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Research Article

Antiproliferative Properties of Leaf Extract of *Hopea ponga* (Dennst.) Mabberly

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Abstract

Objective: The present study aimed to evaluate the antiproliferative properties of hydroalchoholic extract of *Hopea ponga* leaves.

Methods: The antiproliferative capacity of extract was assessed by employing different assays such as by brine shrimp lethality assay, MTT assay and trypan blue assay in HeLa cells. Results were compared with standard vincristine.

Results: *In vitro* antiproliferative study of the hydroalcoholic extract of *H. ponga* was evaluated by brine shrimp lethality assay, MTT assay and trypan blue assay in HeLa cells. The results indicated that the hydroalcoholic extract of *H. ponga* possessed good antiproliferative property when compared to the standard drug vincristine.

Conclusion: The result suggests that the hydroalochoholic extracts of *H. ponga* plays an important role in antiproliferative activity. The activity may be due to the presence of flavonoids. It is further suggested that in vivo studies and characterisation of fractionated extracts May be conducted in this extract, to establish its effect. *Hopea ponga* (Dennst.) Mabberly. *H. ponga* has been used as a source of timbre for construction and agricultural implements shows its potential as being used as antiproliferative, anti-inflammatory and antioxidant, so it can be concluded that this species should be conserved and explored further.

Keywords: *Hopea ponga*, anti-proliferative activity, cytotoxicity assay.

1. Introduction

The medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health. Cancer is a growing public problem which remains the second major cause of death after cardiovascular diseases. Cancers begin as the result of an abnormality in the genes of one or more cells in the body. That abnormality may either be inherited; the faulty gene being passed from one generation to the next, or acquired; a normal gene being damaged or mutating for some reason [1].

The key to effective chemotherapy and chemoprevention is the identification of chemotherapeutic and chemopreventive agents that can effectively inhibit cancer development without toxic side effects [2]. Many plant-derived compounds have been an important source of several clinically useful anticancer agents. These include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide derived from epipodophyllotoxin, and paclitaxel. The present study clearly indicated that hydroalcoholic extract of *H. ponga* appreciable *in vitro* cytotoxic potential against HeLa

(cervical cancer) cells in MTT assay and trypan blue assay. [3]

2. Materials and methods

2.1 Collection and preparation of test plant sample

The leaves of *Hopea ponga* (Dennst.) Mabberly were obtained from Alakyampaalam, Pariyaram, Kannur Kerala (India) in the month of March 2015 and authenticated by Dr. Ratheesh Narayanan M.K, Govt Department of Botany, Payyanur College, Kannur, Kerala. A voucher specimen APSC/COL/05/15 was deposited in the Department of Pharmacology, Academy of Pharmaceutical Sciences, Pariyaram Medical College, Kannur, Kerala. After authentication the plants were collected, cleaned and dried in shade at room temperature. The dried leaves were pulverized in a mechanical grinder to obtain coarse powder.

2.1.1 Hydroalcoholic extract

The powdered plant (500g) was sieved through sieve No.10 and the powder was subjected to defatting with petroleum ether for 6 hours. The filtered powder was then subjected to cold maceration with ethanol: water (70:30) for 7 continuous days. The hydroalcoholic extract was prepared by mixing with the help of a sonicator. It was then filtered through a muslin cloth and marc was discarded. The filtrate was concentrated using a rotary vacuum evaporator and stored in a refrigerator until further use.

2.2. Cytotoxicity assays

2.2.1. Preliminary screening by brine shrimp lethality assay

Brine shrimp lethality assay is a convenient method for general screening for toxicity of the extracts or compounds towards brine shrimp (*Artemia salina*) and it can give an indication regarding possible cytotoxicity of the test samples. [4]

Procedure [5,6]: Artificial sea water was prepared by dissolving 38 g of NaCl (3.8%) in 1000 ml of distilled water and was filtered off to obtain a clear solution. The dried cysts of the brine shrimps were hatched in artificial seawater with constant aeration and light for 48 hours. The extract was dissolved in sea water and transferred to test tubes to obtain concentrations of 1.25, 2.5, 5, 10, 20 and 40 µg/ ml in 5 ml artificial sea water with 20 nauplii in each test tube. Standard drug vincristine sulphate was used as positive control at concentrations of 0.312, 0.625, 1.25, 2.5, 5 and 10 µg/ml. Experiments were conducted in triplicate and the average value was noted. Artificial seawater was used as the control. After 24 h incubation at 25-30°C, the number of viable nauplii was counted using a magnifying glass.

The percent (%) mortality was calculated using the following formula

$$\% \text{ mortality} = \frac{Nt \times 100}{N_0}$$

Where, Nt = Number of dead nauplii after 24 hrs of incubation, No= Number of total nauplii transferred (n = 20).

The percentage of mortality was plotted against concentration. Using the linear regression equation of the graph, the concentration that would kill 50% of the larvae i.e. median lethal concentration (LC_{50}) was determined using Graph Pad Prism software.

