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Comparison of methanol extracts of leaf, stem bark and stem of *Holoptelea integrifolia planch* for anti-cancer and anti-angiogenic activity

[1] Vidhyakini Danthala, [2] Krishna Mohan Gottumukkula, [3] Dinesh Reddy Salla, [4] Bindu Madhav Reddy Aramati, [5] Sandhya Rani M

[1] M.Pharm student, Centre for Pharmaceutical Sciences, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, Telangana, India

[2] Professor, Centre for Pharmaceutical Sciences, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, Telangana, India

[3] M.Pharm student, Srivenkateshwara College of pharmacy, Osmania University, Hyderabad, Telangana, India

[4] Assistant Professor, School of life sciences, University of Hyderabad, Hyderabad, Telangana, India

[5] Asst. professor, Centre for Pharmaceutical Sciences, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, Telangana, India

Abstract
The search for potential cure next to angiogenesis-dependent diseases is focused on plants because the active compounds derived from natural sources are inherently better tolerated in the body and have lower side effects than the synthetic drugs. The leaf, stem bark and stem powders of *Holoptelea integrifolia* were extracted separately by various solvents with an increasing order of their polarity. Preliminary phytochemical screening of methanol extracts of leaf, stem bark and stem of *Holoptelea integrifolia* reveals that among all the 3 extracts of Stem methanol extract of *Holoptelea integrifolia* (HISME) has shown greater accumulation of phytoconstituents and they are alkaloids, flavonoids, tannins, amino acids, proteins, terpenoids, steroids and carbohydrate followed by Leaf methanol extract of *Holoptelea integrifolia* (HILME) has shown presence of alkaloids, glycosides, tannins, amino acids, proteins, terpenoids, steroids and carbohydrates and Bark methanol extract *Holoptelea integrifolia* (HIBME) has tannins, steroids and carbohydrates. The *in vitro* cytotoxic assay-MTT assay for 3 methanol extracts *of H.integrifolia* against RAW 264.7, MCF-7cell lines has performed, data reveals that HILME-10µg/ml (63.59± 0.244) has shown highest cytotoxicity against RAW 264.7,HISME - 1µg/ml (83.89± 0.07) has shown highest cytotoxicity against MCF-7 cell lines. HILME, HIBME and HISME were undergone for *in ovo* chick chorioallantoic membrane (CAM) assay to determine their anti-angiogenic potential. Among all the 3 extracts 2 extracts exhibited inhibition of angiogenesis with the highest activity observed by HILME (64.84± 0.98- 100µg/mL) followed by HIBME ((63.92± 1.16-100µg/mL) it has a comparable activity with known anti-angiogenic agents Sunitinib 1µM (83.1± 0.75), Sorafenib 1µM (77.62± 0.98). The results suggests that *Holoptelea integrifolia* leaf and stem bark extracts can be a potential source of anti-angiogenic compound for the design and development of drugs targeting angiogenesis- dependent diseases.

Keywords: *Holoptelea integrifolia*, *in ovo* anti-angiogenic, chick chorioallantoic membrane assay (CAM), cytotoxic activity, MTT, dexomethasone, sunitinib, sorafenib

1. Introduction
Angiogenesis is the physiological process through which new blood vessels generates from pre-existing vessels. To form a new blood vessel, the endothelial cells need to receive the stimulatory signals and matrix metalloproteinase-MMP which cause the degradation of basement membrane, endothelial cells escape and projected throughout the newly created space then organise into fresh capillary tubes, allowing the sprouting vessels to progress towards the source of angiogenesis stimulus and another way of angiogenesis process is intussusception, also known as *splitting angiogenesis*, by this a new blood vessel is created by splitting of an existing blood vessel in two. In this type of vessel formation, the capillary wall extends into the lumen to split a single vessel in two. Diseases that are related to excessive angiogenesis include diabetic retinopathy, cancer, age related macular degeneration. Since the process plays an important role in these pathological conditions, inhibition of angiogenesis is one important approach in their therapy and control. Synthetic angiogenesis inhibitors are available; however, they are costly and have records of resistance to anti-angiogenic therapy. Since phytomedicines have fewer side effects, there is an increased interest in discovering natural compounds that exhibit anti-angiogenic activity to address the complications brought by synthetic drugs.

