

Antiangiogenic Effect of *Tridax procumbens*(L.) Leaf Extract By Chick Chorioallantoic Membrane (CAM) Assay

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ABSTRACT: Angiogenesis is Playing an important role in wound healing, embryonic development, chronic inflammation, tumor progression etc. The present work analysed the anti-angiogenic property of *Tridax procumbens*(L.) (leaf) extracts using chick chorioallantoic membrane(CAM) assay in vivo. The natural anti-angiogenic inhibitors found in the *T. procumbens*(L.) (leaf) has blossomed as a very promising anticancer agent which suppressed neo-vascularization, endothelial cell migration, proliferation and tube formation. The fertilized eggs of *Gallus gallus* were incubated at 38^oC with 70% relative humidity. The developing embryo were treated with benzene extract of *T. procumbens* plant by window method at 48,72 and 96hrs of incubation and were further incubated upto 144hrs and then CAM was studied morphometrically and histologically. The results of the present study indicate strong inhibitory activity of the benzene extract on the number and area of secondary and tertiary vitelline veins. The inhibition of angiogenesis was highly significant at 48hrs. The antiangiogenic role of plant metabolites is discussed.

KEYWORDS: T.procumbens, anti-angiogenesis, chick chorioallantoic membrane assay(CAM).

I. INTRODUCTION

Angiogenesis is the neo-vascularization of blood vessels by endothelial cell migration and proliferation from existing blood vessels which is essential for growth of tumors and metastasis by (23). In (5) and (28), a tumor starts from a single normal cell which divides to make more cancer cells forming cancer metastasis. Tumors grow by neo-vascularization of blood vessels. Without blood supply, tumors can't grow. These neovascularized blood vessels continually supply oxygen and other nutrients allowing the new tumors to grow, without vasculature support tumors become apoptotic described in (28). Therefore, angiogenesis playing a critical role in the growth of cancer and tumor metastasis by (22).

Angiogenesis in the human body is regulated by two sets of counteracting factors, angiogenic stimulators and inhibitors. The author in (21) described, the various angiogenic stimulators/pro-angiogenic factors released from cancer cells such as Vascular endothelial growth factor/Vascular permeability factor(VEGF/VPF), Hepatocyte growth factor(HGF), basic fibroblast growth factor(bFGF), can proliferate and migrate endothelial cells and angiopoietins. Whereas inhibitors of angiogenesis include angiostatin, endostatin and thrombospondin by(13).

According to Nasar(32) a number of natural inhibitors isolated from plants can block the formation of new blood vessels. The anti-cancer activity of plant is due to antioxidants such as polysaccharide, polyphenols(gallic acid, ellagic acid and tannin), flavonoids(anthocyanin, quercetin, isoflavones, catechins, flavonones), carotenoids, and essential oil etc.

The author in (20) and (21) described that, cancer cell can generate various pro-angiogenic factors such as bFGF, HGF, EGF and VEGF/VPF factors can promote the migration, proliferation and tube formation of endothelial cells and these activities are essential steps of angiogenesis and angiopoietins.

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Tridax procumbens L. belonging to family Asteraceae found in a native of tropical Africa, Asia, Australia and distributed throughout in India. It is commonly known as coat button or Kansari(in Hindi) or Ghamra(in local language) by(15) and used in number of pharmacological activities like insecticidal, leishmanicidal, hepatoprotective, antiviral and antioxidant activity. It also possesses anti diabetic, anti plasmodial, and anti microbial properties, hepatoprotective nature besides gastritis and respiratory distress.

The aqueous extract of *T. procumbens*(leaf) inhibit proliferation of *Pseudomonas aeruginosa* by significant increase in leukocyte count and splenic antibody. It also showed immunomodulatory effect in albino rats by(30) and significant antiobesity activity by(31). In ethanolic extract it also showed significant anti-arthritic effect, antidiabetic and antihyper- lipidemic effects in rats using the Freund's model by(25). Muthusamy et al.(27) showed highest antimicrobial activity of *T.procumbens* in methanolic extract against *Symonella typhi* and *S. flexneri*.

The chick chorioallantoic membrane(CAM) assay is an attractive alternative model used in the study of angiogenesis by(16), tumor cell invasion and metastasis by (7) and (1). The author in (2) described that CAM model is highly vascularized, simple and easily available model allowing antimetastatic demonstration of small quantity experimental compound.

