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Angio Suppressive Effect of Clitoria ternatea Flower Extract is Mediated by HIF-1 α and Down Regulation of VEGF in Murine Carcinoma Model

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Abstract

Angiogenesis is a vital process in the progression of cancer as it also play a key role in tumor transition from its dormant state to a malignant stage. VEGF is key growth factor plays an important role in angiogenesis and is regulated by transcription factor HIF-1α. Natural compounds derived from plants have been a prime source for numerous clinically useful anti-cancer agents specially for targeting neo-angiogenesis. Medicinal plants continue to play a central role in the healthcare system of large proportions of the world's population, particularly true in developing countries like India. In the current report, we studied the angio suppressive effect of aqueous extract of *Clitoria ternatea* in EAC cells induced angiogenesis. *In vivo* anti-angiogenic effect of *C. ternatea* was demonstrated by the down regulation of VEGF secretion from Ehrlich ascites carcinoma (EAC) cells and inhibition of blood vessels formation indicating the potential angio suppressive effect of plant. HIF-1α protein, a transcription factor known to be key a regulator in hypoxia-induced angiogenesis was also down regulated by *C. ternatea*. Our invetigation indicated that, HIF-1α nuclear sequestration is repressed by *C. ternatea* through inhibition of nuclear translocation. We hypothesize that decreased levels of HIF-1α in the nucleus of EAC cells upon *MECT* treatment could be responsible for decreased expression of VEGF which is also attributed to the angio-suppressive effects of MECB. *C. ternatea* promises to be a potential anti-angiogenic plant which can be exploited to treat cancer.

Keywords: *Clitoria ternatea*; Ehrlich ascites carcinoma (EAC); Antiangiogenesis; VEGF; Hypoxia; HIF-1α

Introduction

Angiogenesis is referred to the origin of new vasculature from pre-existing blood vessel in the body. This process involves the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels [1]. It is essential for tumor growth and metastasis, because it provides oxygen and nutrients to the growing tumor [2]. Further, angiogenesis is a fundamental requirement of most solid tumors for their out growth and expansion [3] Among all angiogenic growth factors VEGF plays a potent role as an angiogenic modulator which stimulate endothelial cells to secrete plasminogen activator and other proteases that subsequently causes degradation of basement membrane of blood vessel, which in turn allows cancer cells to invade the surrounding tissues. After subsequent migration and proliferation the cells finally differentiate to form a new vessel. Enhanced expression of vascular endothelial growth factor (VEGF) has been observed in many human cancers including rectal, breast, non-small cell lung and ovarian cancers [4]. Higher amount of VEGF has been found in many of medical conditions like brain edema, human synovial fluid, rheumatoid arthritis and malignant ascites [5]. As a result, many studies have indicated that hindering the over expression of VEGF consequently results in the tumor growth retardation and inhibition of ascites [6,7].

Ascites tumor cells have long been known to adapt and grow under severe hypoxia, providing a large number of hypoxic cells [8-11]. The cells in ascites are resistant to apoptosis under extreme hypoxia, under acidosis and at low glucose levels, recapitulating, at least partly, the malignant feature of cancer cells [12,13]. VEGF is a transcriptional target of hypoxia inducible factor-1 alpha (HIF-1α), a transcription factor that controls expression of many genes of cellular signaling pathways and play a major role in governing oxygen homeostasis [14]. Solid tumors suffer hypoxic condition due to the enhanced tumor cell proliferation which rapidly consume the cellular oxygen. The dynamicity of a tumor is verily depends on expression of HIF-1α and hypoxia that regulates angiogenesis and control the process of invasion and metastasis [15]. Under hypoxic condition HIF-1α protein accumulates and translocates to the nucleus where it forms an active complex with HIF-1β and activates transcription of target genes. Over expression of HIF-1α in colorectal cancer and its metastases is considered to be one of the independent indicator of poor prognosis [16]. However driven by the recent progress in the molecular understanding of the tumor hypoxia response, various strategies for molecular targeting of hypoxic cancer cells are emerging, [17] such as blocking HIF-1α signal [18]. Therefore pharmacological strategies designed to inhibit HIF –1 α activity may represent a novel approach in cancer therapy.

