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Screening of Anti-angiogenic effect of C-Phycocyanin isolated from *Spirulina platensis*

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ABSTRACT:

A well-known blue - green algae *Spirulina platensis* is widely used in India for the management of different ailments. This study aims at extraction, purification and antiangiogenic screening of C-Phycocyanin isolated from *Spirulina platensis.* Preliminary analysis was performed for *Spirulina platensis.* Then, it was treated for the extraction of C-Phycocyanin by different extraction procedures, purified and finally characterization was achieved by reverse phase HPLC and by Mass Spectrometry. Antiangiogenic potential was screened by chick chorio allantoic membrane assay (*in vivo*), sponge implantation method (*in vivo*), morphogenesis assay (*in vitro*), cell migration (*in vitro*) and by cell proliferation assay (*in vitro*). The test and the other groups were compared with control group by one way ANOVA, followed by Dunnett's test, to compare means of other groups with control mean. Chick chorioallantoic membrane assay, evaluated the number of branching points and angiogenic score and strong antiangiogenic results were detected for C-Phycocyanin at doses 10-5 M and 10-4 M. Good decrease in the sponge weights, number of new vessels formed, and the amount of haemoglobin were reported at the selected C-Phycocyanin doses in the sponge implantation method. On the endothelial cells, C-Phycocyanin (1-100 nM) exhibited potent reduction in proliferation, migration and in the length of tubes with increasing doses. C-Phycocyanin exhibited potent antiangiogenic effects in a dose dependent manner.

Keywords: C-Phycocyanin, antiangiogenic activity, *Spirulina platensis*, chick chorio allantoic membrane assay, sponge implantation method, cell migration assay and cell proliferation assay, morphogenesis assay.

INTRODUCTION:

Angiogenesis (AG) is the development of new blood vessels from the existing once's. Antiangiogenic (AAG) compounds precisely aim on budding vessels which makes their use beneficial in many diseases resulting due to pathologically excess AG and also, they treat such diseases from the root cause. Treating disease from roots consequences in great therapeutic benefits with minimal adverse effects[1-4].

In spite of huge investigations, the pathology of Alzheimer's disease (AD) is still uncertain. The pathology includes hypoperfusion of brain cells, inflammation, gene polymorphisms, and cerebral lesions[5-9]. In this study we propose that endothelial cells (ECs) influence the constant destruction of cortical cells in AD. In AD, the endothelium of brain releases precursors of β-amyloid plaque and a neurotoxin that specifically damages cortical cells. Endothelial cells in large numbers get activated by AG triggered by cerebral hypoxia, ischemia and inflammation[8]. If AD is an AG-dependent ailment as proposed, then advancement of antiAG agents aiming the abnormal ECs of brain might prevent and treat AD in a better way. Phytochemicals have been used in India since olden times, claiming for their antiAG potentials. A water-soluble phycobiliprotein, C-Phycocyanin (C-PC), is a colored protein present in cyanobacteria, reed algae, and cryptomonads. C-PC is widely used as an antioxidant, anti-inflammatory, hepatoprotective, radical-scavenging, lipid lowering and antiarthritic agent. Spirulina has a good growth and about 25% (w/w) C-PC content in its biomass, and so becomes a good commercial C-PC source[10-14].

We aimed to investigate the isolated constituents of *Spirulina platensis* for their effects on AAG associated with AD. To achieve this aim, molecular and cell biology analyses were performed on selected cell models utilizing variety of assay techniques.

MATERIALS AND METHODS:

Collection and authentication of plant:

Spirulina platensis was purchased from Perrys Neutraceuticals, Chennai and authenticated by Dr. V.Sampath Kumar (Scientist D in-charge), Botanical survey of India, Southeren regional center, Coimbatore, Tamilnadu, India.

Extraction Procedures of C-PC from *Spirulina Platensis* [11]

Distilled water extraction

Spirulina powder (10g) was mixed in distilled water and allowed to deep freeze at −20 °C for 24 hours, then was subjected to centrifugation at 6000 rpm for 10 minutes.

Homogenization method

Wet *Spirulina* powder (10g) was subjected to homogenization using mortar and pestle with glass beads in 50 mM of Sodium phosphate buffer (pH 6.8). The extract was kept at −20 °C for 24 hours and then was exposed to centrifugation at 6000 rpm for 10 minutes. This was re-extracted with buffer for complete recovery of C-PC.