Holoptelea integrifolia Planch, family Ulmaceae also known as Indian elm tree traditionally used for the treatment of inflammation, intestinal worms, vomiting, wound healing, leprosy, diabetes, haemorrhoids, dysmenorrhea, and rheumatism and intestinal cancers. With the established uses

of the plant, there is still a need to discover other potential uses to optimize its therapeutic capacity.

The anti-angiogenic research utilizes the chorioallantoic membrane (CAM) of the chick embryo. The CAM model has many advantages including its low cost, ease of use, high reproducibility, reliability and simplicity. The CAM assay, in comparison with other animal models, is a closed system, which promotes a longer half-life on many compounds because excretion of such compounds is eliminated. This, in turn, allows the use of minute amounts of experimental compounds. Quantification of blood vessels in large amount of CAM models can be used to screen drugs from sample plant extracts. In CAM assay, sunitinib and sorafenib can be used as the positive controls. These are acts by inhibiting receptor tyrosine kinase (RTK) thereby inhibit angiogenesis process.

2. Materials and Methods

2.1 Extraction

The plant material was collected from Osmania University Campus, Hyderabad on march 2017 then aunthenticated by Prof. H. Ramakrishna, botanist, Osmania University, Hyderabad as *Holoptelea integrifolia* (Roxb) Planch. Voucher no. 329. Then processed separately into leaves, stem bark and stem and dried under shade and grounded into coarse powder extracted with various solvents by increasing order of their polarity by using soxhlet continuous process of extraction. Each extract was concentrated using rotary-evaporator under vaccum. The extracts were kept in desiccator until further use.

2.2 Preliminary Phytochemical Screening

Holoptelea integrifolia leaf methanol extract (HILME), Holoptele integrifolia stem bark methanol extract (HIBME), *Holoptelea integrifolia* stem methanol extract (HISME) were subjected to qualitative phytochemical analysis

Detection of Alkaloids

About 50 mg of solvent-free extract was stirred with little quantity of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows.

- **Dragendorff's test:** To 1 mL of test filtrate, two drops of Dragendorff's reagent (Potassium bismuth iodide solution) was added and observed for the Formation of prominent reddish brown precipitate.

Detection of Carbohydrates

About 100 mg of extract was dissolved in 5 mL of distilled water and filtered. The filtrate was subjected to the following tests.

- **Molisch's test:** 1 mL of the test solution was taken and two drops of alcoholic solution of α– naphthol (Molisch's reagent) was added. The mixture was shaken and 1 mL of conc. H2SO4 was added slowly from the sides of the test tube. The test tube were cooled in ice water and allowed to stand. Then the test tubes were observed for violet-purple ring formation at the junction.

Detection of Proteins and Amino acids

About 100 mg of extract was dissolved in 10 mL of distilled water and filtered. The filtrate was subjected to following tests

- **Biuret test:** To 3 mL of test filtrate, two drops of 4% NaOH was added and treated with two drops of 1% CuSO4 solution and observed for the Formation of pink color.

Detection of Steroids and Terpenoids

- **Salkowski test:** To the test extract, 2 mL of chloroform and 2 mL of concentrated sulphuric acid were added, shaken well and observed the coloration of chloroform and acid layers. Chloroform layer as red in color and acid layer as greenish yellow fluorescence.

Detection of Glycosides

About 50mg of extract was hydrolyzed with concentrated hydrochloric acid for 2 hrs. On a water bath, filtered. The filtrate was subjected to the following tests.

a) Test for cardiac glycosides

- **Keller - Killiani test:** The test filtrates were taken and added 2 mL of glacial acetic acid and two drops of 5% ferric chloride solution and mixed. Then 1 mL of sulphuric acid was added. Reddish brown colour appears at the junction of the two liquid layers and upper layer appear bluish green colour.

b) Test for saponin glycosides

- **Foam test:** Filtrates were taken and 20 mL of distilled water was added and shaken for 15 min in a graduated cylinder and observed for formation of a layer of stable foam.