However, there was no systemic report available to investigate anti-angiogenic activity. The present review is aimed to notice anti-angiogenic activity of *Tridax procumbens*(leaf) benzene extract on chick chorioallantoic membrane(CAM) assay, which helps in serving the ailing mankind.

II.STATEMENT OF THE PROBLEM

Traditional Indian medicines(TIM) has been used for centuries in the treatment of various diseases, they still playing an important role in the development of potentially useful new compounds for prevention of cancer. The *T.procumbens* plant being widely used for their potential medicinal properties in wound healing, anti-inflammatory, anti-oxidant properties. However, scientific studies for investigation of anticarcinogenic and apoptic regulation of cancer, reports are scanty. Thus using *T.procumbens* plant in benzene extract will provide more information for the discovery of effective and novel compounds in the treatment of cancer.

A.OBJECTIVE OF STUDY

The objective of this study is to evaluate the anti-angiogenic property of the crude benzene extract from *T.procumbens*(leaf) through chicken chorioallantoic membrane(CAM) assay.

B.SIGNIFICANCE OF STUDY

More than 300 species of herbs and plant derived medicines used in the various treatment, becomes an important source for the development of anticancer drugs. The phytochemicals extract from plant regulated its cancer cell cycle and apoptosis induction. The exploration of anticancer agents from plant sources has started since the 1950s with extensive research and investigation to produce more effective but less toxic drugs. In the medications, scientist engaged to produce new therapeutic cancer drug which require continuous changes to improve their effectiveness. The pharmacological and toxicological data for antitumor effect of *T.procumbens* will provide more information to produce safe and effective anticancer drugs for treatment of cancer in future.

C.STATISTICAL ANALYSIS

The data was expressed as Mean \pm SEM and the statistical significance between groups was analyzed by Fisher and Yates (10), using student 't' test. The values of $p < 0.05$, $p < 0.01$, $p < 0.001$ were considered as significant.

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III. MATERIAL AND METHODS

A.COLLECTION OF PLANT MATERIAL

The freshly selected leaves of *T.procumbens* plant was washed with water and air dried in cool place. Using mortar and pestal fine powder of *T.procumbens*(leaf) plants was prepared. This powder was further used for preparation of acetone extract. The powder was mixed with benzene and kept for 48hrs at (28-33⁰C) further extract was filtered through whatman filter paper no.1. The filtrate was concentrated by evaporation using high speed vacuum evaporator(Buchi type). The yield extract was stored at 4⁰C under refrigerated condition till needed for. The 1mg of benzene extract was dissolved in 1ml dextrose with normal saline(DNS) was purchased from Mark-Bioscience Ltd, Goa(G21730031, Exp.Dec.2015). DNS is a medicated saline used to make proper concentration of extract for treatment on CAM assay.

B.ADMINISTRATION OF THE EXTRACT ON THE CHICK EMBRYO

The fertilized eggs of *Gallus gallus*(murghi) were swabbed with 70% alcohol for sterilization and further incubated at 37⁰C with 70-80% relative humidity. Without damaging the membrane 1ml of prepared extract was injected in 48, 72 and 96hrs developed chick embryos by window method described by(18). The window of the eggs was resealed using cellotape and kept in the incubator at 37⁰C upto 144hrs of development i.e. On completion of CAM ventilation and capillary networking.

C.DOSE SELECTION AND ADMINISTRATION BY WINDOW METHOD

The dose that showed 100% survivality without any mortality on hatching after treatment of *T.procumbens*(leaf) extract were selected. The selected dose were mixed with 1ml volume of DNS and were administered on different developed 48, 72 and 96hrs incubated embryos.

The experiment were prepared in three groups as Sham, DNS control and treated. The normal group of embryo was maintained as normal group. The embryos CAM of 48, 72 and 96hrs development were exposed to 1mg/ml *T.procumbens*(leaf) in benzene extract as described in Table-1 by (18) window method. The windows were resealed using sterilized adhesive tapes and CAM were studied after 144hrs.

The eggs shell were removed and embryos along with yolk were gently placed in PBS containing saline water after 144hrs of development. The area of embryo CAM and blood vasculature were imaged with digital camera and kept further for imaged analysis on computer.