Clitoria ternatea (*C. ternatea*) belongs to Fabaceae family, known as Aparajitha in India is a persistent, herbaceous perennial legume. It is native to south-east Asia and widely distributed through out the world, mainly in tropical countries [19]. The plant is reputed for its folkloric uses in various diseases [20]. The various parts of the plant has been scientifically evaluatedd for various pharmacological activities including antioxidant [21], anthelmintic [22], analgesic, anxiolytic [23], antidepressant, anticonvulsant [24], hypoglycemic [25], larvicidal [26] and cytotoxic [27] activities. This particular study is an evaluation of angio-suppressive activity of methanol extract of *C. ternatea* flowers (MECT) in Ehrlich ascites carcinoma (EAC) model *in-vivo*.

Materials and Methods

Swiss albino mice were procured from Central animal facility maintained in the Department of Zoology at University of Mysore,

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Mysore, India. Fertilized eggs were from the Veterinary college, poultry section, Bangalore. Plant material was collected from rural parts of local place of Mysore. Anti HIF-1α, β-Actin antibodies, secondary antibodies were procured from Cell signaling, USA. ECL kit from GE life science, USA.

Preparation *C. ternatea* plant extract

Preparation of methanol extract from *C. **ternatea*** flowers (MECT) was carried out according to previously reported method [28]. Thus, the dried flowers of *C. ternatea* (at 50°C) were crushed in a blender and powder was subjected for methanol extraction at 64.5°C using soxhlet apparatus. The residual methanol was evaporated at 40°C under pressure. Finally the solid extract was dissolved in minimum quantity of DMSO to get a stock solution of 100 mg/ml and was used for further analysis.

In-vivo culture of EAC cells and treatment with MECT

The experiment was conducted as per the methods reported earlier [29]. In brief EAC cells (5×10^6 cells /mouse) were injected intraperitoneally (i.p) into 8-10 week old swiss albino mice and weight of the animals were monitored every day. Six days after inoculation, MECT (100 mg/kg body weight/ i.p, in 0.1% DMSO) was administered to the peritoneum of EAC bearing mice every alternate day starting from day 6 till day 12 and finally after treatment mice were sacrificed on day 13. EAC cells suspened in ascites fluid were harvested and used for further experiments. Inner lining of the peritoneal cavity in both control and treated mice were inspected for the growth or inhibition of blood vessels and the images were recorded.

Chorioallantoic membrane (CAM) assay

CAM angiogenesis assay was done according to the previously stated methods [30]. The fertilized chicken eggs were incubated at 37°C in a sterile humidified atmosphere for 9 days and then a window was made on the eggshell to reveal the Chorioallantoic membrane. The MECT was placed on sterile discs, which were allowed to dry under sterile conditions. A loaded and air-dried MECT smeared discs and control disc (0.9% physiological saline) were placed on CAM. Windows were resealed with coverslip and the eggs were placed back in the incubator. On 11th day of incubation windows were opened and examined for changes in the microvessel density below and around the disc and were photographed.

Hematoxylin and eosin staining (H&E)

Histopathological studies were made by performing H&E staining. Peritoneum from mice with and without MECT treatment were fixed in formalin. Fixed peritoneum were embedded in paraffin and sections (5 µm) were made and then stained with H&E. Histological examination of the sections were made by counting the microvessel with clearly defined lumens or linear shape observing under high power field (HPF). Finally micro vessel density mean score was obtained from the areas counted using Radical light microscope, attached to CCD camera.

VEGF–ELISA

VEGF ELISA was carried out using the ascites of EAC bearing mice treated with and without MECT *in-vivo* as reported earlier [31]. In brief 100 µl of ascites from control EAC bearing mice and the MECT treated mice were coated in a coating buffer at 4°C. Further wells were coated with anti-$VEGF_{165}$ antibodies and was followed by incubation alkaline phosphatase conjugated secondary antibodies. PNPP was used as substrate and absorbance was measured at 405 nm.