Detection of tannins

- **Gelatin test:** To the test solution of tannin, aqueous solution of gelatin and sodium chloride are added. A white buff coloured precipitate is formed.

Detection of Flavonoids

- **Shinoda test:** A little quantity of extract is dissolved in alcohol and few fragments of magnesium turnings and concentrated hydrochloric acid were added. Observed pink scarlet or crimson red colour.

2.3. *In vitro* Cytotoxic Studies against Raw 264.7 and Mcf-7 Cell lines

Materials: PBS, MTT (5 mg/ml in PBS) – filter and keep in dark, DMSO, 96-well plate.

Procedure:

1. Cells were seeded at 1×10^5 cells/mL in 96 well microtiter plates in Minimum Essential Medium with fetal bovine serum. The cells were incubated overnight for attachment.
2. Solutions of 3 crude plant extracts (HILME, HIBME, HISME) in serial three dilutions and standard drug dexomethsone were added in triplicates then incubated for 24hrs. & 48hrs. at 5% CO_2 and 37°C.
3. Thereafter, the cells were treated with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltratrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO).
4. Four hours later, the entire medium including MTT solution (5 mg/mL) was aspirated from the wells. The remaining formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm using a 96 well

microplate reader (Synergy™ HT, Bio-Tek Instruments, Inc).
5. The cytotoxicity index was determined using the untreated cells as negative control. The percentage of viability was calculated using following formula. % viability=absorbance of experimental well/absorbance o f negative control well X100.
6. Percentage of cytotoxicity was determined by using following formula. % of cytotoxicity= 100- percentage of cell viability of experimental compound.
7. Result was analysed by comparing % of cytotoxicity of test compound with that of standard drug (Dexomethasone).

2.4 Screening of *In-Ovo* Anti-Angiogenic Activity

Chick Chorioallantoic Membrane test (CAM model)

Materials: Fertilized, white leghorn chicken eggs (54), egg trays, hypodermic needle, sterile surgical blade, sterile surgical tape, 5mL syringe, needles, camera, sterilized absorbable gelatin sponge, standard drug (sunitinib, sorafenib), forecep, surgical seizure.

Procedure:

1. Fertilized chicken eggs of day zero of chick embryo development were obtained from Srikrishna devaraya hatcheries pvt. Limited, Medchal, Telangana State, India. Eggs were incubated in a B.O.D. Incubator at 37 °C with 60% humidity.
2. A small hole was made at narrow end of the egg with hypodermic needle; 3mL of egg white was removed to detach the CAM membrane from shell on day 3 of chick embryo development under aseptic conditions. The hole was resealed with adhesive sterile tape and eggs were returned to the incubator until day 7 of chick embryo development.
3. On day 7, a small window (1x1inche) was opened at middle portion of egg; blood vessels were photographed using Olympus VG-170 camera. Numbers of blood vessel branches/egg were counted before treatment. A sterile gelatin sponge (3mm x3mm x1mm- length x width x depth) was inserted on CAM membrane.
4. 50µL of phosphate buffered saline as a negative control for 1 group, 3 test solutions - HILME, HIBME, HISME, with single concentration of 100µg/mL for 3 groups, 50µL of 1mM Sunitinib, 1mM Sorafenib as a positive controls for 2 groups were injected into gelatin sponge of each group.
5. The window was resealed and returned to the incubator for 48 hours until day 9 (n = 6 chicken embryos per sample).on day 9(48hrs after treatment) egg shell was broken carefully into the petridish, gelatin sponge was removed and number of blood vessel branches were counted around the gelatin sponge then photographed.
6. Results was analysed by comparing % of inhibition of test extracts with that of standard and control.
7. Percentage of inhibition of blood vessel growth calculated by using following formula % of inhibition= Average number of blood vessel branches in control group after 48hrs / average number of blood vessel branches in test group after 48hrs x 100.