D.QUANTIFICATION OF ANGIOGENESIS

Angiogenesis was quantified by counting number of secondary vitelline veins(SVV) and tertiary vitelline vein(TVV) and it's associated area for antiangiogenic response. The bifurcation points were used as initiation and termination markers. The area and vasculature networking were measured on stereoscopic microscope and was confirmed by using graph transparencies. The observed alterations are presented in Table 2 and Fig 1 and 2.

E.HISTOPATHOLOGICAL STUDY

After scarification of chick embryo, CAM were dissected out, fixed in 10% formaldehyde, dehydrated and embedded in paraffin wax(MP.56-58⁰C) for histological studies. The blocks were sectioned at 4 μ m on rotary microtome and stained with Hematoxyline and Eosin(HE) method. Examined under microscope for histopathological changes of CAM embryo

F.EVALUATION OF CAM ANGIOGENESIS

The Melkoman and it's coworker (11), described morphometrical evaluation of CAM as-
The total CAM area was calculated as $Area = (1/2A) \times (1/2B) \times \pi$.
Where A is the longest length, B is longest width and $\pi = 3.14$

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The number of secondary and tertiary blood vessels branching points and it's morphometric evaluation were counted manually on computer image. For histological evaluation paraffin block of CAM was sectioned at 5µm thickness by rotary microtome.

IV. RESULT AND DISCUSSION

The anti-angiogenic effect of the *T.procumbens*(leaf) in benzene extract was determined using the chicken chorioallantoic membrane(CAM) assay in vivo. The CAM model is an attractive alternative model used to study angiogenesis by (16), tumor cell invasion and metastasis by (7) and (1). Tufan and Satiroglu (1) explained, it is highly vascularized, simple and easily available model allowing antimetastatic demonstration of small quantity experimental compound.

Table 1: Groups as per treatment of *T.procumbens*(leaf) benzene extracts at different developmental stages of chick embryo in hrs.

Groups as per developmental stages in hrs.	Groups as per time of exposure initiations			Final development and hrs. of sacrifice
	48	72	96	
Group I - 48	—	—	√	144hrs.
Group II - 72	—	√	—	
Group III - 96	√	—	—	

The present study clearly demonstrated that acetone extract of *T.procumens*(leaf) showed strong anti-angiogenic property on 144hrs developed chick CAM embryo. The antiangiogenic activity were evaluated morphometrically and histologically.

Table 2: Effect of benzene extract of *T.procumbens*(leaf) on total number of blood vessels and area of CAM in chick embryos.

Initiation of Treat - ment(hrs)	Groups	Total number of blood vessels		Total CAM area in sq.cm
		Secondary blood vessels	Tertiary blood vessel	
48hrs.	Normal	12±0.72	176±4.72	26.62 ±1.78
	Sham control	11±0.62	175±4.69	26.34±1.86
	DNS(control)	13±0.82	178±5.12	28.27±2.01
	Benzene extract	9±0.044 ^{bpz}	103±2.34 ^{ctz}	20.58±1.29 ^{apy}
72hrs.	Normal	13±1.04	176±5.10	26.65±2.30
	Sham control	12±0.86	174±4.82	26.61±2.01
	DNS(control)	14±1.12	190±5.08	28.96±1.78
	Benzene extract	10±0.64 ^{aqy}	115±3.12 ^{ctz}	22.34±1.29 ^{apy}
96hrs.	Normal	14±0.98	176±4.64	26.72±0.98
	Sham control	11±1.08	175±3.98	26.64±1.19
	DNS(control)	15±1.30	186±4.02	29.02±1.60
	Benzene extract	11±0.78 ^{apx}	118±4.18 ^{bqz}	24.11±1.92 ^{apx}

