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ANALYSIS

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Phenytoin, *Azadirachta indica* and *Rheum palmatum* inhibits angiogenesis, proliferation and invasion of blood capillaries in implanted sponge by various postulated mechanisms: A pre-clinical screening

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ABSTRACT

Angiogenesis is the growth of new blood vessels. It mostly occurs where Vascular endothelial Growth factors (VEGF) gets triggered and leads to Endothelial Cell proliferation and thus leading to formation of new blood vessels. Inflammation and free radical formation and are the imperative factors for the activation of VEGF and endothelial proliferation so the plants were selected which have already proven anti-inflammatory, antioxidant ad anti-proliferative activity. i. e. *Azadirachta indica*, *Rheum palmatum*, *Rosa centifolia.* Recently Na^+ channels are identified on the endothelial cells of the blood capillaries but the exact role of channels yet not been established so we have selected Phenytoin a Na^+ channel blocker. To find out the effect of *Azadirachta indica*, *Rheum palmatum*, *Rosa centifolia* and Phenytoin sodium for its anti-angiogenic property. *In vivo* Sponge Implantation method was used to induce angiogenesis in male Wistar rats by considering various parameters like capillary density, haemoglobin content, weight of spleen, thymus and implanted sponge etc. Among all the test drugs *Azadirachta indica has* shown some promising anti-angiogenic effect in all parameters at the dose of 0.5 mg and 5 mg. Phenytoin and *Rheum palmatum* also have shown protection but not as good as *Azadirachta indica* while *Rosa centifolia* has not shown any protection for any of the parameters. *Azadirachta indica* has shown strong anti-angiogenic property. Phenytoin sodium and *Rheum palmatum* has also shown anti-angiogenic property but not as good as *Azadirachta indica*.

Key Words: Angiogenesis, *Azadirachta indica*, *Rheum palmatum*, *Rosa Centifolia*, Phenytoin Sodium, VEGF, Sponge implantation method.

1. INTRODUCTION

Angiogenesis can be defined as the development of new blood vessels from an existing vascular bed. No metabolically active tissue in the body is more than a few hundred micrometers from a blood capillary, which is formed by the process of angiogenesis. Oxygen is conducted to these tissues by blood capillaries: more capillaries can improve tissue oxygenation and thus enhance energy production; fewer capillaries can lead to hypoxia and even anoxia in the tissues (Adair and Montani, 2010).

In several diseases, excessive angiogenesis is part of the pathology. These diseases include cancer (both solid and hematologic tumors), cardiovascular diseases (atherosclerosis), and chronic inflammation (rheumatoid arthritis, Crohn's disease, diabetes (diabetic retinopathy), psoriasis, endometriosis, and adiposity (Khurana et al. 2005; Feldmann et al., 1996; Cao, 2010). These diseases may benefit from therapeutic inhibition of angiogenesis. In the current study both Herbal (*Azadirachta indica, Rheum palmatum, Rosa centifolia* and synthetic drug (Phenytoin sodium) have been taken to screen for their anti-angiogenesic property (Schumacher et al., 2011; He et al., 2008; Devi, 2012). From the literature review it was found that drugs which have anti-inflammatory, anti-oxidant and anti-proliferative activity, the same drug may show anti-angiogenic activity based upon these observations we have selected these plants. Vascular endothelial cells contain Na^+ channels which are involved into the modulatory activity of capillary endothelial growth. So, we made an attempt to find out the role of Phenytoin a Na Channel blocker for its anti-angiogenic property (Andikopolos et al., 2011). Many *in vivo* models are available to induce angiogenesis like Hind Limb ischemia model, Left Coronary Artery Ligation Method, Matrigel Plug Assay, Sponge Impantation Method, Corneal Neovascularization Cauterization Method, and Sponge Granuloma Angiogenesis Assay (Kim et al., 2001). Among them we have selected Sponge Implantation method as the implanted sponges resembles the tumor formation in human body, thus drugs effectively inhibiting angiogenesis in the sponges can inhibit tumor formation as blood supply to the tumor would be hindered and thus may show anti-angiogenesis (Auerbache et al., 2003).

2. MATERIAL AND METHODS

Materials:

Drugs: *Azadirachta indica, Rheum palmatum, Rosa centifloia,* Phenytoin sodium.

Instruments: Small animal Operation table, Sutures, 5mm cannulas, Polyester sponge, Applicator stick, Inverted microscope, Dissection kit, UV-Spectrophotometer, 0.1mg sensitive analytical balance, Cooling Research centrifuge (R-24), Soxhlet apparatus, Rotary Flash Evaporator, Heating mantle, Clevengers apparatus.

Chemicals:

Ketamine, Xylazine, *Azadirachta indica* and *Rheum palmatum* methanolic, Petroleum ether, anhydrous sodium sulfate, alcohol, Phenytoin sodium injection was used (Eptoin, Mgf by Abbott health care Pvt limited, batch no: AA2D07), Pyruvic acid, Silver nitrate and Potassium nitrate, Phosphate buffer saline, Dilute NaOH, ethanol, Emodin reference standard supplied by J & K Chemicals Ltd, Rhein reference standard (Sigma) and Quercetin reference standard (Phyto neutaceuticals). The entire chemicals used in the study were of AR grade.