3. Results and Discussion

3.1 Qualitative Analysis Results

Table 1: Preliminary phytochemical screening of *H. integrifolia* extracts (+ = present, - = absent)

Phytoconstituents	Alkaloids	Tannins	Steroids	Terpenoids	Amino acids	Proteins	Flavonoids	Carbohydrates	Glycosides
HILME	+	+	+	+	+	+	-	+	+
HIBME	-	+	+	-	-	-	-	+	-
HISME	+	+	+	+	+	+	+	+	-

3.2. *In -Vitro* Cytotoxicity Studies

3.2.1 MTT Assay against RAW 264.7 cell lines

Table 2: MTT Assay- Percentage of cytotoxicity of *H. integrifolia* extracts after 48hrs of drug treatment

S. No	Treatment	Concentration (µg/mL)	48hrs
1	Control	-	0± 0.03
2	Dexomethasone	1µM	77.24± 0.05
3	HILME	1	53.27± 0.43
		5	61.99± 0.34
		10	63.59± 0.24
4	HIBME	1	47.95± 0.15
		5	50.83± 0.40
		10	59.37± 0.05
5	HISME	1	48.37± 0.14
		5	60.45± 0.03
		10	62.10± 0.16

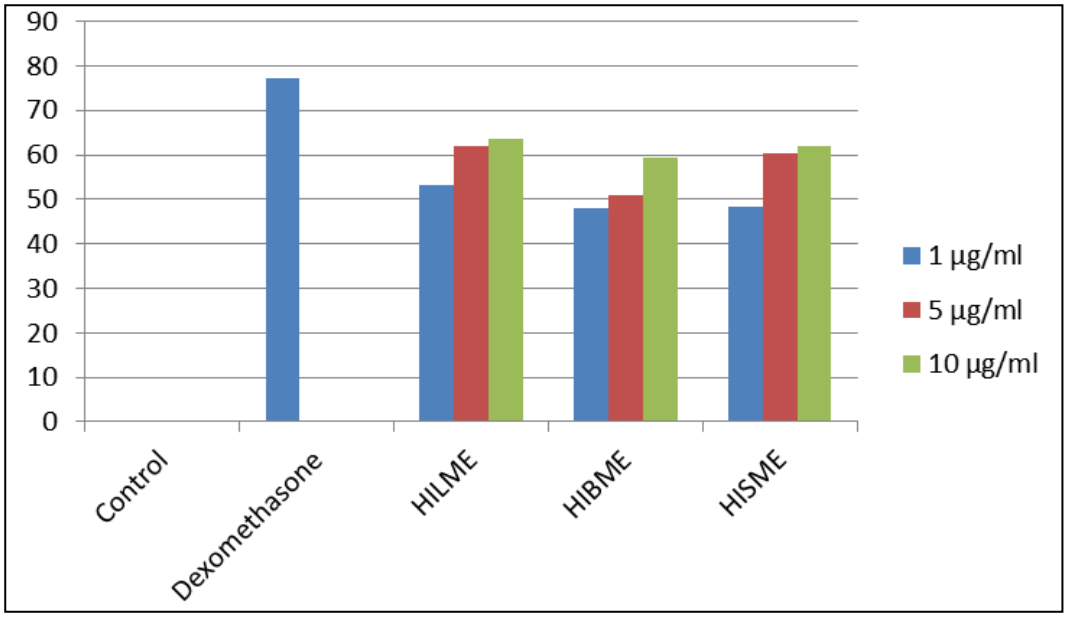


Fig 1: MTT Assay-Percentage of cytotoxicity of *H. integrifolia* extracts after 48hrs of drug treatment.