SDS-PAGE and immunoblot analysis

EAC cells treated and untreated with MECT *in-vivo* were harvested and cytosolic extract and nuclear protein extracts were prepared. For western blots, 50 µg of the protein per lane was separated on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to PVDF membranes, and reacted with the desired antibody. The proteins were detected by the secondary antibody conjugated to horse radish peroxidase and developed by enhanced chemiluminescence.

Statistical analysis

The results of the data were statistically analysed and the values are mean ± standard error (SE) of the three experiments (N=5).

Results

Identification of MECT by liquid chromatography- mass spectrometry (LC-MS)

The major chemical constituents of MECB were identified using LC-MS analysis. As shown in Figure 1, the major peaks from the chromatogram of MECB were identified as flavonoids such as scutellarin (A), apigenin (B), baicalein (C) and luteolin (D). The contents of flavonoids A-D were 36.9%, 6.3%, 12.6% and 9.3%, respectively.

Induction of apoptosis by MECT in EAC cells *in-vivo*

In an attempt to understand the *in vivo* effect of compounds on proliferation of EAC cells, Swiss Albino mice were treated with and without MECT. It was found that MECT treated mice showed significant antiproliferative effect towards EAC cells *in vivo* as monitored by the body weight as shown in Figure 2. In contrast, the weights of untreated EAT bearing mice steadily increased. The percentage of viable cells in control was found to be higher (97%) when compared to MECT treated cells (42%). Trypan blue dye exclusion was the method of choice to test the cell viability. Tumor cells treated with MECT clearly showed nuclear condensation and apoptotic bodies, which is the hallmark of cells undergoing apoptosis wherein control EAC cells very negligible amount of cells were found to be apoptotic.

MECT inhibits angiogenesis *in-vivo*

To investigate the antiangiogenic activity *in-vivo*, MECT was tested in the CAM and the mouse peritoneal angiogenesis assays. In the CAM assay model exuberant growth of blood vessels were observed on CAM of control eggs whereas newly formed microvessels were regressed around the area of MECT implanted disk (Figure 3). Neovascularization

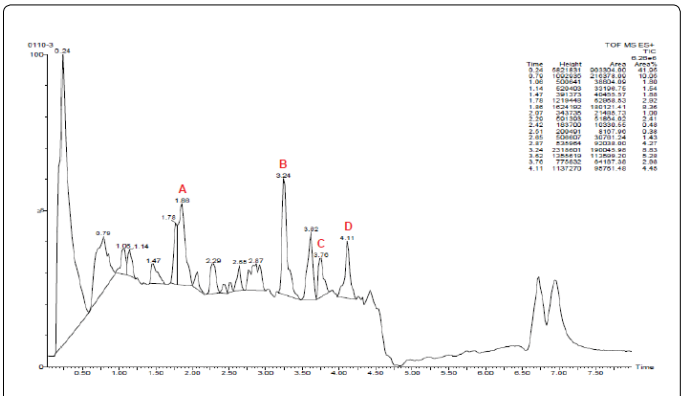

Figure 1: LC-MS analysis of MECT. The major peaks from the LC-MS were identified as flavonoids such as peak A; Scutellarin (36.9%), peak B; Apigenin (6.3%), peak C; Baicalein (12.6%), peak D; Luteolin (9.3%).

