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Research Article

A COMPARATIVE STUDY OF ANTI-ANGIOGENIC ACTIVITIES OF MEDICINAL PLANTS AND ITS THERAPEUTIC POTENTIAL IN ANGIOGENESIS-DEPENDENT DISORDERS

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ABSTRACT

Objectives: The aim of the study is to evaluate and compare the vascular endothelial growth factor (VEGF) inhibitory effect of the medicinal herbs *Ocimum sanctum*, *Andrographis paniculata*, *Syzygium cumini,* and *Ginkgo biloba* using *in vivo* models.

Methods: Both corneal neovascularization assay and chick chorioallantoic membrane assay were used for evaluation. The induced neovascularization was compared with control and extract treated groups.

Results: The acute and repeated dose toxicity tests showed that the extracts are safe on oral administration. Moreover, the results showed the significant anti-angiogenic effect of individual extract and their combination in both *in vivo* models. However, the combination of the extract showed an excellent result ($p<0.001$) by reducing neovascularization in both models.

Conclusion: The above results suggested that combination of the above extracts have promising future in the management of angiogenesis-dependent disorders.

Keywords: *Ocimum sanctum*, *Andrographis paniculata*, *Syzygium cumini*, *Ginkgo biloba*, Angiogenesis, Chorioallantoic membrane, Angiogenesis-dependent disorders.

INTRODUCTION

Angiogenesis is the process involving the growth of new blood vessels from the existing vasculature. It occurs throughout life from the embryo and continuing on through old age both in healthy and diseased condition. Stimulation of angiogenesis can be therapeutic in ischemic heart disease, peripheral arterial disease, and wound healing. Decreasing or inhibiting angiogenesis can be therapeutic in cancer, ophthalmic conditions, rheumatoid arthritis, and other diseases [1]. Hence, the control of angiogenesis could have therapeutic value, and research is going on regarding this for past few years. Anti-angiogenic drugs exert its beneficial effect by disabling the agents that activate and promote cell growth or by directly blocking the growth of the new blood vessels. In recent years, the role of vascular endothelial growth factor (VEGF) in angiogenesis has been studied widely and that shows its significance in regulating both physiological as well as pathological angiogenesis. VEGF activates two tyrosine kinase receptors, VEGF-1 and VEGF-2 and these regulate physiological and pathological angiogenesis. VEGF-2 is expressed mostly on vascular endothelial cells. Activation of VEGF-2 stimulates endothelial cell proliferation, migration, and survival, as well as angiogenesis and microvascular permeability [2,3]. VEGF plays a vital role in embryonic vasculogenesis and angiogenesis. It was observed that *in vivo* administration of VEGF causes marked increase in circulating endothelial progenitor cells kinetics due to mobilization of bone marrow derived endothelial progenitor cells which ultimately results in increased differentiation of endothelial progenitor cells *in vitro* [4]. Apart from this, *in vivo*, VEGF augments corneal neoangiogenesis which implies the importance post-neovascularization [5]. VEGF also plays a critical role in glomerular development and function and is highly expressed within the glomerulus [6]. VEGF also acts as essential mediator in the process of endochondral ossification [7]. Sprouting angiogenesis is initiated in poorly perfused tissues when oxygen sensing mechanisms detect a level of hypoxia that demands the formation of new blood vessels to satisfy the metabolic requirements of parenchymal cells [8,9]. So, the development of new agents that modulate VEGF function has led to the novel treatment in both malignant and non-malignant conditions. Anti-VEGF agents are potential drugs for treating angiogenesis-related disorders such as diabetic retinopathy and cancer nowadays [10,11]. The widespread use of the FDA-approved monoclonal antibodies such as bevacizumab and ranibizumab in proliferative diabetic retinopathy and metastatic colorectal carcinoma, proved their efficacy in the management. However, considering local and systemic side effects, this could be an adjunctive treatment. VEGF inhibitors from plant sources are matter of great interest and show excellent results nowadays. Advantages of anti-VEGF compounds derived from natural sources are that they are safer and their action may be mediated through multiple cell-signaling pathways, and thus, reduces the chances of developing resistance by cancer cells [12]. Studies showed that medicinal plants such as *Ocimum sanctum*, *Andrographis paniculata*, *Syzygium cumini,* and *Gingko biloba* possess greater anti-VEGF activity [13] and could be a potential target in managing pathological angiogenesis mediated disorders such as diabetic retinopathy and cancer. So, the present study aimed to demonstrate and compare anti-angiogenic property of these medicinal plants *in vivo* models including the corneal neovascularization assay and the chick chorioallantoic membrane assay (CMA) and assess their potential role in angiogenesis-dependent disorders.