3.2.2 MTT Assay against MCF-7 cell lines

Table 3: MTT Assay- Percentage of cytotoxicity of *H. integrifolia* extracts after 48hrs of drug treatment

S. No	Treatment	Concentration (µg/mL)	48hrs
1	Control	-	0± 0.0039
2	Dexomethasone (Dexo)	10µM	87.53± 0.005
3	HILME	1	67.77± 0.01
		10	64.64± 0.004
		100	49.94± 0.007
4	HIBME	1	81.94± 0.124
		10	28.16± 0.762
		100	80.34± 0.050
5	HISME	1	83.89± 0.077
		10	81.76± 0.0016
		100	66.05± 0.2007

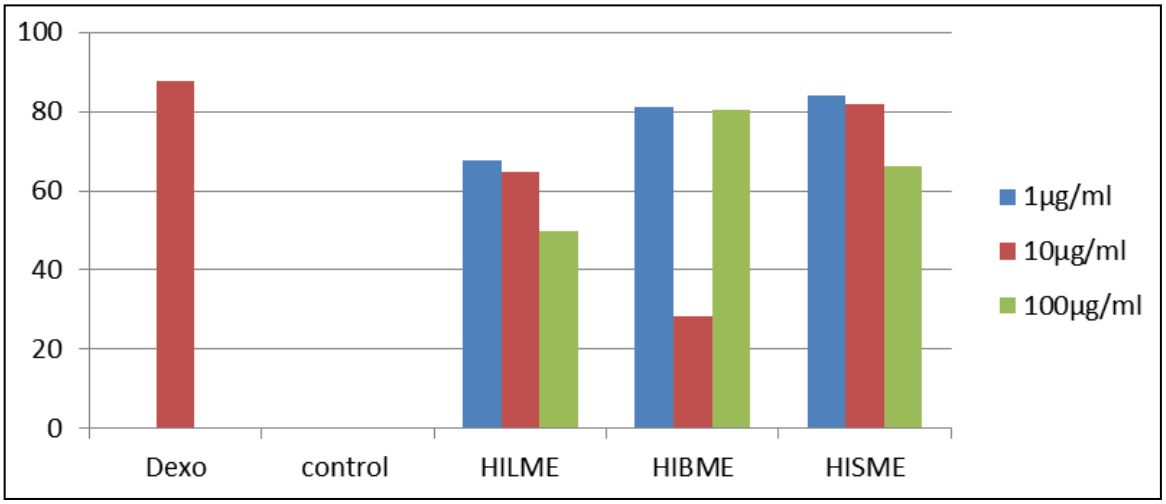
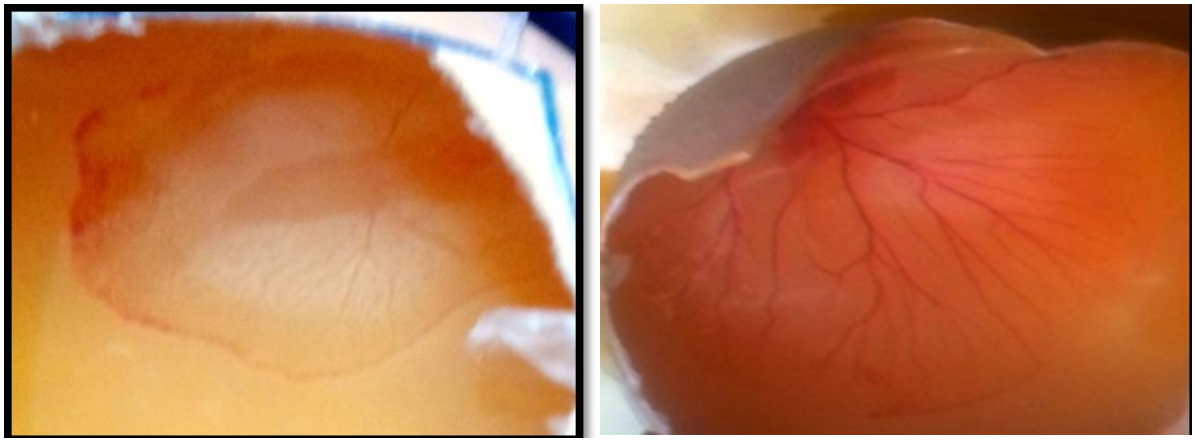


Fig 2: MTT Assay- % of cytotoxicity of *H. integrifolia* extracts after 48hrs of drug treatment.

