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Antiangiogenic activity of hydroalcoholic flowers extract of Lantana Camara: In vitro, in ovo and in vivo evaluation

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Abstract---In current research, *Lanata camara*, flower hydroalcoholic extract; was evaluated for anti-angiogenic properties. The anti-angiogenic potency was evaluate by chick chorioallantoic membrane (CCAM) (*in ovo*), endothelial cell proliferation (ECP), transwell migration (TMA) and Matrigel cord-like morphogenesis (MCLM) assay (*in vitro*) along with sponge implantation method(SIM) (*in vivo*). The results compared to standard anti-angiogenic drug bevacizumab (BEV) and also with the angiogenic factor vascular endothelial growth factor at 500 nM concentration. In CCAM test results, the quantity of branching points(BPS) and angiogenic score(ANS) were found significant at 10^{-5} M and 10^{-4} M. Significant reductions in mass of sponge, quantity of blood vessels produced, and hemoglobin content(HB)wasfound at various concentrations of *Lanata camara* flower hydroalcoholic extract in SIM method. The results of human umbilical vein endothelial cells (HUVEC) the test samples (1-100 nM) displayed major inhibition of proliferation and migration and a decline in network length of cord-like tubes (CLT) in a dosage-dependent pattern. *Lanata Camara*, flower hydroalcoholic extract has significant anti-angiogenic activity by inhibition of cell proliferation, migration, and CLTdevelopment, that results from blocking of the cell proliferation on endothelial cells. Further studies on *Lanata Camara*, flower hydroalcoholic extract for repurposing this lead molecule in treating diseases that are caused by extreme angiogenesis.

Keywords---anti-angiogenesis, human umbilical veinendothelial cells, Lantana Camara, flower hydroalcoholic extract.

Introduction

Angiogenesis is the physiological procedure by which novel blood vessels are formed from existing vessel, formed in the former stages of vasculogenesis (Kirtikar and Babu, 2005). Increase in blood capillary number increases the tissue oxygen conducting ability thus improving energy production while lesser number of capillaries led to ischemia, hypoxia, and anoxia in tissues. Hence angiogenesis is vital for both normal physiology and in pathological circumstances. (Lakshmi et al., 2021; Ramachandran et al., 2011; Nadkarni 1999)

The endothelium is slender membrane that forms inner lining of heart and blood vessels. Endothelial cells (EC) release components that manage vascular relaxation and retrenchment along with enzymes that manage blood clots, immunity and platelet adhesion. These form functional integrity that is vital in sustaining vessel walls and circulation majority of which are regulated by ion channels. (Suman 2013; Kato-Noguchi and Kurniadie, 2021)

Ion channels other than producing electrical signal transduction also regulate the vesicular ion concentration and pH, and cell volume. Hence ion channel malfunction lead to diseases various tissues. In spite of key role played in various diseases, there exists certain drugs that target the ion channel as therapeutic inhibitor for treating diseases arising due to unwarranted angiogenesis. The traditional medical remedies of India are rich sources of valuable medicinal plants with no scientific information available regarding their activities. These plant parts are to be studied for their biological effectiveness and chemical compositions (Deshmukh 2010; Peringattulli 2011). Hence *Aervalanata* flowers, of India origin were chosen for the study. The flowers were treated and screened for antiangiogeni, anticancer activities to validate the traditional claim.

Lantana camara (*L. camara*) is a herbal medicine which is also used as firewood (Ganjewala et al., 2009). They are also used in treating cancer, chicken pox, asthma, ulcer, blood pressures, etc (Mondal et al., 2021). The leaf extracts display antimicrobial, insecticidal and nematocidal activities along with immunosuppressive and antitumor potencies. The literature also reported the antifungal, anti proliferative potency of *L. camara* (Kamili et al., 2017; Sanjib et al., 2013). The present research is aimed at evaluating the phytochemical, free radical scavenging and antimicrobial activities of leaves extract of the *L. camara*. No literature was available on the anti-angiogenic potency of isolates of *L. camara* (Atmani et al., 2003). Hence this plant was chosen for screening new anti-angiogenic drug.

Material and Methodology

Authentication of plant

The flowers of *L. camara* were obtained from Medchal district, Hyderabad, Telangana. The plant was authenticated by Botanical Survey of India, Deccan Regional center, Sai Hill colony, Attapur, Hyderabad, Telangana 500030.