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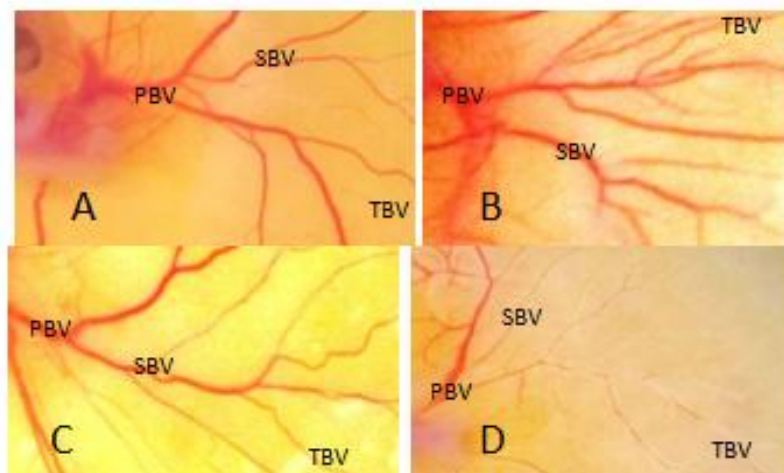
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(Results expressed as mean } S.E. of 5 embryos. p-values-a<0.05, b<0.01, c<0.001 vs. Normal embryos. p<0.05, q<0.01, r<0.001 vs. Sham control embryo. x<0.05, y<0.01, z<0.001 vs.DNS control embryo).

A.MORPHOMETRIC EVALUATION

For morphometric evaluation of CAM embryo, number of intersection points of PBV, SBV and TBV were counted. In normal embryos secondary and tertiary vessels number showed constant at 48, 72 and 96hrs of treatment. In sham control embryos number of blood vessels slightly decreases but marginal increased in branching pattern was observed in DNS control embryos. These results of DNS control indicates enhancement in proliferation of SBV and TBV. Treatment of benzene extract of *T.procumbens*(leaf) on CAM embryo showed marginal reduction in the number of blood vessels at all hrs of treatment but significant depletion in number of secondary blood vessels were observed in the embryos treated at 48hrs and 72hrs as(30.77% and 28.58%% respectively). This trend in the reduction of blood vessels remained same for TBV at 48 and 72hrs of treatment as (42.14% and 39.48%respectively) with normalized in number of blood vessels was observed at 96hrs of treatment. While total area of CAM was depleted at 48 and 72hrs by(27.21%and 22.86%respectively). Significantly decrease in number of blood vessels was noted at 48hrs treatment of *T.procumbens* (leaf) in benzene extract (Plate 1, Table 2 and Fig. 1-3).

Plate 1: Morphometric evaluation of chick CAM by effect of *T.procumbens*(leaf) extract.



A -piece of normal CAM
B-piece of DNS control CAM
C-piece of sham control CAM
D-piece of *T.procumbens*(leaf) extract treated CAM
PBV-primary blood vessel
SBV- secondary blood vessel
TBV- tertiary blood vessel

B.HISTOLOGICAL EVALUATION

As described in Materials and Methods T.S of CAM was stained with hematoxyline and eosin (HE) technique. Histologically normal and sham control CAM embryo showed three layers as . ectoderm, mesoderm and endoderm. The ectoderm showed, thin walled blood capillaries along with capillary plexus(CPL) and red blood cells(RBC) beneath the chorioallntoic epithelium(Plate 2 A and C). While in the DNS control CAM, blood vascularization significantly increased in the mesodermal layers along with capillary plexus(CPL)(Plate-B).