Freezing and Thawing method

C-PC was separated by repeated freezing and thawing of wet biomass (10g). It was then mixed with 50 mM Sodium phosphate buffer (pH 6.8) and deep frozen −20°C for 24 hours. This was repeated many times and then was subjected to centrifugation at 6000 rpm for 10 minutes.

Acid extraction process

Wet *Spirulina* powder (10g) was taken in two test tubes. In one 2 ml concentrated hydrochloric acid and in the other 5 ml of the concentrated hydrochloric acid was added. The test tubes were kept at room temperature for 24 hours, then were subjected to centrifugation at 6000 rpm for 10 minutes.

Heating

Wet *Spirulina* powder (10g) was added into a test tube and kept inside the water bath, which was treated at 60°C for 10 minutes, then were subjected to centrifugation at 1000 rpm for 10 minutes.

Every One Hour Freezing and Thawing

Wet *Spirulina* powder (10g) was added a beaker along with the 50 mM sodium phosphate buffer and kept at −20°C for 1 hour and thawed at 4°C for 1 hour. This was repeated for about 3 to 6 times, then were subjected to centrifugation at 1000 rpm for 10 minutes.

In all the above procedures the supernatant obtained after centrifugation was used for C-PC estimation.

Purification of C-PC

Among the above 6 procedures, C-PC extracted by repeated freezing and thawing was found to be highly effective. Amount of C-PC was estimated as reported by Bennett and Bogard (1e973) and purity was calculated using:

$$\text{Purity} = A_{620}/A_{280}$$

CHARACTERIZATION OF C-PC

RPHPLC

Reverse phase HPLC using C5 column was performed to characterize C-PC. Photo diode array detector was set at 620 and 226 nm and the absorption spectra were analysed[12].

Mass spectrometry

Triple stage quadrupole and ion trap mass spectrometers are used presently, as it was sensitive and selective. It reported maximum identity with C-PC [12].

Antiangiogenic activity:

***INVITRO* ASSAYS:**

HUVECs culture

Human umbilical vein EC (HUVECs) were cultured on gelatine dishes in M199 supplement with 1e5% fetel calf serum, 50 U/ mL penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamycin, 2.5 mg/mL amphotericin B, 5 U/mL heparin, and 150-200 mg/mL EC growth supplement. ECs were used between passages 1 and 3. Three repeats for each experiment were derived and each time using different isolates and/or passages of ECs[15-17].

EC proliferation assay

HUVECs were seeded in 24 well plates at a density of 6000 cells/cem2 and incubated overnight in Dulbecco's modified Eagle's medium. ECs were subjected to various doses of C-PC, bevacizumab, VEGF, or vehicle and were left to proliferate for 48 hours. After 48 hours ECs were trypsinized, and count was calculated by Neubauer heemocytometer[15].

Transwell migration assay

Ability of ECs to migrate across a membrane was evaluated by 6.5-mm diameter transwell chambers with polycarbonate membrane inserts (8 mem pore size). ECs were serum starved overnight. ECs were trypsinized, 1×105 ECs were added per well with 100 mL of serum-free medium having 0.2% bovine serum albumin with of various doses of C-PC, bevacizumab and VEGF. ECs were left for migration for 4 h, after which the nonmigrated ECs left on the top of the well were removed. ECs migrated to bottom of the filter were fixed in Carson's solution for 30 min and were stained with toluidine blue. Migrated ECs were scored and averaged from 8 random fields[16].

Matrigel cord-like morphogenesis assay

The development of cord-like morphology by HUVECs were measured in growth factor-reduced matrigel. The EC groups were plated in 96-wells precoated with 45 mL of matrigel in each well. ECs were incubated for 8 hours, cord-like morphology was measured[17].

***INVIVO* ASSAYS:**

CAM assay

Chorioallantoic membrane (CAM) assay *in ovo* AG procedure for measurement of potential of AG agents. Eggs were harvested from hatchery on Day '0' and observed for damage. After randomly grouping into control, VEGF

(negative control), bevacizumab (standard AG agent), and three C-PC groups (six eggs/ group). Ethanol was used for disinfection and then incubated at 37°C. On Day '3', 2-3 ml of albumin was withdrawn from the narrow end of the eggs and kept back for incubation. On Day '7', a sponge with test/ standard/ control doses (3 mm×3 mm×1 mm) were placed on top of CAM and then incubated till Day '14'. On Day '14', CAM was separated, stained with 10% formalin and examined under trinocular-microscope. Vessel branching points/ square area were counted. AG index is the mean ± standard error of mean (SEM) of branching points[18-21].