Experimental Design and setting:

The animals:

Healthy seventy eight, 4-8 weeks old, male Wister rats weighing in between 150-200 gm were used in this study. National animal ethical guidelines were followed strictly for handling; care and maintenance. The protocol has been approved by the IAEC (Institutional Animal ethical Committee) and CPCSEA bearing reference no: CPCSEA/1657/IAEC/CMRCP/Col 11-13/21.

The animals were divided in thirteen groups by keeping 6 animals in each group. Animal grouping and treatment schedule is mentioned in Table 1.

Collection and Authentication of test drugs: Fresh leaves of *Azadirachta indica, Rhubarb* and Rosa *centifloia* were collected from the campus premises of CMR College of Pharmacy. The Plants were authenticated at Sri Venkateswara University, Tirupathi. Plant specimen is deposited in the department of Botany for the further reference.

***Azadirachta indica*:**

Extraction procedure: The fresh Neem leaves were shade dried for 3-4 days and then was coarsely powdered. Test drug of Neem was prepared by macerating 50gms of dried leaf powder in a beaker. To this 250 ml of methanol was added and kept for 24 h with periodic shaking. Filtered and the filtrate was collected. The procedure was repeated three times. The collected filtered was dried by evaporation process in a china dish and the product was collected and weighed (Marugathavalli et al., 2012).

***Rhubarb centifolia*:**

Extraction procedure: Test drug of Rhubarb was prepared by Soxhlet extraction of the dried roots. Soxhlet extraction of 25 g of the dried coarsely powdered roots in 300 mL of Methanol for 8 h followed by removal of the solvent on rotary evaporator gave a yellow solid material (Ashnagar, et al., 2007).

Rosa centifloia:

Extraction of Oil: For the extraction of essential oil from *R. centifolia*, solvent extraction was performed and concrete & absolute oils were obtained. 20kg flowers of the cultivar *R. centifolia* were used for the extraction of oil. The extraction process was carried out using petroleum ether as organic solvent. When the entire aroma was taken out by solvent, then the process of distillation was carried out. The apparatus to be used in each process was thoroughly washed and dried. Distillation was done in rotary evaporator.

Concrete oil recovery: Dissolved organic residue in the petroleum ether was collected in a flask and dried over by adding anhydrous $NaSO_4$. The last traces of petroleum ether were removed by bubbling nitrogen gas through the oil. Concrete oil was taken in preweighed 100 ml flask and the weight of concrete oil was determined by again weighing the flask. Percentage yield of concrete oil was calculated on the basis of petal weight.

Absolute oil recovery: Concrete oil was dissolved in minimum volume of absolute alcohol to remove the natural waxes present in the essential oil and was then filtered through a Whatmann filter paper # 43. Alcohol was removed by distillation and by passing nitrogen gas through the oil. Percent yield of absolute oil was also calculated on the basis of petal weight. The results are mentioned in table 2.

HPLC Analysis of Rhubarb extract:

The HPLC method showed the separation of Rhein and Emodin in the extract of the Rhubarb root (Rheum palmatum) using gradient analysis on a reversed phase column and UV detection. The autosampler temperature was set to 4 ºC to avoid decomposition of the samples**.** Refer fig 1.

HPLC Analysis for Neem leaves extract:

An Agilent 1200 HPLC system with a DAD UV–VIS detector was used. The separation was performed on a reversed-phase column (ZORBAX C18 21.2 9 150 mm, 5 lm) eluting with methanol–water (0:100–100:0) at a flow rate of 1 ml/min and UV detection (280 nm). The active compound Quercetin in methanolic leaf extract was identified by comparison with pure standard. Refer fig 2 and 3.

Sponge Implantation in Rats:

It was carried out by anaesthetizing the rats by a cocktail of Ketamine (80mg/kg) *i.p* and Xylazine (5mg/kg) *i.p* and sponges were implanted *s.c.* All the sponges were soaked in 3 mg Pyruvic acid and then implanted. Sponges of 2cm diameter and 8mm thickness were sterilized by soaking them in 70% ethanol for 3 hours and then soaking them in Phosphate buffer saline solution and finally by exposing them in U.V. The skin was cut by surgical blade and sterile sponge was implanted *s.c* by creating a subcutaneous air pocket and 5-mm polyethylene cannula was installed inside each sponge disc through needle puncture in the skin and was sutured back by 5/0 silk sutures. Daily administration of test drugs of doses 0.03 mg, 0.38 mg and 3.85 mg into the sponge site through the installed cannula for 13 days i.e., a total of 0.5 mg, 5 mg and 50 mg at the end of 13 days. After recovery of the animal from anaesthesia they were allowed to have normal diet and water, there after the animals were housed individually in cages. Tramadol at a dose of 0.9 mg/kg was injected *i.m* twice a day in the morning and evening, Amikacin at a dose of 5 mg/0.5kg was injected *i.m* in the morning. The analgesic and antibiotic drugs were given for the first three days post-operation in order to prevent further pain Tramadol at a dose of 12.5 mg/kg *p.o* was given for one week. On 14^{th} day the animals were sacrificed by CO_2 over dose and the sponges were dissected (Ref. fig 4) out and wet weight was calculated followed by histopathology and haemoglobin count of each sponge (Lee et al., 2001).

Wet Weight of the Organs: On the 14^{th} day of the study after sacrificing animals Thymus gland and spleen were also taken out. While dissecting out much traction pressure was avoided to reduce errors in weight calculations. All the dissections and weight calculation was done by single investigator to avoid person to person variation.

Haemoglobin counts in the R.B.C's of the blood vessels on the sponge were calculated.