METHODS

Swiss albino mice were purchased from King's Institute, Guindy, Chennai. Brown leghorn chicken eggs were purchased from local hatcheries. VEGF was purchased from Sigma-Aldrich, Bangalore. Leaves of *O. sanctum*, *A. paniculata*, *S. cumini* seeds, and *G. biloba* extract were purchased and got authenticated by Director, National Institute of Herbal Sciences, Chennai. The study was approved from the Institutional Animal Ethical Committee (001/02/IAEC/2014/SBMCH).

Plant extraction

Dried leaves of *O. sanctum*, *A. paniculata,* and *S. cumini* were extracted by hot maceration method with little modification. In brief, weighed quantity of the leaves macerated in 1:40 proportion in water at 800°C for 2 hrs. Resultant solution was filtered through muslin cloth and filtrate was dried at 60°C to constant weight. The extract thus obtained was stored in refrigerator until further use.

Acute toxicity studies of extracts

Acute toxicity studies were carried out following OECD 423 guidelines [14]. Following toxic class method, both extracts were evaluated to 2000 mg/kg dose. No any signs of toxicity were observed even at this dose. So, the extracts were classed as unclassified. Therefore, in further studies dose of 250 mg/kg was used.

Repeated dose toxicity studies of extracts

Repeated dose toxicity of both the extracts was carried out at 250 mg kg, 500 mg/kg, and 1000 mg/kg dose to evaluate any adverse effect of chronic use following OECD 408 guidelines. Adult Swiss albino mice weighing between 30 and 40 g were selected as test animals. 5 male and 5 female mice were used for each group. Animals were treated for 90 days with a daily dosing and observed for biological, hematologic, and behavioral parameters as per OECD guidelines [15].

Phytochemical investigations

Aqueous leaf extract of *O. sanctum*, *A. paniculata*, *G. biloba,* and seed extract of *S. cumini* revealed the presence of alkaloids, glycosides, triterpenoids, steroids, saponins, flavonoids, and tannins.

***In vivo* models of angiogenesis**

Corneal neovascularization assay

The mice were divided into 6 groups each consists of 6 animals. 160 ng of VEGF was implanted in to each mouse corneal micropocket under anesthesia (mixture of ketamine 75 m/kg and xylazine 5 mg/kg i.p.). The neovascularization after 1 week was documented for the control (drinking water with 1% ethanol) as well as for all the extract fed groups (extract drinking group 250 mg/kg) by using simple stereo microscope. Control mice were compared with each test group in these following aspects.

(1) Areas of VEGF-induced neovascularization (2) Vessel count.

Chick CMA

Chorioallantoic membrane (CAM) represents hypoxic model of angiogenesis. The developing embryo undergoes hypoxia to develop new blood vessels, which partly mimics angiogenesis in tumors fertile eggs were obtained and 70% ethanol was sprayed on the eggs for disinfecting the surface. They were kept in humidified incubator at 37.5 c (±1.5 c). Eggs were rotated 4-6 times a day to ensure uniform vessel development. On embryonic day 4, a window was made on the shell over the embryo to express CAM. Eggs were sealed again with cellophane film and incubated for further few days. On the 6th embryonic day, 0.9% sodium chloride (normal saline) solution or paper discs impregnated with solution of extract (10 mg/ml) were placed on intact CAM. These eggs were again incubated for further 24 hrs. On the 7th embryonic day, the eggs were observed:

1. For number of primary and tertiary vessels formed
2. Nature of blood vessels
3. Area of the CAM (neovascularization).

The results were documented on 7th day by noting down the inhibition of neovascularization compared with the control.

Statistical analysis

The data were expressed as mean and standard error of mean. For comparing all groups, we used One-way Analysis of Variance (ANOVA) and multiple comparisons between two groups Dunnett-test used. Statistical significance is to be considered as $p < 0.05$ level. Statistical analysis was done using SPSS for Windows (V 17).

RESULTS

Corneal neovascularization assay (Figs 1-3)

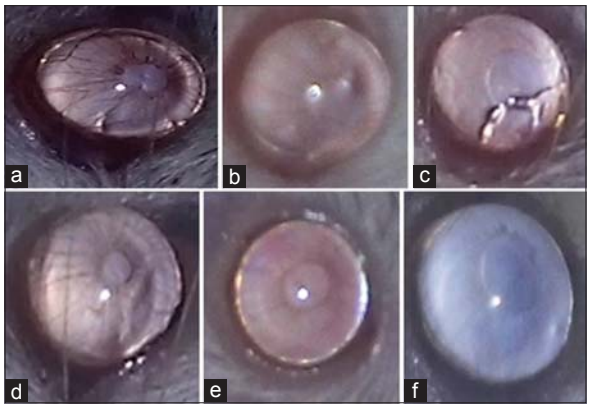

Fig. 1: Effects of aqueous extracts in vascular endothelial growth factor (VEGF)-induced neovascularization in mice. Photographs show mice corneas of different groups. (a) Cornea of control, (b) *Ocimum Sanctum* treated cornea, (c) *Andrographis paniculata* treated cornea, (d) *Syzygium cumini* treated cornea, (e) *Ginkgo biloba* treated cornea, and (f) All extracts treated cornea. The extracts significantly inhibited corneal neovascularization induced by VEGF versus control groups that drank water alone