Table 1: AG score on 1-4 for branching points

Branching points	Score
≥35	4
between 25 and 34	3
between 15 and 24	2
<15	1

Sponge implantation assay

Rats were anesthetized by a mixture of ketamine (80 meg/keg) and xylazine (5 meg/keg). Two sterilized sponges (2 cm diameter and 8 mm thickness) were implanted subcutaneously (s.c.) on the mid-dorsal line of animal's body. Standard and test doses were injected on sponges for 13 days to the respective groups. On Day '14' animals were sacrificed and sponges were separated out. The sponge weight, amount of hemoglobin and number of vessels per sponge were quantified. [22-24]

Procedure for estimating hemoglobin content: After soaking the sponges in double distilled water they were homogenized over ice platform for 5 min. The homogenate was subjected to cooling centrifugation at 10,000 rpm for 5 min and the supernatant was used to estimate hemoglobin content by Sahli's hemoglobinometer (g/dL)[23].

Procedure for determining number of blood vessels formed per sponge: Sponges were immersed in saline at 4°C for 1 hour and were soaked in 75% ethanol for 30 min and then moved into 10% formalin. Paraffin sections (10 pm) were made and were stained with hematoxylin-eosin. The prepared slides were then observed under a trinocular microscope. Circular spaces amidst the fibroblast regions present were counted as they represent vessels formed in the sponges[24].

RESULTS

Extraction and purification of C-PC

Among the 6 procedures, C-PC separated from repeated freezing and thawing was reported as the most effective (Figure 1 & 2, Table 2). Cell mass was separated by centrifuging at 5000 rpm for 10 min and the supernatant is the crude extract. Amount of C-PC was calculated and purity was determined by formula:

$$\text{Purity} = A_{620} / A_{280} \times 100$$

Figure 1. C-PC extracted by different methods.

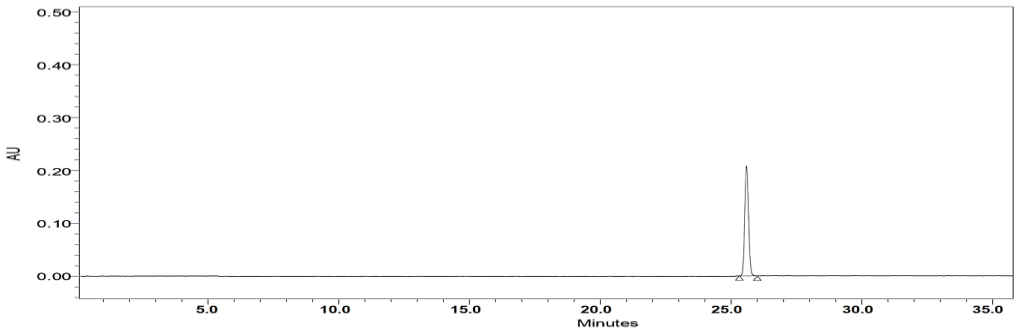
Figure 2. Crude Sample and C-PC

Table 2. OD values for purification of C-PC

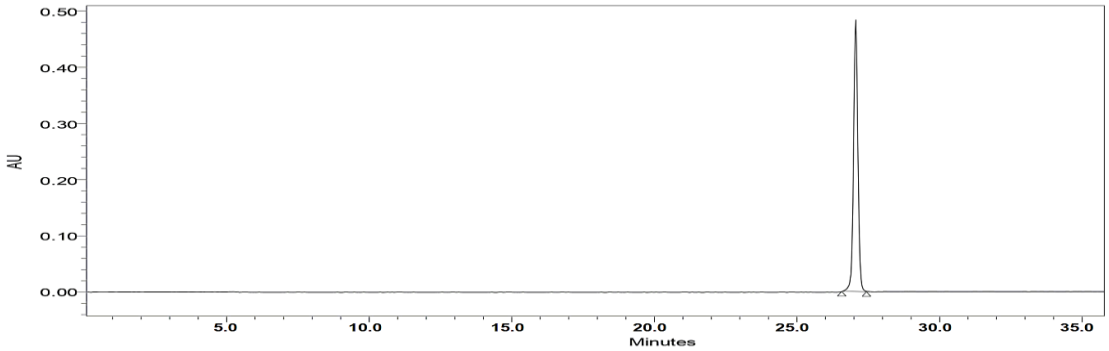
Method	Optical Density (OD)		Purity (A620/A280) x 100
	A620	A280	
Normal freezing and thawing extract	2.58	3.42	75.43
Homogenized freezing and thawing extract	2.66	3.36	79.16
Water extract	2.77	3.51	78.91
1 hour freezing and thawing extract	2.48	3.61	68.69