Physico-chemical Characterization of *L.camara*

The % moisture in dry powder was calculated as follows

$$(\text{LOD}) (\%) = \frac{\text{Loss in weight Moisture}}{\text{Weight of powder (g)}} \times 100$$

The % ash value of dry powder was calculated as follows

$$\text{Ash Value} = \frac{\text{Weight of total of ash}}{\text{Weight of powder (g)}} \times 100$$

The % yield (Extractive values) of fractions

About 0.2 kg of dry powder was obtained by extraction procedure and the total quantity of hydro alcohol extract obtained (dried) was 0.15 kg.

Extraction and fractionation

The powdered flowers were extracted with ethanol-water (1:1) by maceration for 72 h. The liquid extract was concentrated under vacuum to yield dry extract (hydroalcoholic extract 11.47% w/w with respect to dry material) and preserved in a desiccator till further experiments. The hydroalcoholic extract was further used to evaluate its in-vitro anti-angiogenic activity.

Preliminary phytochemical screening

The screening carried out to spot the attendance of various constituents (Kamili et al., 2020; Velikovic et al., 2007).

Qualitative Phytochemical tests

Total phenolic contents (TPC)

The TPC of *L.camara* were analyzed by Folin-Ciocalteu colorimetric technique with gallic acid and evaluated spectrophotometrically at 765 nm (HITACHI. Model: U-1100 573415). The data tabulated as gallic acid equivalent (GAE) mg per g of dried extract. (Kamili et al., 2020)

Estimation of total flavonoids content (TFC)

The TFC was estimated as per procedure reported in Sakanaka et al (Ammineni et al., 2015; Siener et al., 2004; Bhakta and Ganjewala 2009). The samples evaluated spectrophotometrically at 510 nm.

Estimation of total tannins (TT)

The TT was estimated by Van-Burden and Robinson WC method and samples evaluated spectrophotometrically at 120 nm and expressed as mg per g of dried fraction (Saraf et al., 2011; Kamili et al., 2020; Chidrawar et al., 2015)

Antiangiogenic activity:

CAM assay

The eggs procured from hatchery on 0th day and examined for no damage. The eggs indiscriminately categorized into control (CR), VEGF, bevacizumab (BEV), and 3 test concentrations (conc.), each comprising of six eggs. The eggs were subjected to disinfection with ethyl alcohol followed by incubation at 37°C. On 3rd day a narrow crevice was drilled at narrower side of egg, about 2-3 mL of albumin taken out with the help of 18-gauge hypodermic needles. The crevice was then conserved with sterilized tape and eggs placed back in incubator. On 7th day, a small opening was made through the shell and a sterilized gelfoam piece was positioned on membrane. The CR was provided with saline while test and standard were infused with doses. The eggs subjected to incubation till 14th day. On 14th day, the CAM tissue underneath the sponge was detached positioned in 10% formalin followed by staining with hematoxylin-eosin, and inspected. The vessel branches per square area were calculated. The resulting angiogenesis index expressed as mean $\pm$ standard error of mean (SEM) of fresh branching points. The conc. (10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M) were chosen in accordance with previous study results. Formerly, the solution of 10⁻⁵ M caused submaximal efficiency of extract. (Ammineni et al., 2015)

SIM

In this procedure, the surgical process was carried out by solo investigator to augment the procedure reproducibility. The sponges were entrenched subcutaneously (s.c.). All apparatus were sterilized in an autoclave at 121°C for 25 min. The sponges (2 cm x 8 mm) subjected to sterilization using 70% ethyl alcohol for 3 h followed by heating to 70°C for half an hour. The procedure carried out in rats under anesthetized conditions using a mixture of ketamine (80 mg per kg) and xylazine (5 mg per kg). The skin incised using surgical blades, a sterilized sponge was entrenched s.c. through air pockets and stitched using 5/0 silk sutures.

Two sterilized sponges were fixed in middle-dorsal line of body. On recovery from anesthesia, the animals were provided with normal diet and water. The subjects were caged in isolation. The Tramadol (0.9 mg per kg) was administered intramuscularly (i.m.) two times per day; gentamycin (2 mg per kg) was injected i.m. once in morning. The analgesic and antibiotic drugs were administered for 3 days. Standard drug and test extract were applied on sponges of each group for 13 days post implant. On 14th day the animals sacrificed, the sponges weighed and evaluated".