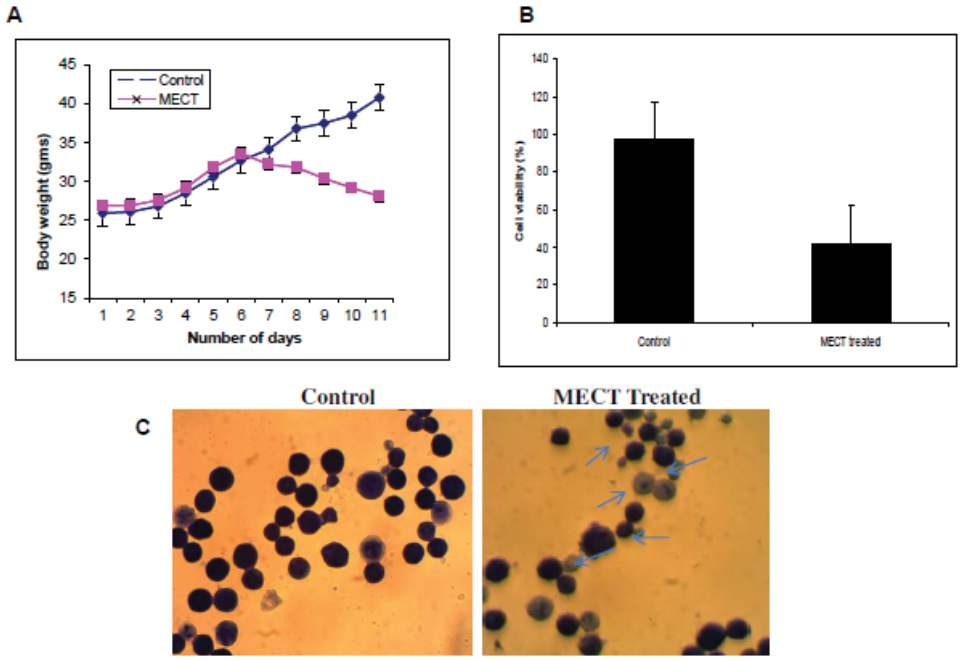

Figure 2: MECT inhibits ascites carcinoma cells proliferation *in-vivo*. EAC cells were grown in the mice peritoneum and administered with 100 mg/kg (i.p.) of MECT for three doses. A. Body weight of tumor bearing mice depicting the tumor regression. B. Reduction in ascites secretion. C. Giemsa stain showing the apoptotic morphology of MECT treated EAC cell.

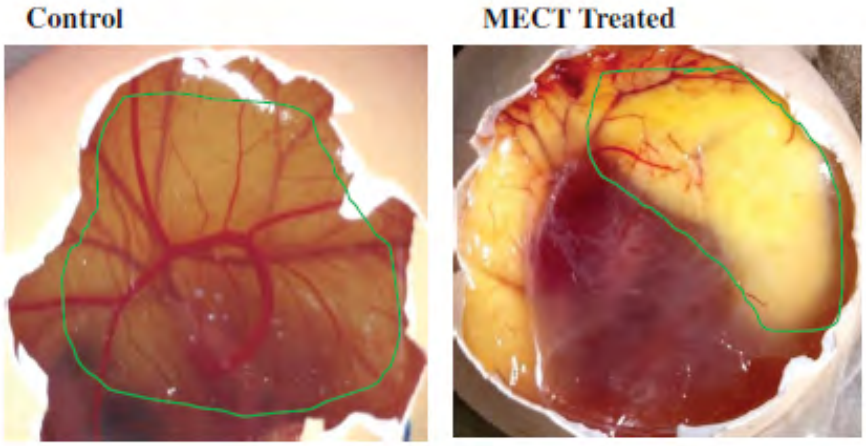

Figure 3: MECT inhibits *in-vivo* angiogenesis as seen in CAM assay. Pictures demonstrate the blood vessel branch points on CAM of both MECT treated and control egg during 11 days of fertilization. Inhibition of angiogenesis in CAM of MECT treated egg is evident. The experiment was done using minimum five eggs in each group. All photographs were taken at same magnification with unselected variability in vessel size.

is apparent in the inner side of peritoneal of EAC bearing mice and it's a reliable model to study *in-vivo* angiogenesis. Therefore the peritoneal lining of MECT treated mice was verified for the effect of plant extract on peritoneal angiogenesis. EAC bearing mice upon treatment with MECT showed decreased peritoneal angiogenesis when compared to untreated EAC bearing mice wherein excess vasculature was observed. H&E staining of peritoneum sections from the control mice showed prominent blood vessel growth. In contrast MECT treated peritoneum sections were characterized by a pronounced decrease in micro vessel density and the caliber of detectable vascular channels. Control peritoneum sections showed 22 ± 3 blood vessels where as in case of MECT treated 9 ± 2 blood vessels were observed (Figure 4).