3.3 Pharmacological Activity Results

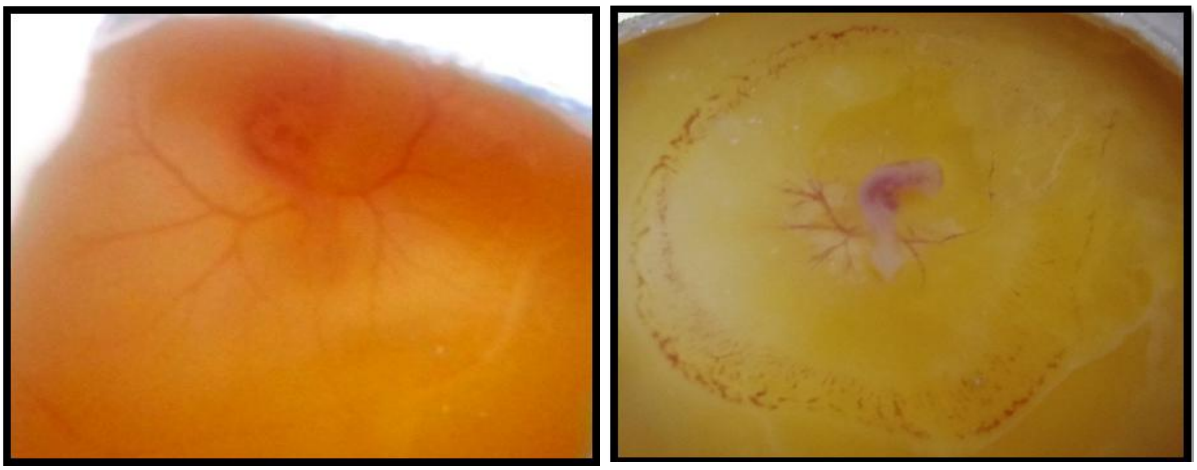
Chick chorioallantoic membrane test results