The dosage calculation is as follows

$$\text{Dose of the animal} = \frac{\text{Surface area of animal} \times \text{Human dose}}{\text{Surface area of human}}$$

Rat surface area=0.025 m²

Human surface area=1.6 m²

Initially maximum conc. of extracts were formulated and diluted serially

Determination of HB: The sponges that were removed from rats, soaked in distilled water and subjected to homogenization at cold conditions for 5 min. The samples subjected to centrifugation at 10,000 rotations per minute for 5 min. The supernatant was estimated for HB(g/dL).

Determination of number of blood vessels/sponge: The sponges were placed in saline at 4°C for an hour followed by soaking in 75% ethyl alcohol for 30 min and stored in 10 % formalin. The paraffin sections (10 µm) were equipped, tainted with hematoxylin-eosin. The slides examined using microscope.

EC culture

The HUVEC were developed on gelatin dishes in M199 complemented with 15% fetal calf serum, 50 U per mL penicillin, 50 mg per mL streptomycin, 50 mg per mL gentamycin, 2.5 mg per mL amphotericin B, 5 U per mL heparin, and 150-200 mg per mL. The cells placed among passages 1 and 3. Each trial was repeated thrice with various pools (isolates) and/or passages of cells each time.

ECP

The HUECs were seeded in 24-well plates at 6000 cells/cm² followed by overnight incubation in Dulbecco's modified Eagle's medium. The cells were exposed to various concentrations of hydroalcoholic extract of *L.camara* (HLC), BEV, VEGF, or vehicle and permitted to proliferate for 48 h. Post incubation period, the cells trypsinized, and the number estimated with the help of Neubauer hemocytometer.

TMA

The ability of EC to drift via a pore-bearing membrane was evaluated with the help of 6.5-mm diameter transwell chamber fitted with polycarbonate membrane insert of 8 µm sized pores. All test samples were furnished overnight. About 1×10⁵ cells were placed in each trans well containing 100 µL of serum-free medium with 0.2% bovine serum albumin in the control and in the presence of different concentrations of HLC, BEV, and VEGF.

The cells were allowed to migrate for 4 h, after which the non-migrated cells at the top of the transwell filter were removed with a cotton swab. The migrated cells on the bottom side of the filter were fixed in Carson's solution for 30 min at room temperature and then were stained with toluidine blue. The migrated cells were scored and averaged from eight random fields per transwell as previously described elsewhere (Gotsman et al., 2010)".

MCLM

The formation of CLS by HUVECs was evaluated in growth factor-reduced Matrigel. The cells were placed on 96-wellplates coated with 45 mL of Matrigel/well. After incubating for 8h, cord-like configuration development was observed and evaluated.

Results

The % ash value was < 5% which is within the pharmacopoeia range. The % moisture in the dry powder was < 10% which is also within pharmacopoeia range (Ghisalberti 2000).

The % yield (Extractive values) for fractions- Total weight of hydro alcohol extract was (dried) 12 g (6%).

Qualitative analysis results

The results indicate modest amounts of phytochemicals as shown in table 1.

Estimation of TPC

The TPC value for *L. camara* was (40.859±0.017) as mg GAE per g dry sample.

Estimation of TFC value

The TFCof *L. camara* (53.112±0.199) were mg RU per g dry sample.

Estimation of TT

The TT for *L. camara* (0.860±0.038) mg/g of the dried fraction in dry sample.

Results of the chick CAM test

The chick CAM assay (*in ovo*), HLC exhibited significant anti-angiogenic action at all the 3 concentrations selected. A marked drop in sponges mass and inhibition in development of novel vessels, and steepdecline in HB were observed, that was similar to standard response (*in vivo*). (Figure 1; Table 2).

Results of SIM

A smalldecline in sponge weight and statistically significant vessel growth inhibition and a drop in HB were observed at 1.0 mg per kg and 10 mg per kg of extracts. The sphericalplaces amidst the fibroblast regions were noted as they signify newlyformed vessel. In VEGF group high number of vessels wasformed, while in standard only few vessels were formed owing to potent anti-angiogenic activity. The test extractscause a dosage-dependent decline in blood vessel formed (Figure 1 and Figure 3; Table 3).