MECT treatment inhibits the VEGF production and inhibits the nuclear translocation of HIF-1α

To determine whether MECT inhibits VEGF mediated angiogenesis *in-vivo*, we tested the effect of MECT on production of VEGF in EAC bearing mice. ELISA result confirms that MECT decrease *in-vivo* VEGF secretion in dose dependent manner. Figure 5A compares the amount of VEGF at different growth period for untreated and MECT treated EAC cells. In control EAC mice, the increment in the amount of VEGF was observed over the growth period, whereas in MECT treated mice

the ascites showed rather decreased amount of VEGF upon treatment indicating a dose dependent effect of MECT on VEGF secretion in EAC cells. In order to investigate the underlying mechanism of anti-angiogenesis of MECT we studied the expression of transcription factor HIF-1α known to be responsible for VEGF regulation. We observed that *in-vivo* treatment of MECT expressed HIF -1α protein levels in cytosolic fraction than in the nuclear extract where as in untreated increased expression of HIF-1α in the nuclear extract than in the cytosol indicating inhibition of nuclear translocation of HIF-1α. HIF-1α expression (Mean ± SE) in cytosolic extract of control and MECT treated was found to be 58.76 ± 1.77, 278 ± 4.61 respectively where as in nuclear extract which is 303 ± 4.98 and 77 ± 1.79 (Figure 5B).

Discussion

Angiogenesis is an essential step in solid tumour development, invasion, and metastasis. Angio-supression is one of the strategy adopted in cancer treatment which would promise a better therapeutic approach [30]. In the present study, the angiogenic activity of MECT has been investigated by murine carcinoma model and chick CAM assay. The experimental results of MECT on EAC model showed that administration of plant extract on day 7, 9, and 11 after tumor cell inoculation produced effective antitumor response (>25% ILS). MECT also caused reduction in ascites volume and tumour cell proliferation, exhibiting a potent anti-angiogenic activity against EAC *in-vivo*. *C. ternatea* effectively reduced the ascites secretion in EAC model without showing harmful effects on mice. Numerous studies have demonstrated angio-supressive action of naturally occurring phytoconstituents both *in-vivo* and *in-vitro* [31,32]. EAC cells treated with MECT exhibited apoptotic morphologies such as cellular shrinkage, nuclear condensation, and intra nucleosomal fragmentation. These results indicate that MECT induce apoptosis in EAC cells and inhibit tumour cell proliferation. The administration of MECT directly into the tumour growing site i.e., peritoneal region may bring about immediate and direct action on the tumour cells. In the control mice extensive angiogenesis in peritoneal lining was observed which may be attributed to the secretion of the angiogenic factors from the rapidly growing tumor cells. VEGF play a major role inmalignant ascites formation [19]. Upon ttreatment MECT significantly decreased peritoneal angiogenesis in EAC bearing mice suggesting its possible inhibitory effect on secretion of angiogenic stimulators. Neovasculature in tumors can be assessed based on micro vessel density (MVD) counts which reflects angioarchitectural properties of the tumor. The aim of anti-angiogenic therapy also focus on reduction of intercapillary distance to such an extent which limits tumour growth [20]. H&E stained peritoneal lining of both control and MECT treated mice were examined for the MVD count. As a result reduced amount of microvasculature was seen in the peritoneal linings of treated mice when compared to untreated EAC bearing mice. In addition CAM assay revealed MECT induced suppression of newly formed microvessel at the region of implanted disc. VEGF quantification provides additional support by proving dose dependent action of MECT in reducing VEGF secretion *in-vivo* and the effect was compared with untreated EAC bearing mice where increased VEGF level was observed over the growth period. Further, VEGF is been considered as a key inducer of angiogenesis in tumour condition [21]. As part of the study that involves phytochemical identification from *C. ternatea* flower was performed by LC-MS analysis. The major peaks in the chromatogram (Figure 1) represents the flavonoids Scutellarin (36.9%), Apigenin (6.3%), Baicalein (12.6%), Luteolin (9.3%) from the flower. Flavonoids being ubiquitous dietary phytoconstituents in nature have been shown to pocessess many pharmacological activities including anticancer property. Scientific reports have elucidated the significant chemopreventive role of flavonoids against ovarian, lung, breast, prostate and colon cancer etc. [33,34]. Recent study has shown that scutellarin, apigenin, baicalein, luteolin affect apoptotic signalling molecules in various leukemia cell lines and thus have