Characterization of C-PC

To characterize C-PC, reverse phase HPLC was done using a C_5 column. Photo diode array detector at 620nm and 226nm exposed 2 peaks at 25.612 min and 27.024 min. When absorption spectra of these 2 chromatogram peaks were analysed, it was found that A_{620}:A_{280} for the first peak RT = 25.612 min, it was due to phycocyanobilin (PCB) chromophore, indicating the peak corresponds to α subunit of C-PC. While the A_{620}:A_{280} for second peak RT = 27.024 min, it was due to the presence of 2 PCB chromophores, and therefore this peak corresponds to β subunit of C-PC (Figure. 3).



A) Chromatogram at 620nm with retention time of 25.612



B) Chromatogram at 226nm with retention time of 27.024

Figure 3. RTHPLC peaks of C-PC showing α and β subunit peaks.

Characterization of C-PC by mass spectrometry

Triple stage quadrupole and ion trap mass spectrometers were used, as they are very sensitive and selective. It showed maximum identity with C-PC (Figure.4).

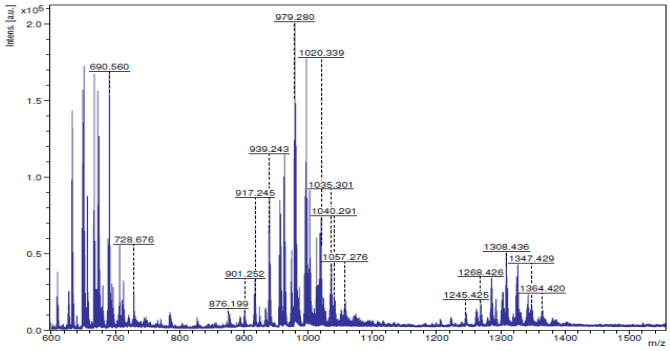


Figure 4. Mass Spectrophotometric peaks of C-PC

Results of CAM assay

Effect of C-PC treatment on the two evaluation parameters, number of branching points and angiogenic score, are presented in Figure 5a, Figures 7a and 7b, Table 3. The results of three doses of C-PC, the standard anti-angiogenic drug bevacizumab, and VEGF were statistically compared with the control results. Significant AG actions were reported with all three test doses selected: 10-6 M, 10-5 M, and 10-4 M, in a dose dependant manner.

Results of the sponge implantation method

The parameters evaluated in this method are weight of the sponge, number of vessels per sponge, and hemoglobin content of the sponge. A reasonable decrease in weight of sponges and a significant inhibition in neovascularization and hemoglobin content were reported at 1.0 mg/kg and 10 mg/kg of C-PC. In VEGF group highest neovascularization were identified, whereas with the standard antiangiogenic agent very few microvessels were noticed due to strong anti-angiogenic action, and the C-PC groups caused a dose-dependent decrease in neovascularization (Figure 5b and Figure 7c,7d,7e; Table 4).

Results of the EC proliferation, transwell matrigel and morphogenesis assays

In the cell proliferation assay VEGF resulted in elevated proliferation (increase of 50%), whereas bevacizumab and the three doses of C-PC showed significant inhibition of proliferation (inhibition by 50%, 80%, 70%, and 60%, respectively). In addition, test doses of C-PC significantly inhibited cell motility through transwell by about 50%. Significant inhibition of tube morphogenesis was observed with C-PC (inhibition by 70%, 60%, and 50%, respectively) (Figures 6 and 8; Table 5).