Histopathology Procedure: The sponges were bisected and fixed in formal saline at 4°C for 1 h and then immersed in 75% ethanol for 30 min and finally kept in 90% ethanol. Paraffin sections (10 pm) were prepared and stained with haematoxylin and eosin. Refer fig 5.

Procedure for determining Hemoglobin content: The sponges after they were removed from the body of the rats, were soaked in double distilled water and homogenized completely over ice platform for 5min and then the liquid obtained was centrifuged at 10,000 rpm in Cooling centrifuge machine for 5 minutes and the supernatant liquid obtained was put in the cell count machine and the hemoglobin content was estimated as g/dL.

Procedure for determining Number of blood vessels formed per sponge: The sponges were bisected and fixed in formal saline at 4°C for 1 h and then immersed in 75% ethanol for 30 min and finally kept in 90% ethanol. Paraffin sections (10 pm) were prepared and stained with haematoxylin and eosin. The prepared slides were then seen under trinocular microscope at magnification of 400X and the number of circular spaces midst the fibroblast growth regions present were counted as they represent the blood vessels formed in the sponges.

Statistical Analysis

The data were analyzed as mean ± SEM. P value less than 0.05 ($P \leq 0.05$) was considered as the significant level in different groups using one-way ANOVA followed by Dunnet's test.

3. RESULTS

Before the start of the experiment, healthy rats in a narrow weight range (150-200 g) were selected; so, there was no significant difference between the animal's weights in different animal groups. After the implantation of sponges on the 14th day the rats showed a significant decrease in weight of the sponge, thymus and spleen as well as decreased number of blood vessel count over the sponges and haemoglobin content in the sponges when compared to pyruvic acid treated groups.

Number of Blood vessels: With treatment of 0.5 mg Phenytoin sodium, 0.5 mg Rhubarb and 0.5 mg Neem extracts there was a significant decrease in the number of blood vessels when compared to pyruvic acid treated group. (27±0.73, 19±0.69 and 14±0.4 vs. 38±0.79) and when treated with 5 mg Phenytoin sodium, 5 mg Rhubarb and 5 mg Neem extracts there was a significant decrease as well **(**16±0.87, 13±0.6 and 10±0.6 vs. 38±0.79). The best results were shown by 50 mg Neem extract where the number of blood vessels reduced from 5±0.57 to 38±0.79. Rose extracts didn't show any significant decrease in number of blood vessels. For results refer fig 6.

Hemoglobin content: With treatment of 0.5 mg Phenytoin sodium, 0.5 mg Rhubarb and 0.5 mg Neem extracts there was a significant decrease in the Hemoglobin content on the sponges when compared to pyruvic acid treated group. (0.51±0.03, 0.4±0.03 and 0.4±0.03 vs. 0.78±0.06) and when treated with 5 mg Phenytoin sodium, 5 mg Rhubarb and 5 mg Neem extracts there was a significant decrease as well (0.3±0.03, 0.03±0.07 and 0.03±0.07vs. 0.78±0.06). The best results were shown by 50 mg Neem extract where the number of blood vessels reduced from 0.02±0.05 to 0.78±0.06. Rose extracts didn't show any significant decrease in number of blood vessels. For results refer fig 7.

Wet weight of Spleen, Thymus gland and Sponge: With treatment of 0.5 mg Phenytoin sodium, 0.5 mg Rhubarb and 0.5 mg Neem extracts there was a significant decrease in the wet weight of Spleen (651.83±11.06, 530.83±12.05 and 504±14.79 vs. 1027.8±11.1), Thymus (253.16±11.95, 180±14.93 and 160.5±12.01 vs. 454.83±12.15) and Sponge (359.16±11.25, 437±14.84 and 341.66±18.46 vs. 513.3±11.52) when compared to pyruvic acid treated group. When treated with 5 mg Phenytoin sodium, 5 mg Rhubarb and 5 mg Neem extracts there was a significant decrease as well in Spleen (534.5±12.06, 448±12.87 and 426.66±12.53 vs. 1027.8±11.1), Thymus (168.33±12.15, 103±12.25 and 92.16±12.24 vs. 454.83±12.15) and Sponge (428.5±12.49, 312±12.68 and 302.83±15.55vs. 513.3±11.52). The best results were shown by 50 mg Neem extract where the wet weight of spleen reduced from 1027.8±11.1 to 313.5±9.28, wet weight of thymus gland reduced from 454.83±12.15 to 49.83±11.56 and wet weight of sponge reduced from 513.3±11.52 to 231.66±10.83.Rose extracts didn't show any significant decrease in number of blood vessels. With the treatment of pyruvic acid the organs and the sponges significantly showed increase in weight but after the treatment there was a significant decrease in the weight and the weight of the organs gradually returned to their actual weight with increase of dose. But there was no significant change in the decrease of weight when treated with 50 mg Phenytoin sodium, 50 mg of Rhubarb, 0.5 mg, 5 mg and 50 mg of Rose extract. For results refer fig 8.

4. DISCUSSIONS

We had several findings in the study. Methanolic extract of Neem was able to induce apoptosis and had anti-proliferative effect through several mechanisms. Nimbolide is a plant derived limonoid of *A. Indica.* Down regulation of lipid peroxidation, simultaneously increased the level of Glutathione (GSH) and GSH-dependent enzyme which exerts one of the anti-proliferative pathway. Nimbolide a triterpene was found to be highly effective in several cancer cell lines like U937, HL-60, THP1, B16 and bowe. Nimbolide can modulate the expression of genes of MMP-9, VEGF, ICAM-1, which are required for tumor metastasis. Nimbolide down –regulates the expression of MMP-9, VEGF, and ICAM-1 which is responsible for its anti- proliferative effect (Dinda, et al., 2013).