Results of the ECP and TMA and LCLM assay

Cell proliferation, migration, and CLT formation are the basic steps in the vessel formation. To further test the link between the active constituents of HLC and anti-angiogenesis, EC-base assay that cause proliferation and mobilization were carried out. The ECP of VEGF results in superior proliferation (above 50%), whereas BEV, 3 dosages of HLC specify inhibition of proliferation (by 50%, 80%, 70%, and 60%, respectively). Additionally, test dosages of HLC inhibited cell movement through transwell compartments that were equivalent to the vehicle controls. Significant inhibition was observed in cord-like tube formation assay with the test dosages (inhibition by 70%, 60%, and 50%, respectively) (Figures 2 and 4; Table 4).

Discussion

The anti-angiogenic action of HLC owing to presence of alkaloid, glycosides and triterpenoids which act on various cellular mechanisms resulting in angiogenesis. (Al-Snafi 2019) In the CAM studies, the HAL displayed strong anti-angiogenic potency at all 3 concentrations. A noteworthy decline was observed in sponge weight, blood vessels number, and HB at 1 mg per kg and 10 mg per kg. The data confirm that HAL possesses noteworthy inhibition of sprout development and branches in a dosage-dependent pattern. Intonation of EC response to HLC was major at 1 μ M, 10 μ M, and 100 μ M doses on EC proliferation, migration, and CLT development assays HLC. This extract serves as a fine chemical model that could be structurally customized for additional site-specific, cost-effective drug in anti-angiogenic treatment.

Conclusion

The present research scientifically proves the anti-angiogenic activity of hydro-alcoholic extract of *Lanata Camara* by *in-vitro* and *in-vivo* analysis. The investigation reveals the phytochemicals of the extract are responsible for antiangiogenic activity.

Conflict of Interest

The authors declare no conflicts of interest”.

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Tables

Table 1: Qualitative phytochemical analysis of leaf extracts of *L. camara*.

Constituents		Presence
Alkaloids	Dragendorff's test	-
Phenolic	Mayer's test	+++
		+++
Flavonoids		+++
Tannin		++
Saponin		++
Terpenoids		++
Phlobetanin		-
Coumarin		-

+++ : Strongly positive, ++ : Moderately positive, + : Weakly positive, - : Negative

Table 2: Tables representing the impact of HLC on branch points in CAM assay and ANS in CAM analysis

S.NO	Treatment	No. Of BPS	Angiogenic score
1	Control	38.0±1.24***	3.0±0.1***
2	Bevacizumab (1µM)	13.3±1.41*	1.17±1.167*
3	HLC (1µM)	32.1±1.78***	2.7±1.11***
4	HLC (10µM)	26.7±1.8***	2.0±0.35***
5	HLC (100µM)	1.5±1.19***	1.00±1.0***
6	VGEF (500pM)	70.3±2.74***	4.00±2.0***

"All the results were expressed as mean ± standard error of mean; n=6. ***p<0.001, **p<0.01, *p<0.05 vs control ns: Non-significance".

Table 3: Tables representing the impact of HLC on sponge weight, total vessels per sponge and HB per sponge in SIM

S.NO	TREATMENT	weight of sponge (g)	number of vessels per sponge	Hemoglobin content per sponge (g/dl)
1	Control	1.28±0.147	39.2±0.91	1.32±0.122
2	Bevacizumab(1μM)	0.688±0.0557*	17.5±2.11*	0.433±0.0494***
3	HLC (1μM)	1.7± 0.554 ^{ns}	32.0± 1.971 ^{ns}	0.97±0.25*
4	HLC (10μM)	0.75±0.15**	25.1±1.51***	0.77±0.22**
5	HLC (100μM)	0.50±0.11**	18.0±1.66***	0.47±0.77***
6	VGEF (500pM)	2.28±0.0946**	70.8±2.33***	2.88±0.079***

All the results were expressed as mean ± standard error of mean; n=6. ***p<0.001, **p<0.01, *p<0.05 vs control ns: Non-significance”.

Table 4: Tables representing the effect of HLC on MEC responses to HLC, BEV and VEGF

S.NO	TREATMENT	Proliferation	Migrated Cells	Network length
1	Control	100.0±1.00***	99.0±0.11***	100.0±0.11***
2	Bevacizumab(1μM)	50.3±1.29*	21.7±1.157*	20.33±0.17*
3	HLC (1μM)	69.5± 1.34 ^{ns}	65.8± 1.291 ^{ns}	64.5± 1.00 ^{ns}
4	HLC (10μM)	79.3±1.45***	80.0±0.30***	76.99±1.30***
5	HLC (100μM)	58.8±2.11***	58.3±1.01***	49.3±2.01***
6	VGEF (500pM)	149.0±2.00***	250.0±2.04***	243.03±2.04***

“All the results were expressed as mean ± standard error of mean; n=6. ***p<0.001, **p<0.01, *p<0.05 vs control ns: Non-significance.