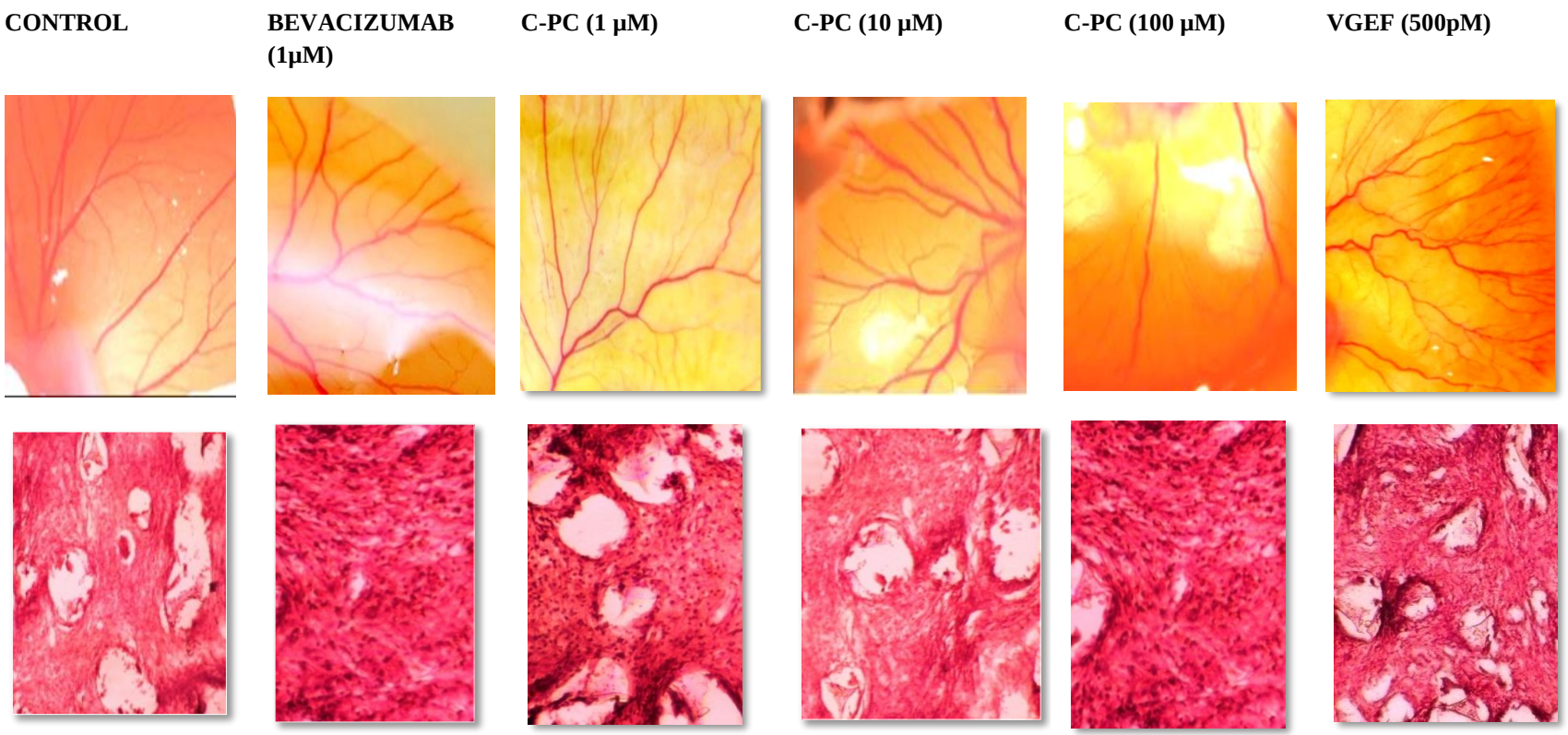


Figure .5: Photographic images showing results of a) CAM assay; b) Sponge implantation assay.