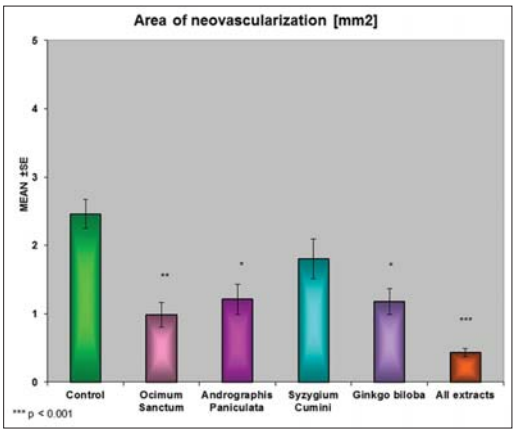

Fig. 2: Corneal neovascularization assay: Comparison of area of neovascularization in herbal extract treated groups

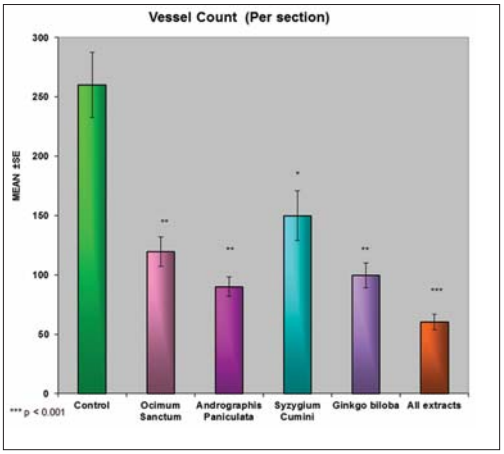

Fig. 3: Corneal neovascularization assay: Comparison of tertiary vessel count in herbal extract treated groups

Chick CAM assay (Figs 4-6)

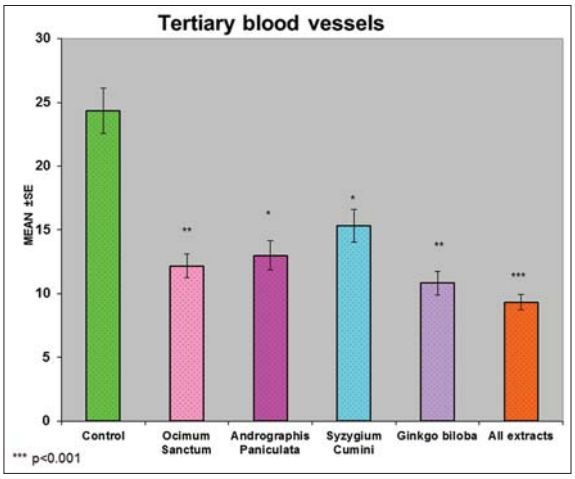

Fig. 4: Chick chorioallantoic membrane (CAM) assay: Comparison of tertiary vessels count of CAM treated different extracts

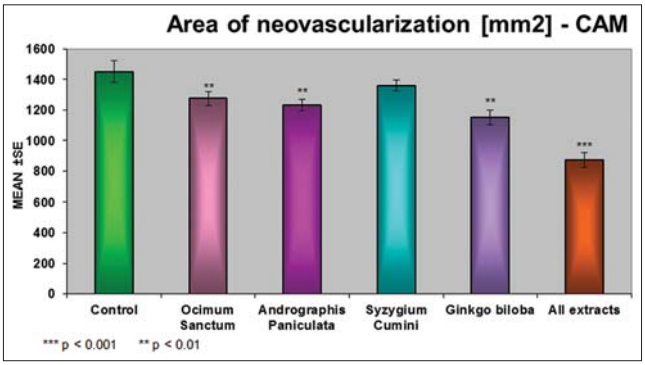

Fig. 5: Chick chorioallantoic membrane (CAM) assay: Comparison of area of neovascularization of CAM treated different extracts

