes colorless. The extract was filtered through a cotton plug, followed by whattman filter paper (no.1). The extract was evaporated under reduced pressure using rotovac evaporator.

Chicken Chorioallantoic Membrane Assay (CAM Assay) [29-32]

In its original form the CAM of day 7-9 chick embryo s was exposed by making a window in the egg shell, and the sterile filter paper loaded with the compounds to be tested was carefully inoculated into the nerve region of the chick. The dosage of the extracts to be tested was taken initially will be around 1000ppm. (1mg in 1ml of 0.2% of DMSO). Then the window was sealed, eggs were re incubated and the grafts were recovered after an appropriate length of incubation time, may be of 48 hours. The grafts were ten scored for growth and vascularization on a 0 to 4 basis, but more recently, imaging techniques such as the measurement of bifurcation points in a designated area around the test material have improved the qualification of the assay. A positive control (VEGF), an angiogenic inducer and a negative control (DMSO) were done simultaneously to compare the activity of the ligands used. All the experiments were done in triplicates. The images of each treated CAM were captured under dissecting microscope. Imaging of the vascularized eggs was performed using a digital camera with 3x magnification objective (Canoneos 500 with a Canon mp-e 65 2.8 macro objective). For illumination, a mercury arc lamp was used which provided a high fraction of blue and UV light to obtain good contrast values between yolk and vessels. The pictured image section had a size of 5 × 5 mm.

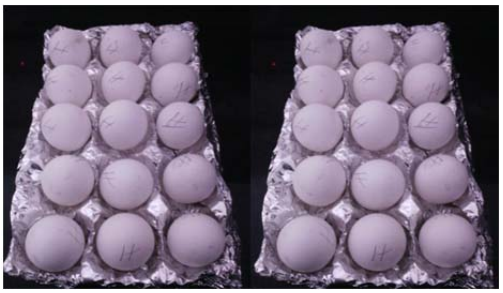
RESULTS AND DISCUSSION

Anti-angiogenesis activity of crude ethanol extract was tested in vivo CAM model. We examined the 5th day old embryo after treatment for number of vessels and their reduction. The extract loaded on the sterile filter paper was removed and observed for its changes are shown in Table 1. The most of the eggs in this extracts formed lacuna on the nerve line. It strongly elicited an antiangiogenic response as shown in Figure 1. Furthermore, it is supported in Figure 2. Ethanol extract of root bark of *Crataeva magna* Lour DC showed higher anti-angiogenesis activity. Over the recent years, more attention has been focused on the anti-angiogenic and anti-tumor effects of non-toxic compounds from natural products. Several reports have shown that crude plant extracts are more effective pharmacologically than isolated active compounds. This may be due to the synergistic effects of various components present in the extracts.[33] Plants contain tremendous amount of Phytochemical constituents such as phenolic and flavonoid compounds, these have a great potential in promoting and maintaining a good health.[34] Antiangiogenic compounds are gaining more and more interest as a new approach in the prevention and treatment of cancer and inflammatory diseases.[35] The CAM assay is a sensitive, easily feasible and cheap *in vivo* test for investigations of the antiangiogenic potential of individual compounds and plant extracts. [36] The assay does not only provide information on the efficacy of test samples *in vivo* but also on their toxicity *in vivo*. To the best of our knowledge, their antiangiogenic property is being reported here for the first time. In this direction, root bark of *Crataeva magna* Lour DC is being actively explored as a source of new chemical substance that can inhibit angiogenesis. Independent of this effect in this study, it is clearly elucidated that antiangiogenic activity of root bark of *Crataeva magna* Lour DC by performing *in vivo* antiangiogenesis assay. It has been observed that extract significantly formed lacuna on the nerve line in CAM. The observation in this study suggests that extract of root bark of *Crataeva magna* Lour DC exhibits a strong antiangiogenic activity. It may have the potential to be a useful deactivator of numerous serious diseases characterized by regulated angiogenesis.

Table 1: Observation of the extract with lacuna formation

Extract	Replicates	Observation	
		Budding of blood vessels	Plaque formation
1000ppm	R1	-	+++
	R2	-	++
	R3	-	+++
750ppm	R4	-	++
	R5	-	++
	R6	-	+
500ppm	R7	-	-
	R8	-	-
	R9	-	+
250ppm	R10	-	-
	R11	-	-
	R12	-	-
Positive control (VEGF)	R13	4	-
Negative control	R14	0	-

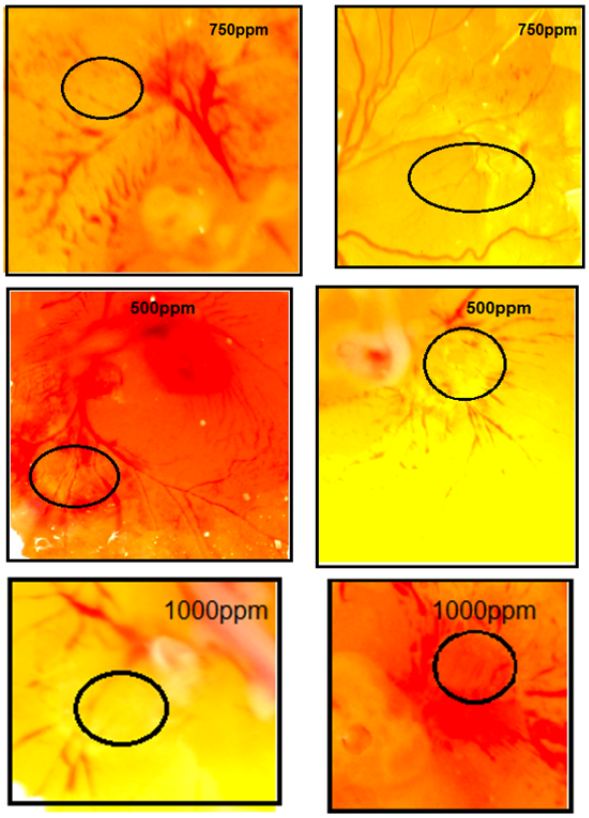
+++= strong lacuna formation
++ = lacuna formation



Eggs for CAM Assay

Under Incubation

Figure 1: Extract loaded filter paper impregnated in the nervous region of the 5thday old chick embryo



Positive control

Figure 2: Plaque formation

CONCLUSION

We estimate medicinal plants in general and especially traditional plants as valuable and indispensable resources for the development of new drugs and the rational use of phytotherapy. This point of view is supported by a comprehensive survey of the NCI, USA, showing that the vast majority of clinically established cancer drugs during the past three decades were based on natural products. [37] The present study reports for the first time the inhibition of angiogenesis by root bark of *Crataeva magna* Lour DC extract by blocking the VEGF expression leading to inhibition of endothelial cell proliferation, migration on matrigel matrix. This plant may provide a new source of antiangiogenesis agents which can be considered as potential candidates in the treatment of angiogenesis related diseases.

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