2.2.2 MTT assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. [7,8]

Procedure: After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 370C for 4h.The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO. Measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

$$\% \text{ Cell Inhibition} = 100 - \frac{\text{Abs (sample)} \times 100}{\text{Abs (control)}}$$

Using the linear regression graph plotted between % cytotoxicity and concentration, IC50 was determined using GraphPad Prism software.

2.2.3 Cytotoxicity evaluation in HeLa cells by Trypan blue assay

Trypan blue is a vital stain used to selectively color dead tissues or cells blue. Trypan blue is recommended in dye exclusion procedures for viable cell counting based on the principle that live (viable) cells actively pump out the dye by efflux mechanism whereas dead (non-viable) cells do not. Hence in this assay, white transparent cells are viable cells and blue cells taking up the dye are dead cells [9,10].

Procedure: 700 µl of a cell suspension was transferred to 24 well plates and incubated for 24 hrs in 5% CO_2.After incubation, 300 µl of varying concentrations of extract and standard (25-100 µg/ml) was added and incubated for 24 hrs.100 µl of cell suspension was taken in an Eppendorf tube and to that 100 µl of 0.4% trypan blue solution was added and mixed thoroughly. It was allowed to stand for 5-15 minutes. A small amount of trypan blue-cell suspension mixture was transferred to both chambers of a hemocytometer using a Pasteur pipette. All the chambers were filled by capillary action and not overfilled. From

chamber 1 of the hemocytometer, the cells in the 1 mm centre square and four 1mm corner squares were counted. Non-viable cells stained with blue colour. Viable and non-viable cells were counted separately.

$$\% \text{ cytotoxicity} = \frac{\text{number of non-viable cells (stained)}}{\text{Total cells (stained and unstained)}} \times 100$$

Using the linear regression graph plotted between % cytotoxicity and concentration, IC_{50} was determined using Graph Pad Prism software.

4. Results

The phytochemical screening subjected to detect the presence of some secondary plant metabolites following standard procedures as shown in Table 1. The percentage yield of hydro alcohol extract was found to be 3.12 %.

Table 1: Phytochemical screening of hydroalochoholic extracts of *H. ponga*

Sl. No	Constituents	Hydroalcoholic extract
1.	Alkaloids	-
2.	Carbohydrates	+
3.	Proteins and Amino acids	-
4.	Flavonoids	+
5.	Saponins	+
6.	Triterpenoids	-
7.	Steroids	-
8.	Tannins and Phenolic compounds	+
9.	Glycosides	-

(+) present (-) absent

4.1 Cytotoxicity assay

Preliminary screening by brine shrimp lethality assay

The hydroalcoholic extract showed concentration dependent mortality with an LC_{50} value of 12.49 µg/ml. Vincristine was found to have an LC50 value of 2.48 µg/ml. The findings are tabulated in Table 2 and depicted in Figure 1.

Table 2: Preliminary screening by brine shrimp lethality assay of hydroalochoholic extracts of *H. ponga*

Groups	Concentration	% mortality	LC_{50} (µg/ml)
Control	-	0	-
Hydroalcoholic extract	1.25	18.33±1.66	12.49
	2.5	23.33±1.66	
	5	33.33±4.41	
	10	66.66±1.66	
	20	78.33±1.66	
	40	86.66±1.66	
Vincristine	0.312	20±2.89	2.48
	0.625	31.66±1.66	
	1.25	38.33±1.66	
	2.5	38.33±1.66	
	5	86.66±1.66	
	10	100	

Values are in Mean ± SEM, n=3

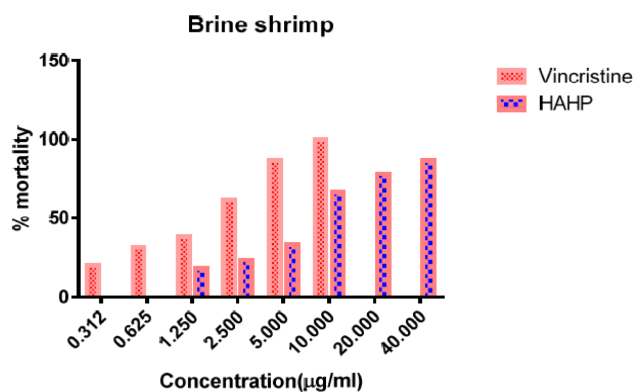

Figure 1: *In vitro* cytotoxic activity of *Hopea ponga* by brine shrimp lethality assay

HAHP- Hydroalcoholic extract of *Hopea ponga*

4.3 Cytotoxicity evaluation in HeLa cells by MTT assay

The hydroalcoholic extract showed concentration dependent cytotoxicity on HeLa cells with an IC_{50} value of 95.06 µg/ml. Vincristine was found to have an IC50 value of 42.75µg/ml. The findings are tabulated in Table 3and depicted in Figure 2

Table 3: Evaluation of cytotoxicity by MTT assay of hydroalochoholic extracts of *H. ponga*.