1. Effect of Phosphate buffered saline (negative control) on blood vessel growth.



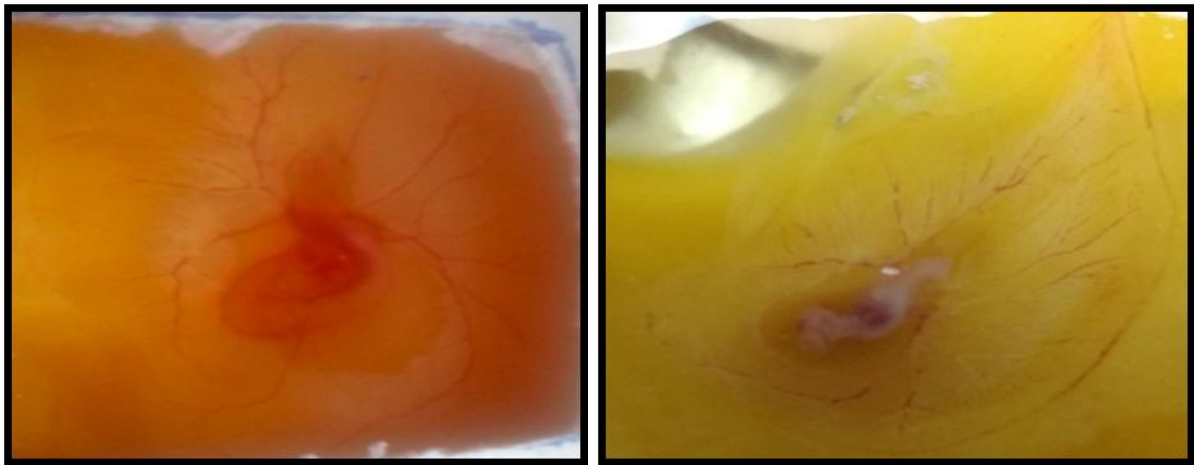
Before After

2. Effect of Sunitinib 1 µM on blood vessel growth



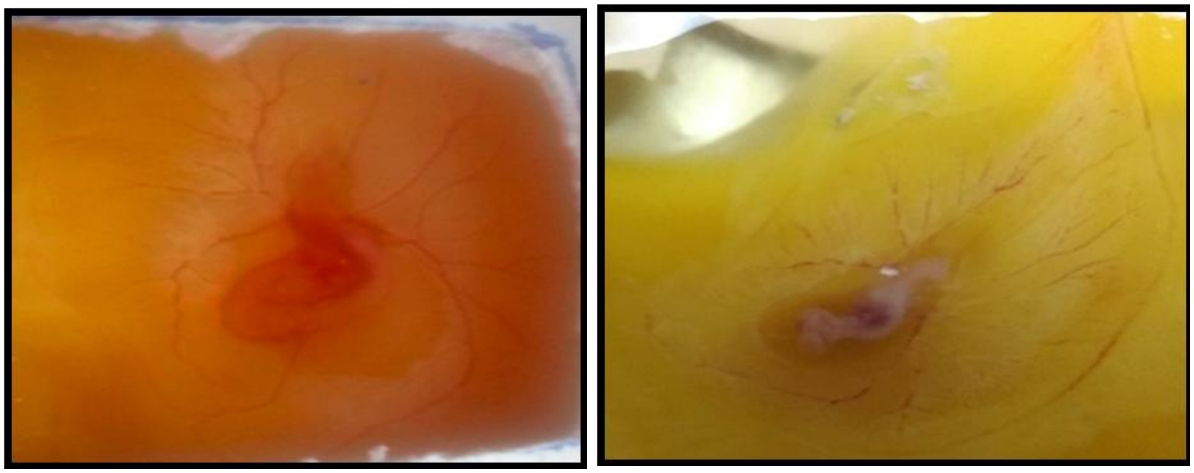
Before After

3. Effect of Sorafenib 1 µM on blood vessel growth.



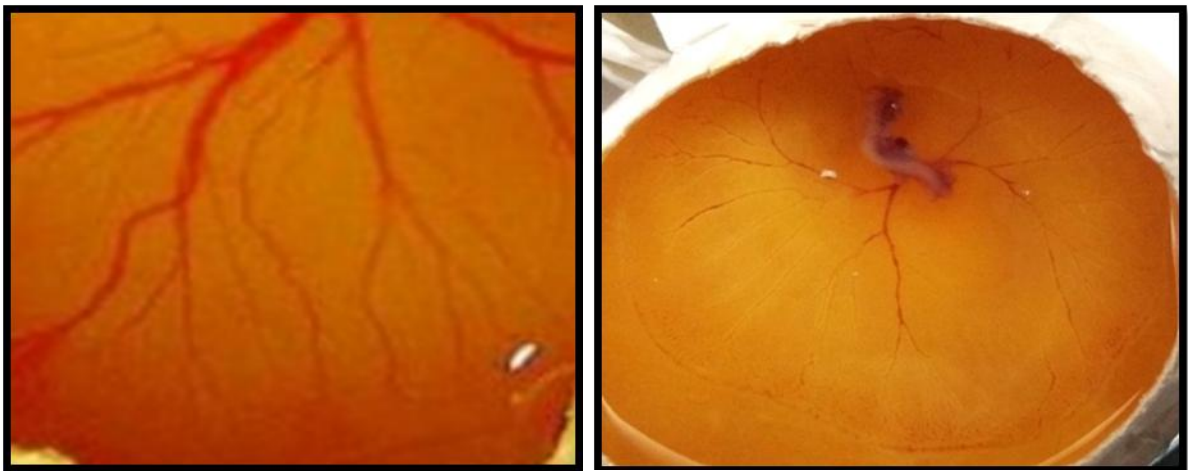
Before After

4. Effect of leaf methanol extract of *H. integrifolia* (HILME- 100µg/mL) on blood vessel growth.



Before After

5. Effect of bark methanol extract of *H. integrifolia* (HIBME- 100µg/mL) on blood vessel growth.



Before After

6. Effect of stem methanol extract of *H. integrifolia* (HISME- 100µg/mL) on blood vessel growth.

Before After After

Fig 3: Effect of various plant extracts and standards on blood vessel growth -Before and after drug treatment with 1) PBS, 2) Sunitinib 1µM, 3) Sorafenib 1µM 4) HILME (100µg/mL), 5) HIBME (100µg/Ml), 6)HISME (100µg/Ml).