Quercetin is another anti-proliferative constituent of Neem. Quercetin inhibits several important steps of angiogenesis, including proliferation, migration and tube formation. It inhibits the enzymatic action of Protein kinase C, an enzyme essential for signal transduction on the cell membrane and cell growth (Yi, et al., 2008). Inhibition of cell proliferation is also due to suppression of endothelial nitric oxide synthase(eNOS) and early M- phase cell cycle arrest (Jackson and Varema, et al., 2006).

Quercetin undergoes auto-oxidation by intracellular reactions and in presence of oxygen radical species to Quinonoid intermediates. These compounds may damage DNA directly by strand scission thus contributing to cytotoxic effect. Quercetin inhibits angiogenesis also by direct inhibition of VEGFR-2(Vascular endothelial growth factor receptor-2) on the endothelial cell surface. It inhibits VEGFR-2 phosphorylation in a dose dependent manner (Pratheeshkumar, et al., 2012).

Rhubarb which has been used as a traditional Chinese medicine since ancient times and today is stil present in various herbal preperations, has a anti- neoplastic potentials, due to its anthraquiones like Rhein, emodin and aloe-emodin.the most abundant anthraquinone of Rhubarb,

Emodin, was capable of inhibiting cellular proliferation through tyrosine kinases, phosphoinosotal 3-kinase(PI3K), protein kinase C,(PKC) , NF-kappaB and mitogen activated protein kinase (MAPK) signaling cascades.Aloe- emodin is another major compound in Rhubarb found to have anti- tumor properties demonstrated through the P^{53} and its downstream P^{21} pathway.Rhein inhibits the uptake of glucose in tumor cells and causes changes in membrane associated functions, leading to cell death. Presence of all the three major anthaquinones was confirmed by compairing our test sample of rhubarb wih standard Rhein and Emodin at 250nm and 300nm respectively (He, et al., 2011).

In the angiogenesis sodium channels also plays the important role as an initiating factor which is triggered by hypoxic conditions. Vascular endothelial growth factor (VEGF) is essential for EC functioning under normal and pathophysiological conditions. Binding of VEGF to its major receptor, VEGFR2 Flk1/KDR), activates at least three parallel intracellular signaling cascades involving phospholipase C1 (PLC1), Src kinase, and phosphoinositide 3-kinase (PI3K) The final result is mobilization of downstream effectors, such as protein kinase C (PKC), endothelial nitric-oxide synthase, mitogen-activated protein kinases (MAPKs), and focal adhesion kinase. Voltage-gated sodium channels (VGSCs) are plasma membrane proteins that allow influx of Na^+ upon membrane depolarization. VGSCs comprise an α subunit (VGSCα), associated with one or more auxiliary β subunits (VGSCβs) . In mammals, nine VGSCα isoforms have been identified: Nav1.1– Nav1.9. Based on their sensitivity to the selective VGSC blocker tetrodotoxin (TTX), the isoforms are defined as TTX-resistant (TTX-R; Nav1.5, Nav1.8, and Nav1.9; IC50 in the μmol/liter range) and TTX-sensitive (TTX-S; Nav1.1–Nav1.4, Nav1.6, and Nav1.7; IC50 in the nmol/liter range). VGSC expression is not restricted to neurons and cardiac muscle cells; a number of "non-excitable" cells, including ECs, also express functional VGSCs

It was previously demonstrated that up-regulation of functional VGSC expression in metastatic tumor cells, and VGSC activity was found to enhance cellular invasiveness in a variety of human cancers, including prostate, breast and colon. Based on the parallels between the processes involved in angiogenesis and tumor cell invasion and the similarities between endothelial and neuronal guidance, it was hypothesized that VGSC activity may be involved in the angiogenic properties of ECs. VGSC expression has been reported in human umbilical vein endothelial cells (HUVECs). However, the subtype(s) of channel present has not been determined, and the status of α-subunits, which can significantly influence VGSC function and act independently as cell adhesion molecules, is not known. Finally, although it has been speculated that ion channel activity may be involved in angiogenesis the specific functional roles of the VGSCs expressed in ECs (and non-excitable cells generally) and the associated molecular signaling mechanism(s) are unclear. VEGF-induced proliferation, chemotaxis, and tubular differentiation and decreased adhesion to substrate thus significantly reduced by Phenytoin sodium which is a sodium channel blocker and thereby showing anti-angiogenic effect.

5. CONCLUSION

Methanolic extract of *Rheum palmatum* showed the presence of Rhein, Emodin and aloe-emodin which are potent anti-oxidants thus showing anti-oxidant properties at a dose of 0.5 mg and 5 mg. The methanolic extract of *Azadirachta indica* showed the presence Catechin, Epicatechin, Gallic acid which are potent anti-oxidants and anti-inflammatory agents, Nimbolide which is potent anti-proilferative agent showed good anti-angiogenesis activity by dose dependent manner in sponge implantation model. Sodium channel activation leads to angiogenesis. Phenytoin, a Na^+ channel blocker showed good anti-angiogenesis property by use dependent blocking the Na^+ channels and thus inhibiting new blood vessel formations at a dose of 0.5mg and 5 mg. The decrease in angiogenesis was proved by observing the parameters like decrease in weight of Spleen, Thymus and implanted Sponge and decrease in number of blood vessels on the sponges and decrease in content of Haemoglobin from the R.B.C's in the blood present in the blood vessels in the sponges. By estimating all the significance values it was conformed that *Azadirachta indica* has shown potent effect by inhibiting angiogenesis. Phenytoin and *Rheum palmatum* has also shown significant values and thus proving to be effective anti-angiogenic drug. It could thus be suggested that Phenytoin, Rhubarb and Neem may have a therapeutic potential in the angiogenic disorders.