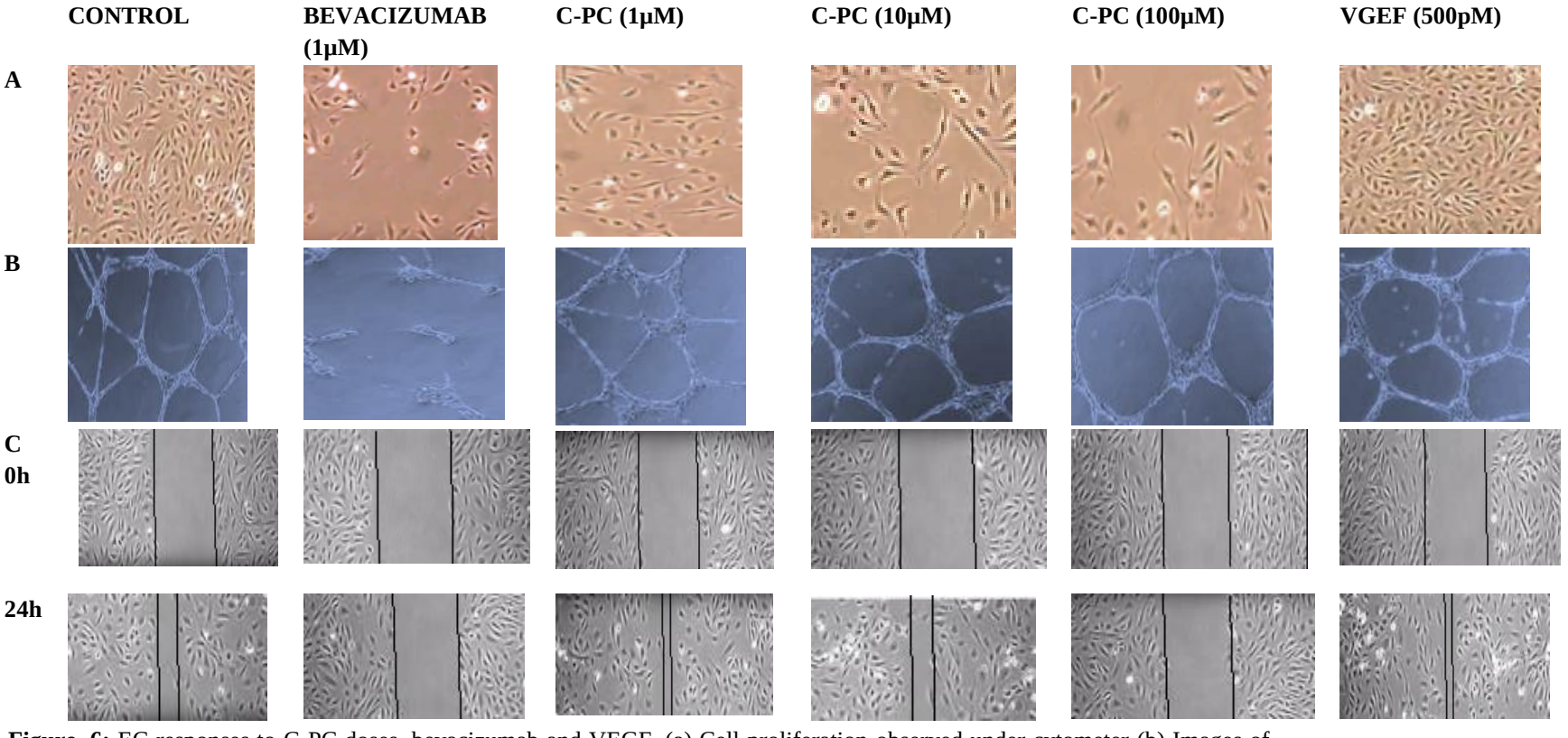
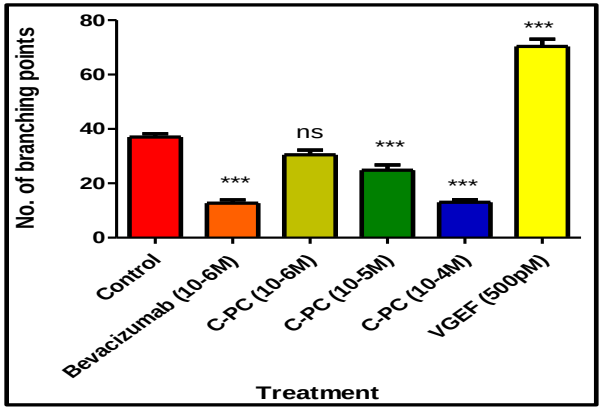
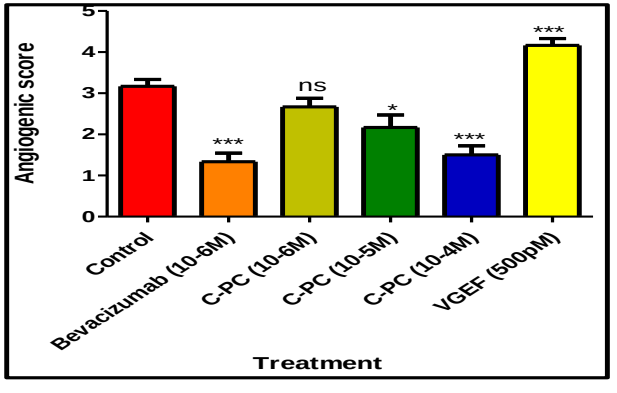


Figure .6: EC responses to C-PC doses, bevacizumab and VEGF. (a) Cell proliferation observed under cytometer (b) Images of tube morphogenesis after being treated for 2 hours following VEGF stimulation. (c) Cell migration observed after 24 hours of treatment