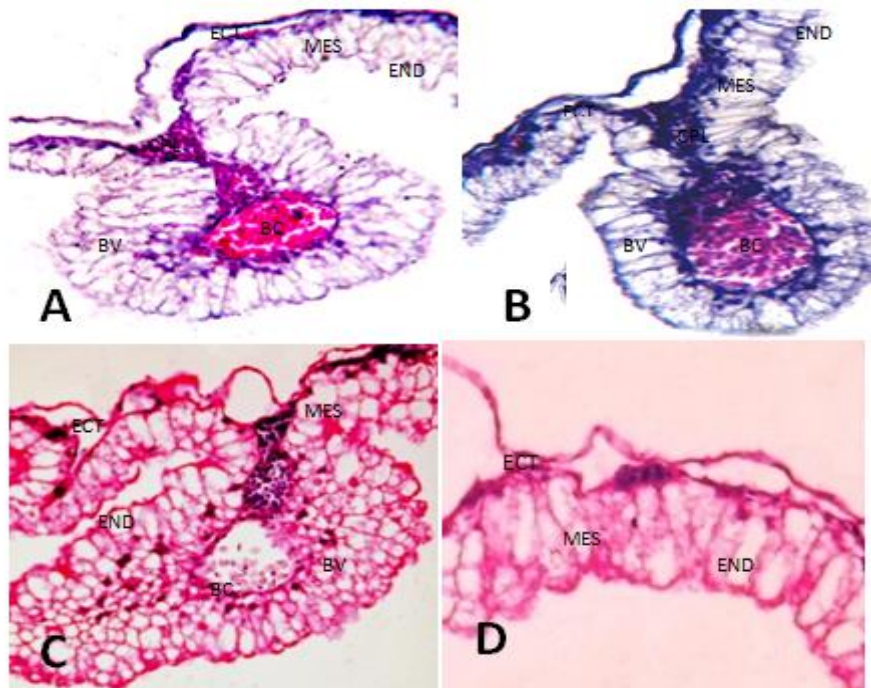
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Administration of *T.procumbens*(leaf) extract at 48hrs developed CAM, showed significantly depletion in endothelial cell proliferation with obliteration of blood vessels(Plate 2 D). The thickness of CAM was significantly decreased at 48hrs than 72 and 96hrs. The sham controlled embryo CAM showed slight decrease in endothelial blood vessels along with number of capillary plexus in the mesodermal layer but in DNS controlled CAM embryo significantly increased in number of capillary plexus along with numerous blood vessels were noted in the mesodermal layers(Plate-B).The Kerbel and Folkman (26) explained, angiogenesis is a sprouting of new blood vessels essential for tumor progression, dissemination and sprouting of new blood vessels during tumor formation which is regulated by both pro and anti-angiogenic factors. In (14) author showed, tumor induced process of angiogenesis by secreting several growth factors like VEGF, bFGF and HGF. Anti-angiogenic substances prevent and treat cancer area leading to the formation of formation. Now a days scientists are being tested for herbalists drugs targeting for blocking it's central role in promoting tumor growth. The *T.procumbens* L. contains compound like α -pirene(C10H16), β -pirene(C10H16), polyphenols, triterpenoids, flavonoids, alkaloids and essential oil.The author Manjamalai et al.(3) showed, anti-cancer effect of the α and β pirene in human cancer cells. The author in (29) explained, the flavonoids such as polyphenols and triterpenoids extracted from *T.procumbens*(leaf) exhibit anti-cancer effects against solid tumors and inhibited several epithelial cancers.

Plate-2: Histological evaluation of T.S. of chick CAM showing anti-angiogenic effect by *T.procumbens*(leaf) benzene extract.



A-Normal CAM, B-DNS control CAM, C-Sham control CAM, D- *T.procumbens* (leaf) extract treated CAM, BV- blood vessel, BC- blood cell, CPL- capillary plexus, ECT-ectoderm, END-endoderm, MES-mesoderm.

Figure 1 : *T.procumbens*(leaf) benzene extract influenced alterations in number of secondary blood vessels (on 144hrs of development).

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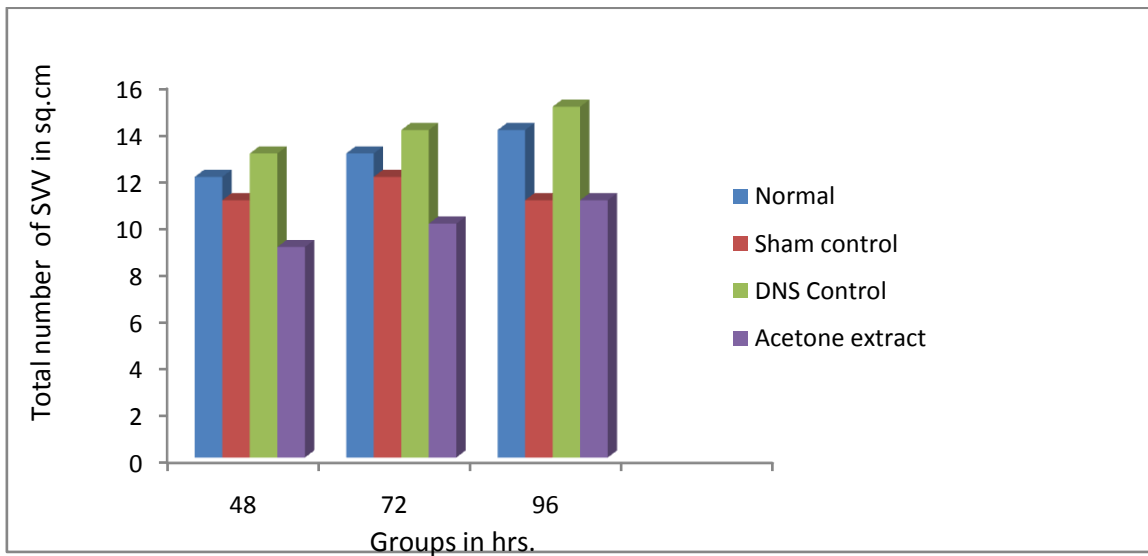