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Table 1

Treatment schedule for Sponge Implantation Method

GROUP NO.	TREATMENT (Sponge implantation method)	DOSE		R.O.A
		In mg	In ml	
I	Pyruvic acid	3 mg	0.059 ml	*s.c* Implanted
II	Water for injection+ Phenytoin	0.03 mg	0.015 ml	*s.c*
III	Water for injection+ Phenytoin	0.38 mg	0.076 ml	*s.c*
IV	Water for injection + Phenytoin	3.85 mg	0.12 ml	*s.c*
V	Water for injection + *Rheum palmatum*	0.03 mg	0.015 ml	*s.c*
VI	Water for injection + *Rheum palmatum*	0.38 mg	0.076 ml	*s.c*
VII	Water for injection+ *Rheum palmatum*	3.85 mg	0.12 ml	*s.c*
VIII	Water for injection + *Azadirachta indica*	0.03 mg	0.015 ml	*s.c*
IX	Water for injection+ *Azadirachta indica*	0.38 mg	0.076 ml	*s.c*
X	Water for injection + *Azadirachta indica*	3.85 mg	0.12 ml	*s.c*
XI	Water for injection+ *Rosa centifolia*	0.03 mg	0.015 ml	*s.c*
XII	Water for injection + *Rosa centifolia*	0.38 mg	0.076 ml	*s.c*
XIII	Water for injection+ *Rosa centifolia*	3.85 mg	0.12 ml	*s.c*

Table 2

Percentage yield obtained after extraction process

Name of the Plant	Yield obtained	% Yield obtained
Azadirachta indica	4 gms	8%
Rheum palmatum	10 gms	40%
Rosa centifolia		0.128%

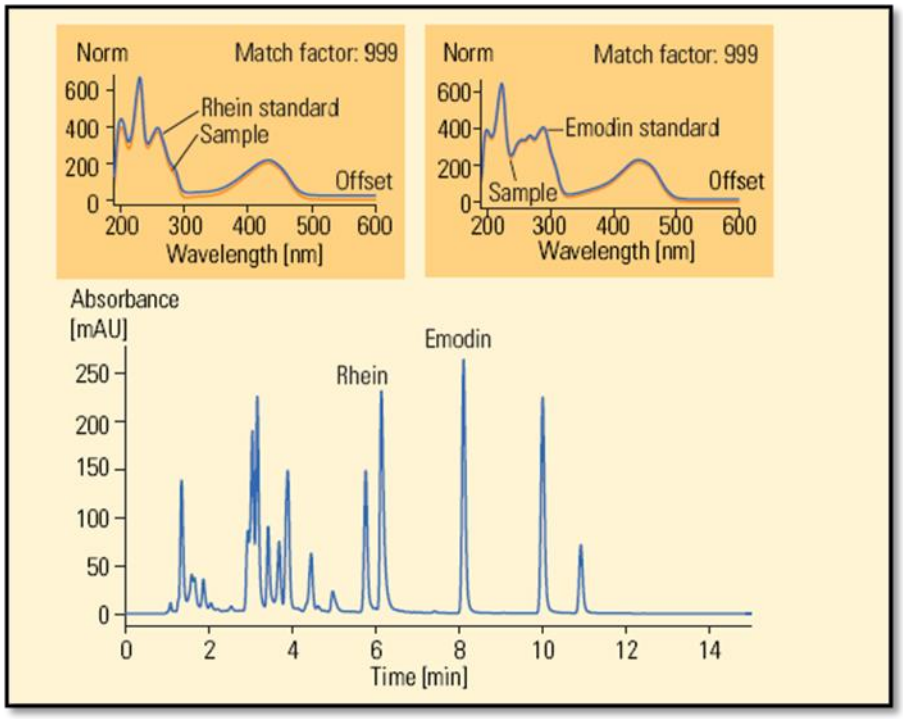

Figure 1
Comparison of sample and standard spectra.

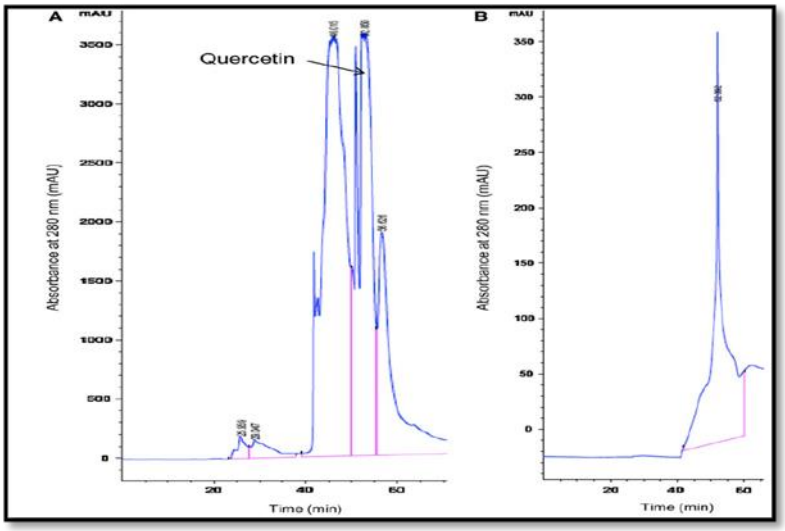

Figure 2
HPLC of Crude Neem leaf extracts (A)