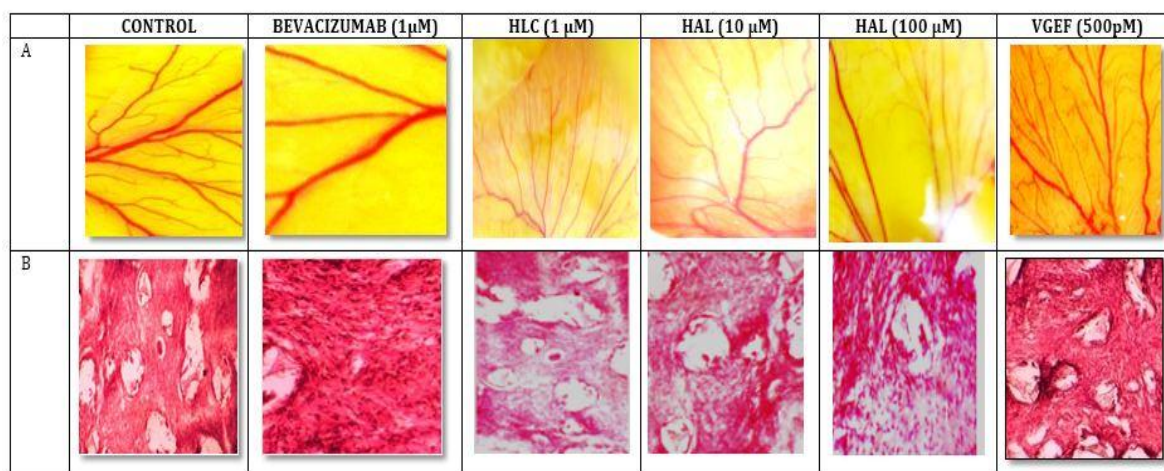


Figure .1: Photographic images showing results of a) CAM assay; b) Sponge implantation assay.

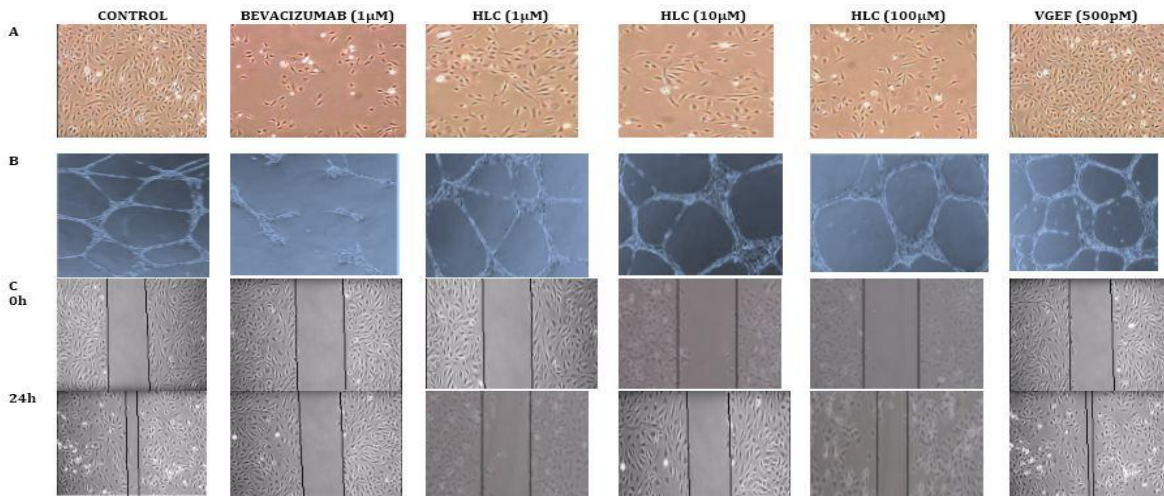


Figure .2: Modulation of endothelial cell responses to HLC, bevacizumab and VEGF. (a) Cell proliferation was determined by cell counting with a hemocytometer. (b) Representative images of tube formation after being treated with HLC for 2 h following VEGF stimulation. (c) Quantitative data of scratch wound-healing inhibition in HUVECs treated with HLC for 24 h under VEGF stimulation.