Figure 2: *T.procumbens*(leaf) benzene extract influenced alterations in number of tertiary blood vessels (on 144hrs of development).

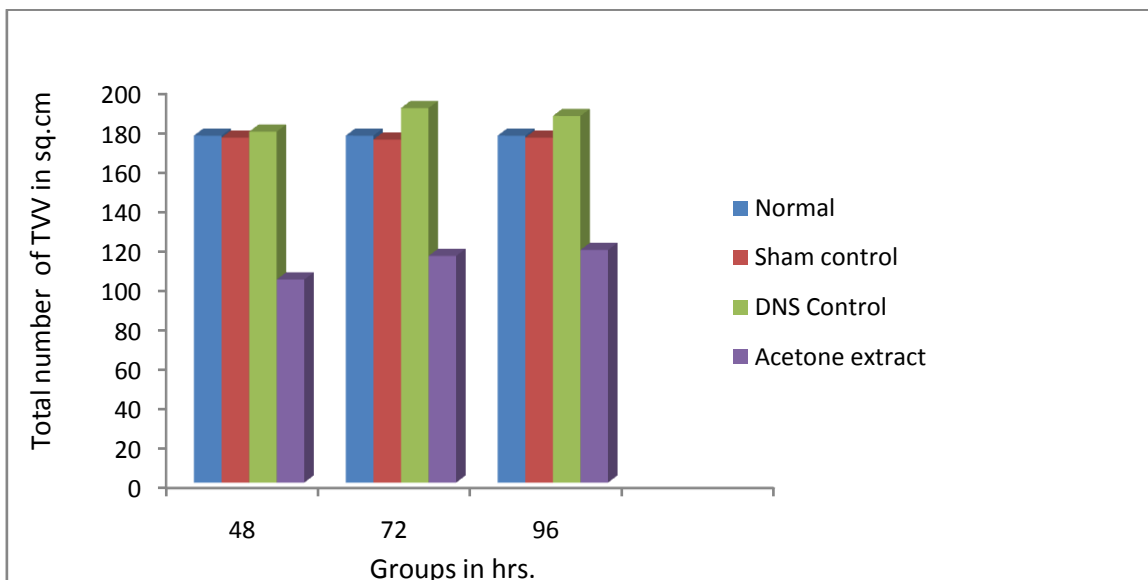
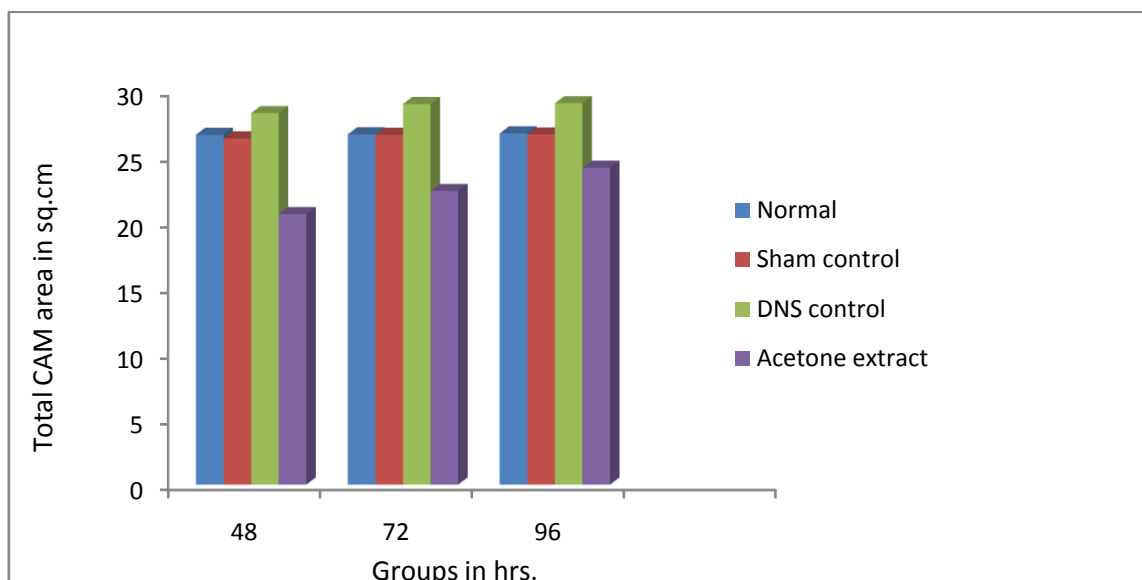