Figure 3
HPLC of Pure Quercetin (B)

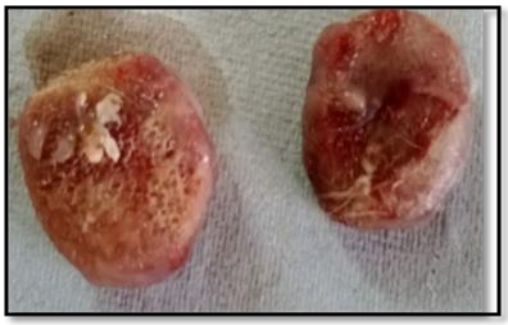

Figure 4
Sponges after removing from rat body.

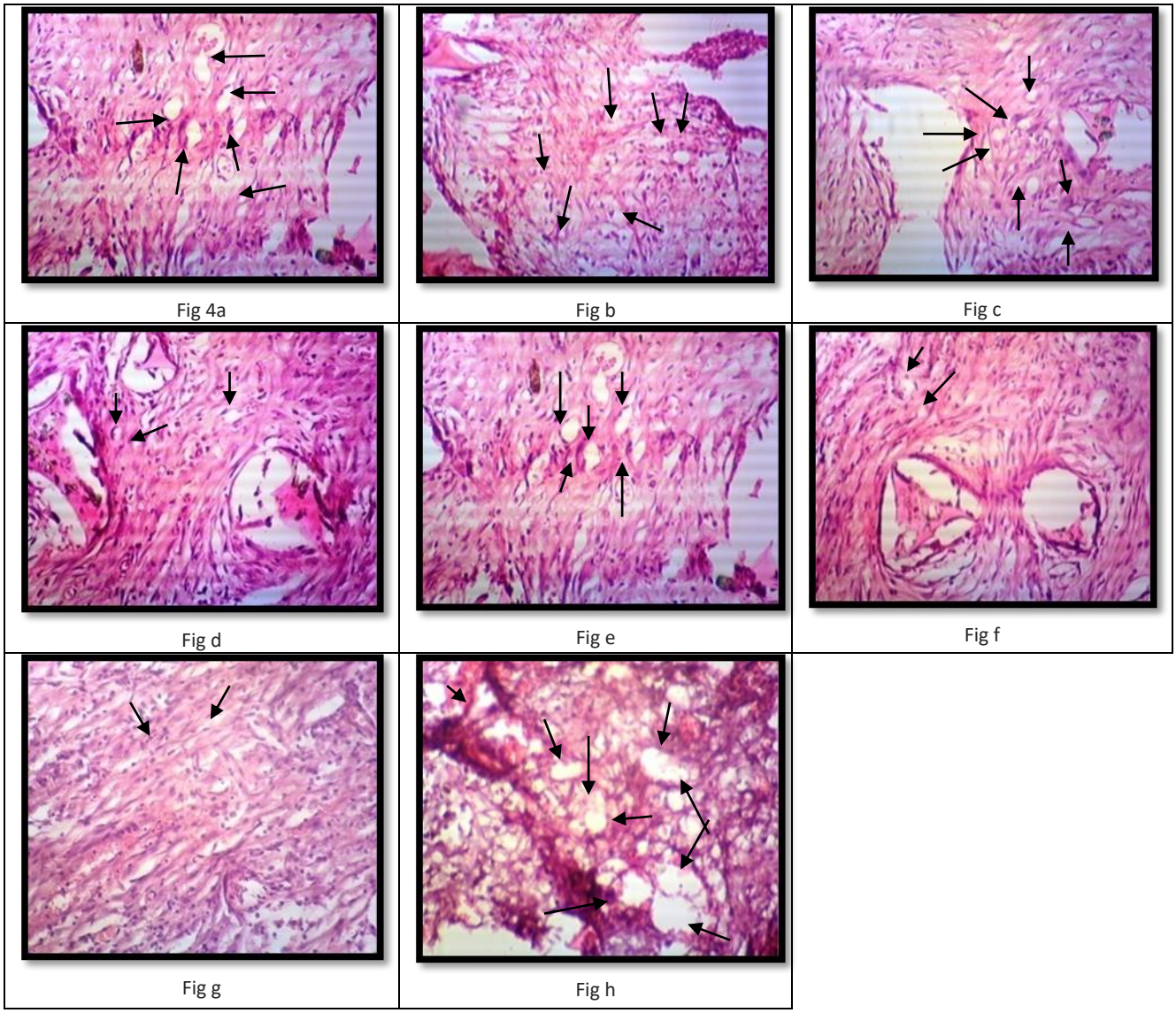

Figure 5
Representative photographic results showing the inhibitory effect of a) Phenytoin sodium (0.03 mg *s.c.*, daily), b) Phenytoin sodium 0.38 mg *s.c.*, daily, c) *Rheum palmatum* (0.03 mg *s.c.*, daily), d) *Rheum palmatum* (0.38 mg *s.c.*, daily), e) *Azadirachta indica* (0.03 mg *s.c.*, daily, f) *Azadirachta indica* (0.38 mg *s.c.*, daily), g) *Azadirachta indica* (3.85 mg *s.c.*, daily) on the 3 mg Pyruvic acid stimulated formation of new capillaries fig h). Image from Trinocular microscope at magnification of 400 X. All sections were stained with H&E. Scale bar, 50 mm. Arrows indicate the newly formed microvessels.