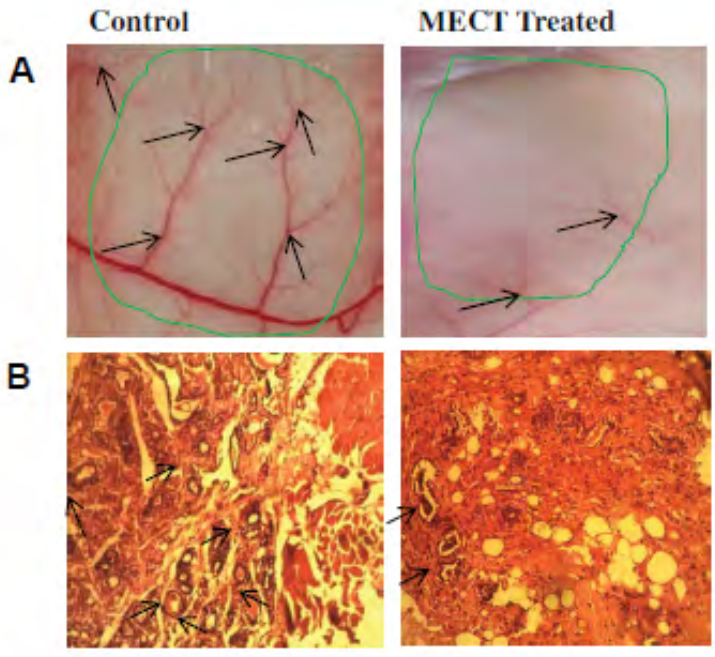

Figure 4: A. Suppression of peritoneal angiogenesis: Peritoneal linings of control mice and MECT treated mice were examined for angiogenesis. Inhibition of angiogenesis in MECT treated mice was evident as illustrated in the picture. B. H&E staining of peritoneum sections of the EAT bearing mice either untreated or treated with MECT *in-vivo*.