A



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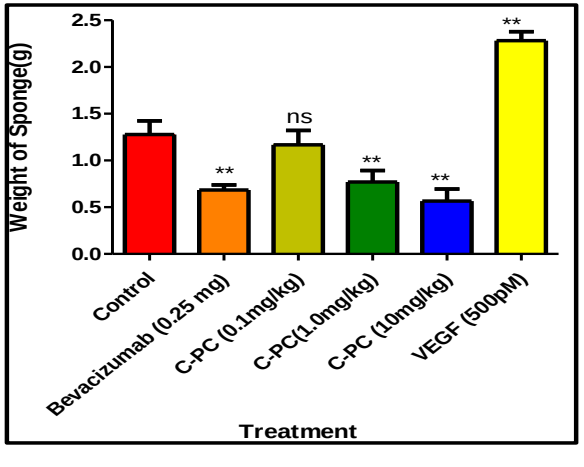

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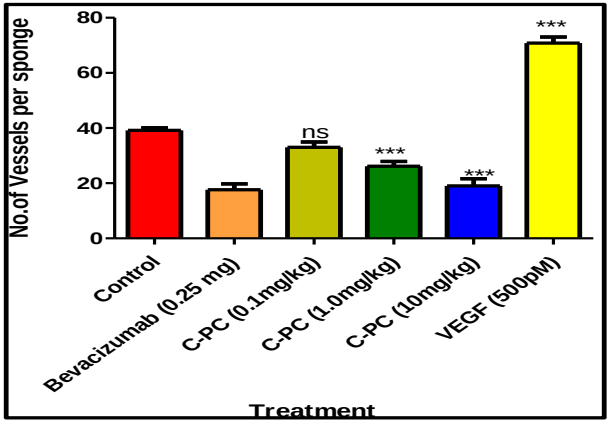

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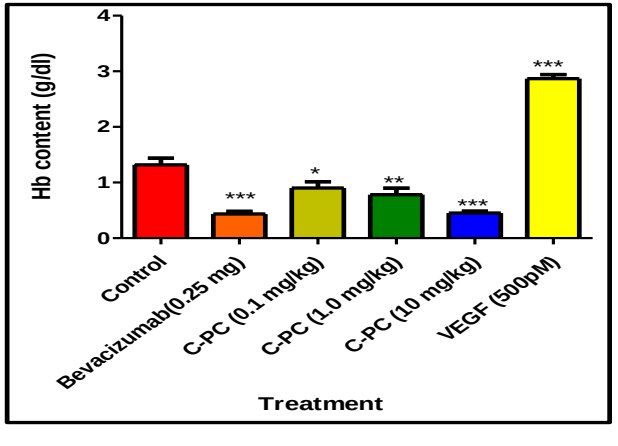

E

Figure .7: Graphs representing EC responses to C-PC, bevacizumab and VEGF. All the results were expressed as mean ± standard error of mean; ne=6. ***pe<0.001, **pe<0.01, *pe<0.05 vs control ns: non-significance.