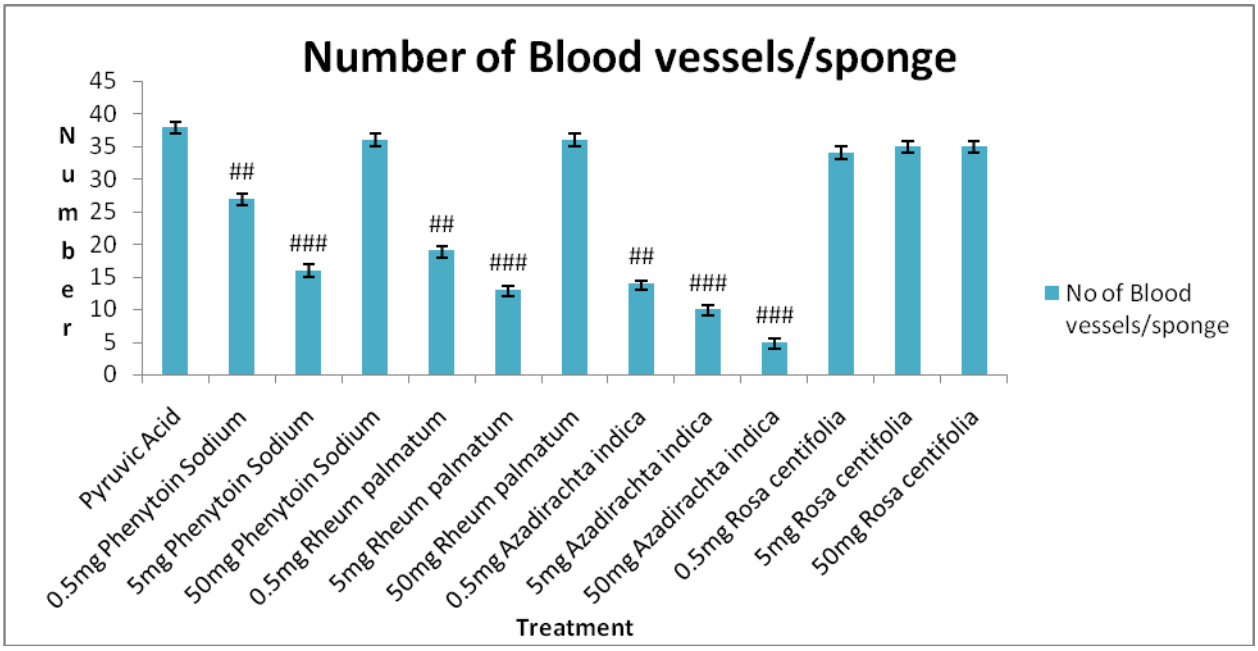

Figure 6

Graph showing the effect of Treatment groups on Number of blood vessels formed per sponge.

All of the data obtained from the experimental groups have been compared to the Pyruvic acid diseased control group. The data was analysed by one-way ANOVA followed by Dunnett test using graph pad prism 6.0software. Values are significant at # p<0.05. Comparison of the test groups were done with Pyruvic acid disease control group.

Values are expressed as mean ± SD; n=6

(p<0.001) compared to Pyruvic acid disease control group, ## (p<0.01) compared to Pyruvic acid disease control group, # (p<0.05) compared to Pyruvic acid disease control group.

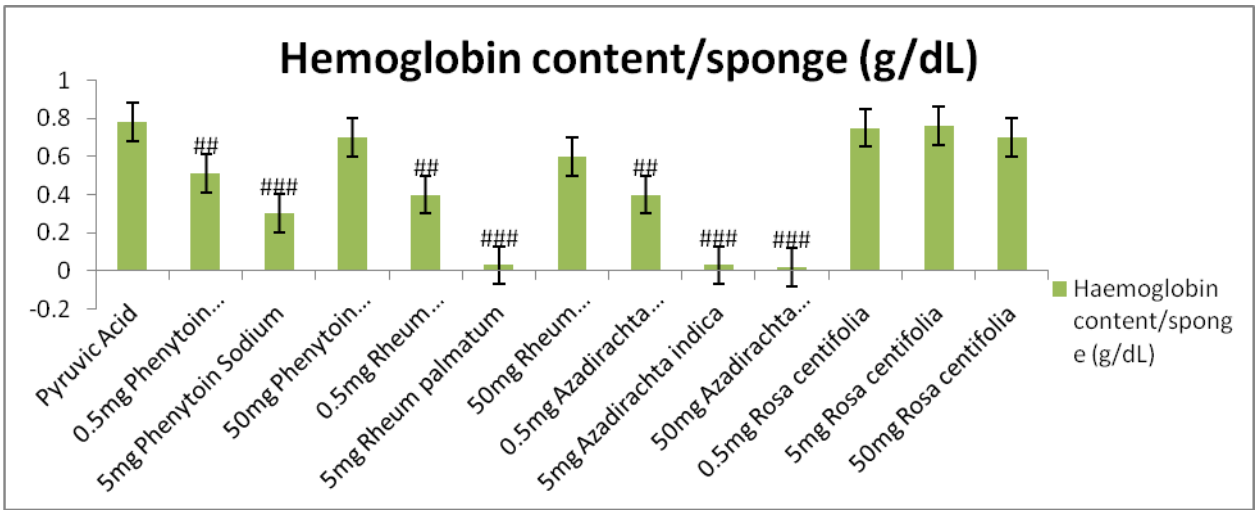

Figure 7

Graph showing the effect of Treatment groups on Hemoglobin content per sponge.