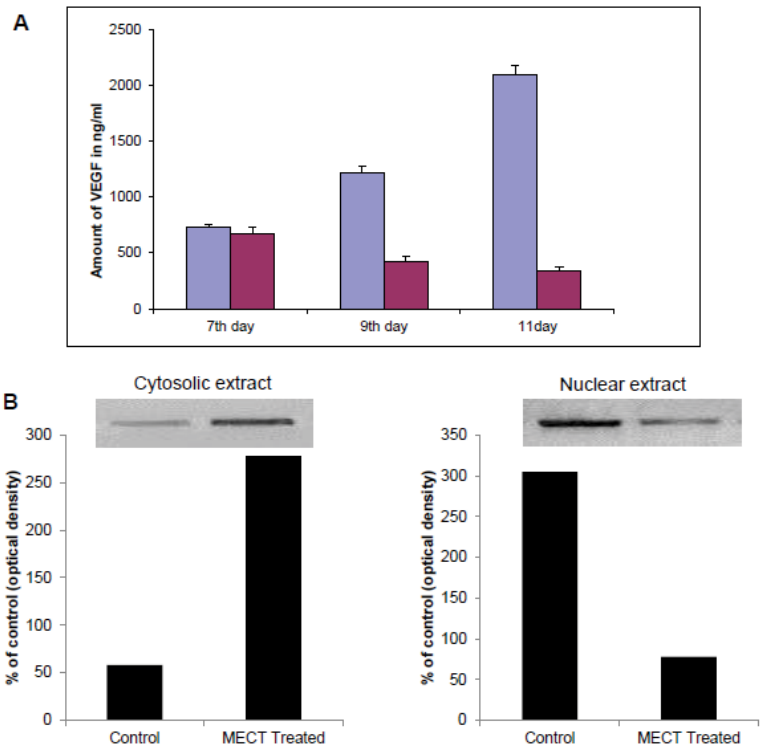

Figure 5: (A) *In-vivo* effect of MECT on VEGF production. EAT bearing mice was injected with 100 mg/kg bodyweight MECT *in-vivo* and ascites was collected after sacrificing the animal every alternate day after six days. ELISA was carried out to quantitate VEGF in the ascites fluid using anti-VEGF165 antibodies. Animals bearing EAT cells and not treated with BP-1 was used as control. **(B)** Effect of MECT on nuclear translocation of HIF-1*α*. Western blot analysis of HIF-1*α* expression in cytosolic extract and nuclear extract of EAT cells. The cells were treated with and without MECT (100 mg/kg body weight, i.p). Proteins were separated by SDS-PAGE followed by immunoblotting and detection with anti-HIF-1*α* antibodies. HIF-1α expression (Mean ± SE) in cytosolic extract of control and MECT treated was found to be 58.76 ± 1.77, 278 ± 4.61 respectively where as in nuclear extract 303 ± 4.98 and 77 ± 1.79. Essentially identical results were obtained in the three independent experiments. GAPDH was used as internal control in the study.

been demonstrated to possess anti-proliferative effect [35]. Whereas individual studies on apigenin [36] and luteolin [37] have shown that these flavonoids supress tumour angiogenesis by down regulating HIF-1α and VEGF secretion. Hence the anti-angiogenic property of MECT may be attributable to its flavonoid content. This report is a clear evidence for the *in-vivo* antiangiogenic effect of MECT as it was shown by peritoneal angiogenesis assay, chorioallantoic membrane (CAM) assay and also from the reduced EAC cell growth and proliferation, ascites volume, and decreased body weight of treated mice. Apart from cancer, angiogenesis plays an important role rheumatoid arthritis, in inducing synovitis, pannus formation [38]. CD147 also named extracellular matrix metallo proteinase inducer (EMMPRIN), induces up-regulation of VEGF and HIF-1α in rheumatoid arthritis (RA) FLS, further promotes angiogenesis. The expression levels of CD147 showed significantly positive correlations with VEGF and HIF-1α levels, as well as with vascular density, in RA synovium. CD174 targeted monoclonal antibodies helps in the reduced expression of HIF-1α and VEGF [39]. Keeping these facts in our studies it clearly evident that MECT treated EAC mice showed decreased VEGF levels and inhibits nuclear translocation of HIF-1α. This anti-angiogenic effect might be due to suppression of CD147 by phyto constituents of the plant which is a key regulator of HIF-1α and VEGF production. Hence we strongly declare that the anti-angiogenic effect of bio-active compounds of MECT in combating RA and which can be further translated for clinical applications.

Conclusion

From all supportive evidence it is certain that MECT treatment could significantly suppress the process of angiogenesis in murine carcinoma model *in-vivo* which was also evident from CAM assay. However, MECT is composed of several flavanoids compounds, major one is scutellarin as determined by LC-MS. The anticancer activity of these individual components or their combination effect is yet to be resolved. Molecular mechanisms studies showed that MECT treated cells very effectively suppress the translocation of HIF-1*α* translocation from cytosol to nucleus there by bringing the anti-angiogenic effect.

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