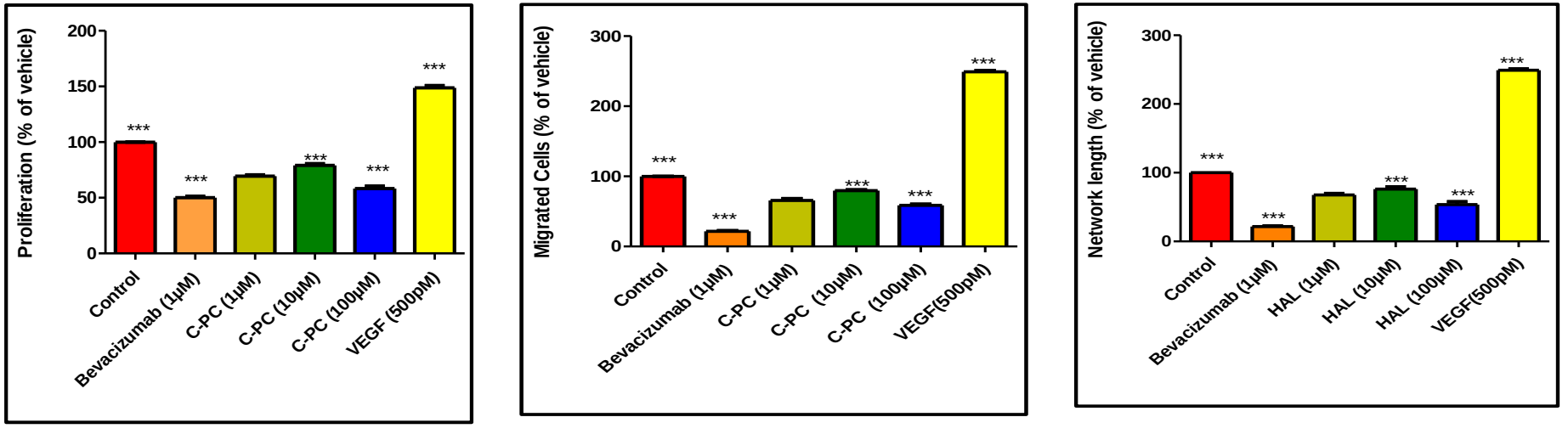

Figure .8: Graphs representing effect of C-PC on (a) number of branching points (CAM assay) (b) angiogenic score (CAM assay) (c) weight of sponge (d) number of vessels per sponge (e) hemoglobin content per sponge. All the results were expressed as mean ± standard error of mean; ne=6. ***pe<0.001, **pe<0.01, *pe<0.05 vs control ns: non-significance.

TABLES:

Table 3: Effect of treatment groups on branching points and AG score in CAM assay

S.NO	TREATMENT	Branching points	AG score
1	Control	33.0±1.34***	3.3±0.3***
2	Bevacizumab(1µM)	13.33±1.31*	1.13±1.137*
3	C-PC (1µM)	31.3± 1.38***	2.63± 1.311***
4	C-PC (10µM)	25.3±1.3***	2.13±0.303***
5	C-PC (100µM)	13.7±1.37***	1.30±1.0***
6	VGEF (500pM)	70.3±2.34***	4.30±3.0***

Table 4: Effect of treatment groups on weight of sponge, number of vessels and hemoglobin content in sponge implantation method

S.NO	TREATMENT	weight of sponge (g)	number of vessels per sponge	hemoglobin content per sponge (g/dl)
1	Control	1.23±0.143	39.3±0.31	1.33±0.132
2	Bevacizumab(1µM)	0.683±0.0357*	17.5±2.31*	0.43±0.0394***
3	C-PC (1µM)	1.13± 0.134^{ns}	33.0± 1.351^{ns}	0.937±0.135*
4	C-PC (10µM)	0.734±0.132**	26.3±1.32***	0.737±0.13**
5	C-PC (100µM)	0.530±0.133**	19.3±2.38***	0.463±0.33***
6	VGEF (500pM)	2.23±0.0346**	70.3±2.33***	2.83±0.039***

Table 5: Effect of treatment groups on EC responses

S.NO	TREATMENT	Proliferation	Migrated Cells	Network length
1	Control	100.3±1.30***	99.3±0.31***	100.3±0311***
2	Bevacizumab(1µM)	50.3±1.29*	21.7±1.157*	20.33±0.17*
3	C-PC (1µM)	69.5± 1.34^{ns}	65.8± 1.291^{ns}	64.5± 1.00^{ns}
4	C-PC (10µM)	79.3±1.45***	80.0±0.30***	76.99±1.30***
5	C-PC (100µM)	58.3±2.13***	58.3±1.31***	49.3±2.31***
6	VGEF (500pM)	149.3±2.30***	250.3±2.03***	243.03±2.03***

DISCUSSION

C-PC anti-angiogenic action might be due to calcium channel blocking.[24-26] Ca2+ ions act as secondary messengers in cell signalling pathways resulting in AG. Deficiency of extracellular Ca2+ results in cell-growth arrest in G1/S phase.[27-32]

In CAM assay, C-PC showed potent anti-angiogenic action at the 3 test doses of 10-6 M, 10-5 M, and 10-4 M. A strong decrease in weight of sponge, neovascularization, and hemoglobin content were reported at 1 mg/kg and 10 mg/kg. The results suggest that C-PC has strong inhibition of sprout formation and branching in a dose-dependent manner. EC response to C-PC was significant at the test doses of 1µM, 10µM, and 100µM during proliferation, migration, and morphogenesis assays, which are the critical steps in arresting the angiogenesis.

CONCLUSION

Spirulina Platensis serves as good commercial source for the isolation of C-PC. C-PC, being a strong blocker of Ca2+ ion influx, resulted in significant anti-angiogenic actions both in *in vitro* and *in vivo* antiangiogenic assays. This phytochemical serves as good template that can be structurally modified for a site-specific effect in anti-angiogenic therapy.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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