Figure 3 : *T.procumbens*(leaf) benzene extract influenced alterations in total area of CAM(on 144hrs. of development).

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The anticancer activities of *T.procumbens* L. reported by Vishnu Priya et al.(24) found to contains compounds such as α -pirene(C10H16), β -pirene(C10H16) strongly showing breast cancer, leukemic cancer and anti-prostate cancer activities. The author in (3) showed, the essential oil of *T.procumbens*(leaf) for chemoprevention of lung cancer development in B16F-10 mice and potentially used in drug development for cancer treatment.

The author Sarkar and Li (9) reported, isoflavones (Genistein) inhibit angiogenesis by arresting G2 cell cycle, suppressing c-fos expression and NF- κ B activation signaling pathway, induction of apoptosis, arresting G2 cell cycle, inhibition of c-fos expression, NF- κ B activation, and suppression of receptor tyrosine kinase activity for VEGF, EGF, and HGF.

The author Davis (4) found that, quercetin is a flavonoid from numerous types of fruits and vegetables inhibiting secretion of MMP-2 and MMP-9 from tumor cells and also suppressed endothelial cell proliferation and migration. It reduces in vitro tube formation of VEGF-stimulated human umbilical vein endothelial cells (HUVECs) by (12) and also inhibit hypoxia induced VEGF expression in NCI-H157 cells by (6) along with STAT -3 inhibition by (19) might be involved in anti-angiogenesis.

According to Sayed(8), an alkaloid isolated from chili pepper inhibit DNA synthesis, VEGF-induced p38 MAPK, p125 also arrest endothelial cells and author in (17) showed blocked activation of STAT3 associated with depletion in proliferation of angiogenesis.

In the present investigation it is observed that secondary and tertiary vitelline veins are most affected and shows maximum inhibition at 48hrs. than 72 and 96hrs. The benzene extract of *T.procumbens*(leaf) showed maximum depletion in the number and area of TVV by benzene extracts.

V. CONCLUSION

Cancer is the life threatening and dreadful disease characterized by the abnormal and uncontrolled proliferation and migration of cells leading tumor formation. Conventional treatment chemotherapy could cause adverse and toxic side effects which fails to control the disease affecting persons life. *T. procumbens* leaves have been traditionally used worldwide for its versatile therapeutic properties as wound healing, anti-inflammatory, anti-diabetic activity. In the present study it revealed that, the leaf extract of *T. procumbens* suppressed endothelial cell migration, proliferation and growth of tumor metastasis. The phytochemicals such as flavonoids, triterpenoids, alkaloids and essential oil present in the leaves of this plant could be responsible for its significant anti-angiogenic activity. This can be an important source in finding new medicine, leading to discover future anti-carcinogenic drug.

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VI. ABBREVIATIONS

VEGF-Vascular endothelial growth factor, VPF-Vascular permeability factor, HGF- Hepatocyte growth factor, bFGF- basic fibroblast growth factor, DNS- Dextrose normal saline, CAM- Chick chorioallantoic membrane, SVV- secondary vitelline veins, TVV- tertiary vitelline vein, PBV-primary blood vessel, SBV- secondary blood vessel, TBV- tertiary blood vessel, HUVEC- human umbilical vein endothelial cells and STAT 3 – Signal Transducer And Activation of Transcription 3, BV- blood vessel, BC- blood cell, CPL- capillary plexus, ECT-ectoderm, END-endoderm, MES-mesoderm.

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