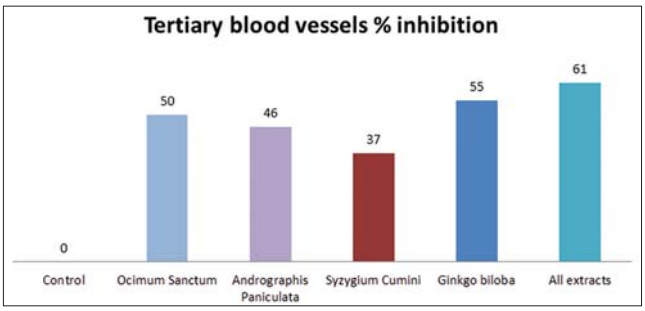

Fig. 6: Administration of extracts *Ocimum sanctum*, *Andrographis paniculata*, *Syzygium cumini,* and *Ginkgo biloba* in the dose of 10 mg/ml reduces angiogenic response to such hypoxia to about 50%, 46%, 37%, and 55%. While their combination was able to reduce it to 61%

DISCUSSION

Both corneal neovascularization and Chick CMA are the standard *in vivo* methods for assessing anti-angiogenic activity [16-18]. In this study, in corneal neovascularization assay, both *A. paniculata*, *G. biloba* ($p<0.05$), and *O. sanctum* ($p<0.01$) significantly reduced the area of neovascularization except *S. cumini*. However, the total no of tertiary blood vessels count was reduced by above three ($p<0.01$) including *S. cumini* ($P<0.05$). However, the combination of all above extracts showed excellent result by significantly reducing both area of neovascularization and tertiary blood vessels count ($p<0.001$). In CAM method, *A. paniculata* ($p<0.05$), *G. biloba,* and *O. sanctum* ($p<0.01$) are more effective in reducing the tertiary blood vessel count and the area of neovascularization ($p<0.01$). However, the combination of above extracts showed a significant reduction in terms of neovascularization area and blood vessel count ($p<0.001$). Hence, the study showed in both *in vivo* models, individually these plants showed significant result but the combination was more effective in reducing neovascularization.

Recent years, anti-angiogenic agents play a major role in angiogenesis-dependent disorders particularly diabetic retinopathy and cancer. Studies showed that these plants possess greater anti-VEGF activity and can be potential target in such disorders. Commercially, two anti-VEGF agents (bevacizumab and ranibizumab) are available for treating proliferative diabetic retinopathy. Off label use of these drugs are useful in both primary treatment and as an adjuvant in laser and surgical treatment for advanced proliferative diabetic retinopathy. Although evidence supports the effectiveness of anti-VEGF therapy for treating diabetic retinopathy their limited half-life necessitates repeated injections. However, repeated intraocular anti-VEGF therapy have side effects such as cataract formation, infection (endophthalmitis), vitreous hemorrhage, and retinal detachment [19]. So, it is necessary to discover new anti-angiogenic agents with longer ocular half-lives or novel delivery mechanisms to prolong the effects of anti-VEGF agents in the eye. Similarly, in cancer treatment, FDA-approved the use of bevacizumab in combination with carboplatin and paclitaxel chemotherapy in metastatic colorectal cancer [20]. Combination of this therapy has been reported to bring about a 25% improvement in survival compared with chemotherapy alone. Several other clinical trials are currently under way studying the use of this monoclonal antibody in the treatment of recurrent colorectal carcinoma, metastatic breast cancer, and cervical cancer [20-22]. But, the toxicities include bleeding, disturbed wound healing, thrombosis, hypertension, hypothyroidism and fatigue, proteinuria and edema, skin toxicity, leucopenia, lymphopenia, and immunomodulation are more specific with these potent angiogenesis inhibitors.

Anti-VEGF agents derived from natural sources show promising result nowadays and have many advantages. They increase the effectiveness and reduce the side effects of the existing therapy. But, considering the cost and repeated intravitreal administration of the current ocular anti-VEGF agents (monoclonal antibodies) in diabetic retinopathy, these natural sources could be an effective oral angiogenesis inhibitor and safely administered with other existing novel therapies. In cancer chemotherapy also anti-VEGF agents derived from natural sources provide significant benefits [23]. They are least toxic and the mechanism of multiple cell signaling pathway reduces the chances of resistance. *O. sanctum*, *A. paniculata*, *S. cumini,* and *G. biloba* contains phytochemicals, that may augment the immune function, inhibit the formation of cancer forming nitrosamines, hinder hormonal activity as well as induce Phase I and Phase II detoxification enzymes, reduce inflammation, thus protecting the body against chronic diseases such as cancer [24]. Moreover, the oral route of administration also favors the additional advantage. Considering these facts the above herbal plants could be a better alternative and safely used in diabetic retinopathy, cancer, and other angiogenesis-dependent disorders.

CONCLUSION

So, the present study proved the anti-VEGF activity of individual plant and the efficacy of their combination in angiogenesis-dependent disorders. But, further experiments will be needed to clarify the major anti-angiogenic constituents of these herbal extracts.

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