Table 4: Percentage of inhibition of angiogenesis after drug treatment along with concentration.

S. No	Sample	Concentration	% of inhibition
1	CONTROL- PBS	-	0± 1.04
2	SUNITINIB(SNT)	1µM	83.1± 0.75
3	SORAFENIB(SRF)	1µM	77.62± 0.98
4	HILME	100µg/mL	64.84± 0.98
5	HIBME	100µg/mL	63.92± 1.16
6	HISME	100µg/mL	-12.3± 0.89

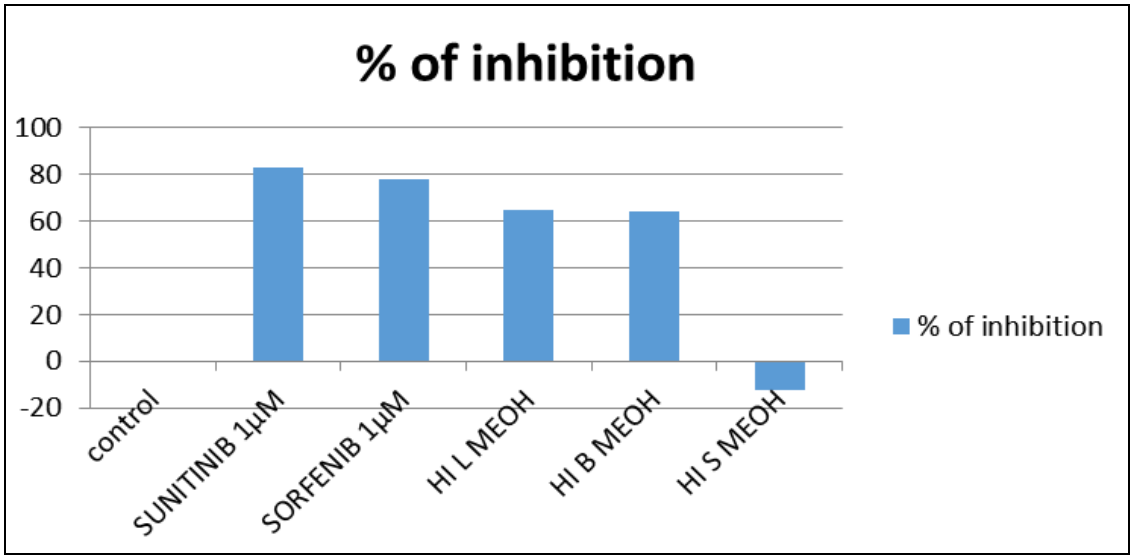


Fig 4: Percentage of inhibition of angiogenesis after drug treatment

4. Conclusions

Our data reveals that HILME-10µg/ml (63.59± 0.244) has shown highest cytotoxicity against RAW 264.7, HISME - 1µg/ml (83.89± 0.07) has shown highest cytotoxicity against MCF-7 cell lines. Among all the 3 extracts *Holoptelea integrifolia* 2 extracts exhibited inhibition of angiogenesis with the highest activity observed by HILME (64.84± 0.98-100µg/mL) followed by HIBME ((63.92± 1.16-100µg/mL) it has a comparable activity with known anti-angiogenic agents Sunitinib 1µM (83.1± 0.75), Sorafenib 1µM (77.62± 0.98). *H. integrifolia* stem methanol extract (HISME-12.3± 0.89), did not show any inhibition, the data suggest that HISME enhanced new blood vessel growth when compared to negative control PBS (0± 1.04). This may possible source for angiogenesis stimulators which are useful in pro-angiogenic therapy to treat various cardiovascular diseases and cardiac is chemia, peripheral artery disease.

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