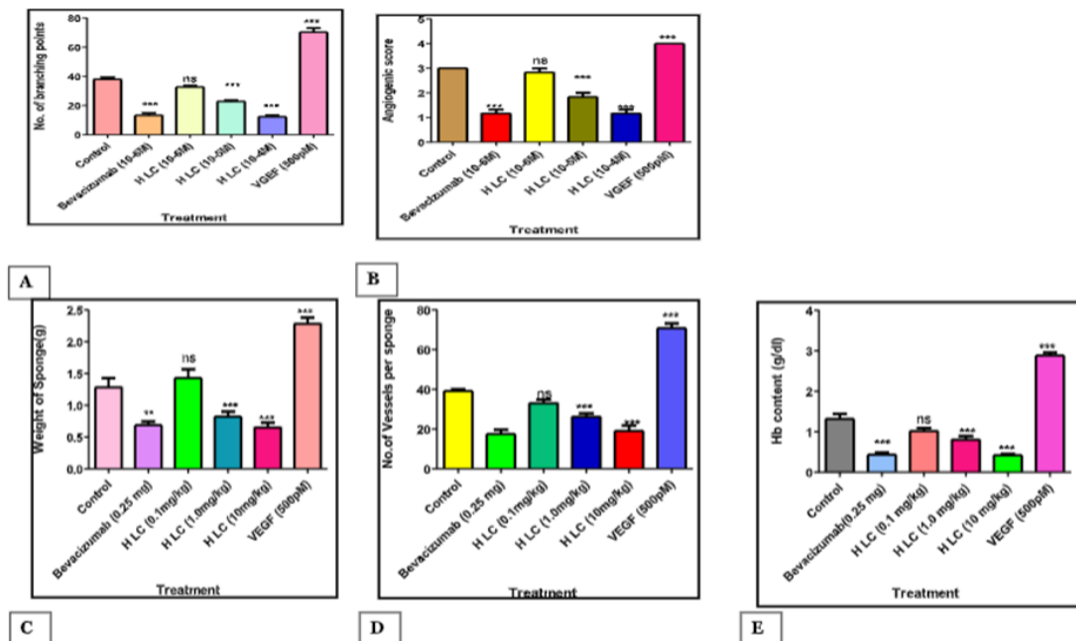


Figure. 3: Graphs showing the effect of HLC on (a) number of branching points in CAM assay (b) angiogenic score in CAM assay (c) weight of sponge (d) number of vessels per sponge (e) Hemoglobin content per sponge in sponge implantation method. All the results were expressed as mean $\pm$ standard error of mean; n=6. ***p<0.001, **p<0.01, *p<0.05 vs control ns: Non-significance.

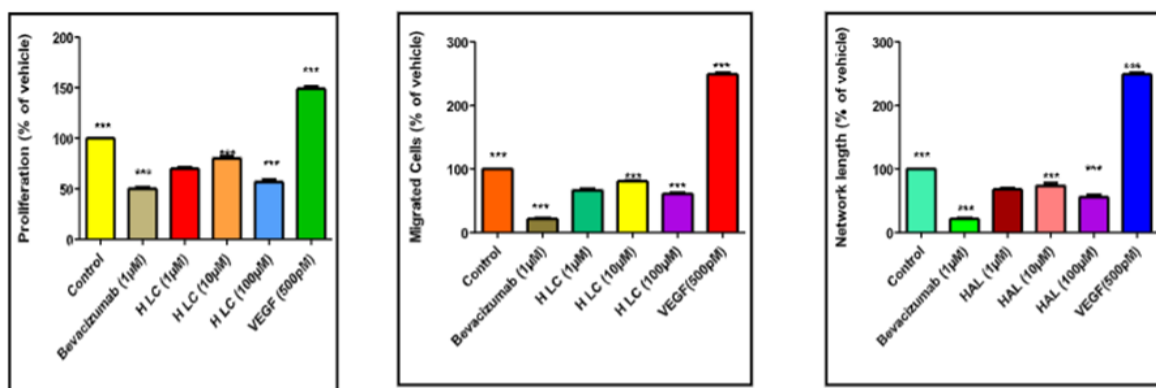


Figure. 4: Graphs showing the effect of HLC on Modulation of endothelial cell responses to HLC, bevacizumab and VEGF. All the results were expressed as mean ± standard error of mean; n=6. ***p<0.001, **p<0.01, *p<0.05 vs control ns: Non-significance.