All of the data obtained from the experimental groups have been compared to the Pyruvic acid diseased control group. The data was analysed by one-way ANOVA followed by Dunnett test using graph pad prism 6.0 software. Values are significant at # p<0.05. Comparison of the test groups were done with Pyruvic acid disease control group.

Values are expressed as mean ± SD; n=6

(p<0.001) compared to Pyruvic acid disease control group, ## (p<0.01) compared to Pyruvic acid disease control group, # (p<0.05) compared to Pyruvic acid disease control group.

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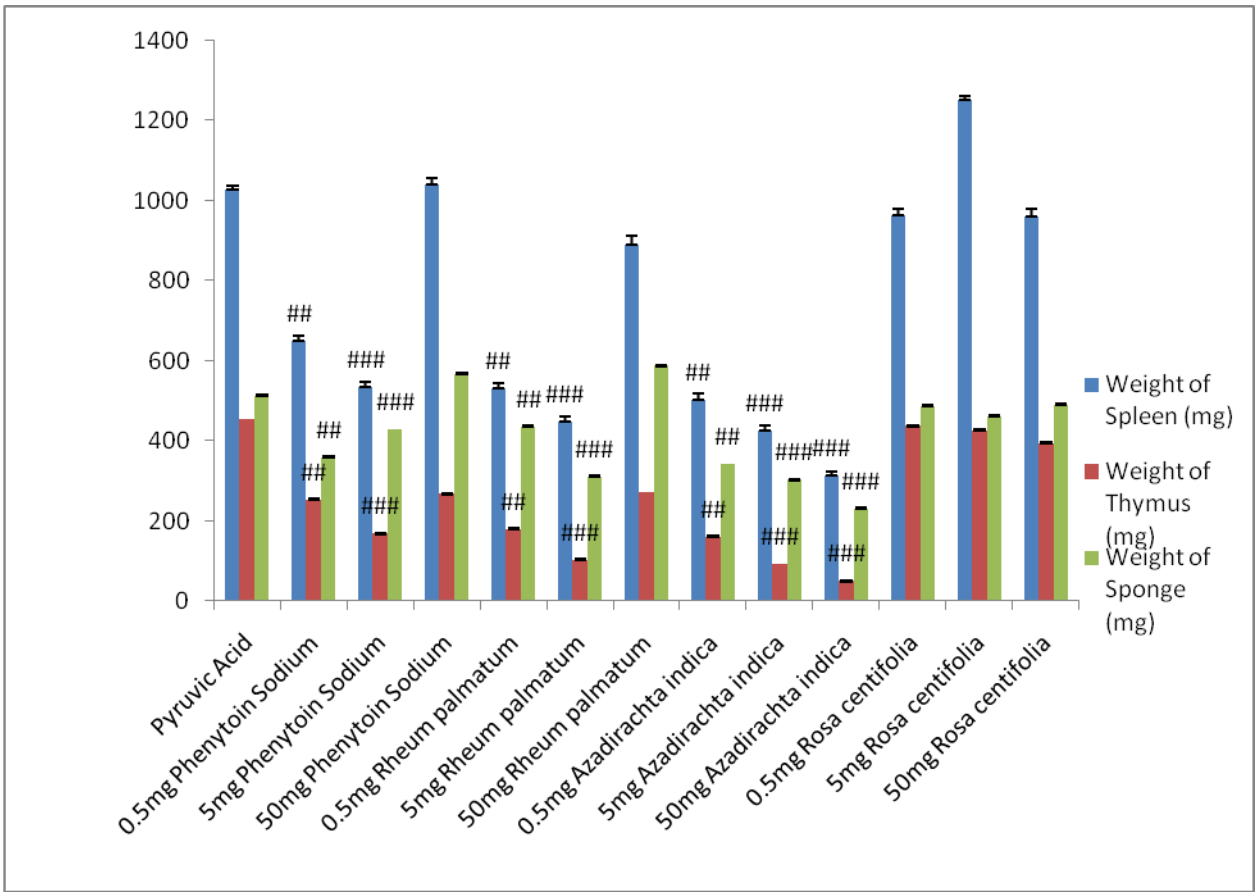

Figure 8

Graph showing the effect of Treatment groups on Weight of Spleen, Thymus and Sponge

All of the data obtained from the experimental groups have been compared to the Pyruvic acid diseased control group. The data was analysed by one-way ANOVA followed by Dunnett test using graph pad prism 6.0software. Values are significant at # p<0.05. Comparison of the test groups were done with Pyruvic acid disease control group.

Values are expressed as mean ± SD; n=6

(p<0.001) compared to Pyruvic acid disease control group, ## (p<0.01) compared to Pyruvic acid disease control group, # (p<0.05) compared to Pyruvic acid disease control group.

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