	Concentration	% Cytotoxicity	IC_{50} (µg/ml)
Control	-		
Hydro Alcoholic extract	12.5	13.78±0.002	95.06
	25	18.71±0.001	
	50	34.90±0.004	
	100	50.98±0.002	
	200	67.17±0.001	
Vincristine	12.5	25.28±0.003	42.75
	25	32.56±0.004	
	50	41.72±0.002	
	100	76.54±0.003	
	200	94.32±0.001	

Values are in Mean ± SEM, n=3

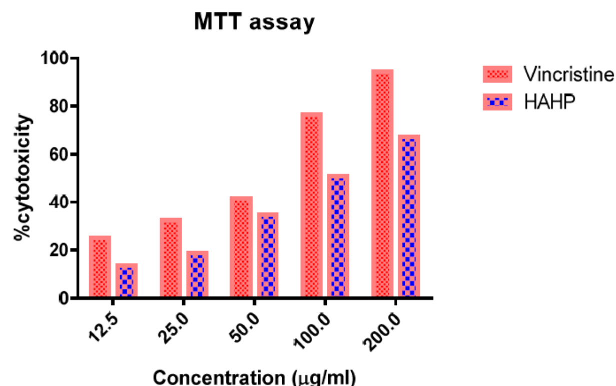

Figure 2: *In vitro* cytotoxic activity of *Hopea ponga* by MTT assay

HAHP- Hydroalcoholic extract of *Hopea ponga*

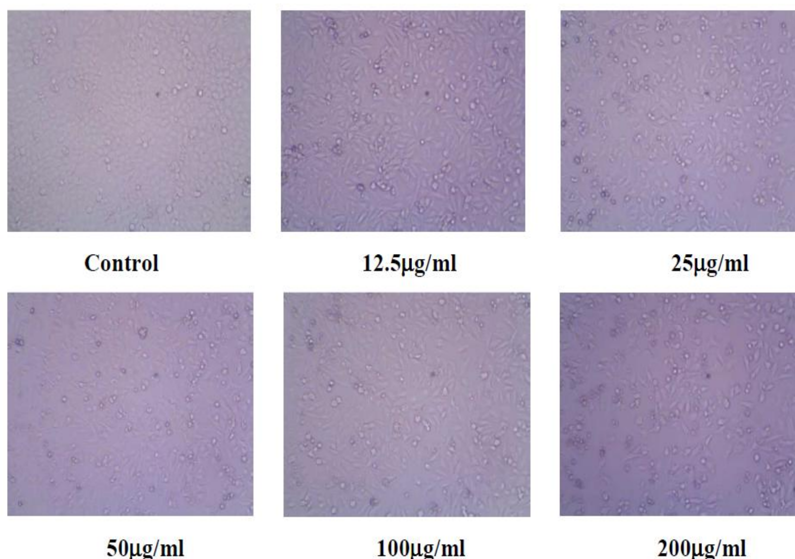

Figure 3: Cell death in HAHP by MTT at various concentrations

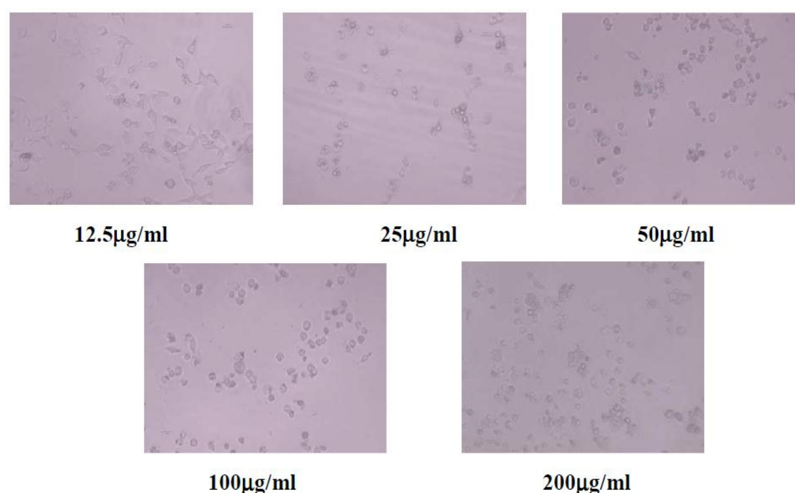

Figure 4: Cell death in Vincristine by MTT at various concentrations

4.4 Cytotoxicity evaluation in HeLa cells by Trypan blue assay

The hydroalcoholic extract showed concentration dependent cytotoxicity on HeLa cells with an IC50 value of 64.18 µg/ml. Vincristine was found to have an IC50 value of 51.12µg/ml. The findings are tabulated in Table 4 and depicted in Figure 5.

Table 4: Evaluation of cytotoxicity by Trypan blue assay

Group	Concentration (µg/ml)	% Cytotoxicity	IC_{50} (µg/ml)
Control	-	0	
Hydroalcoholic extract	25	19.68±2.91	64.18
	50	38.72±2.82	
	75	59.04±3.04	
	100	77.38±1.81	
Vincristine	25	30.75±4.67	51.12
	50	45.94±3.58	
	75	67.77±2.96	
	100	86.34±1.95	

Values are in Mean ± SEM, n=3

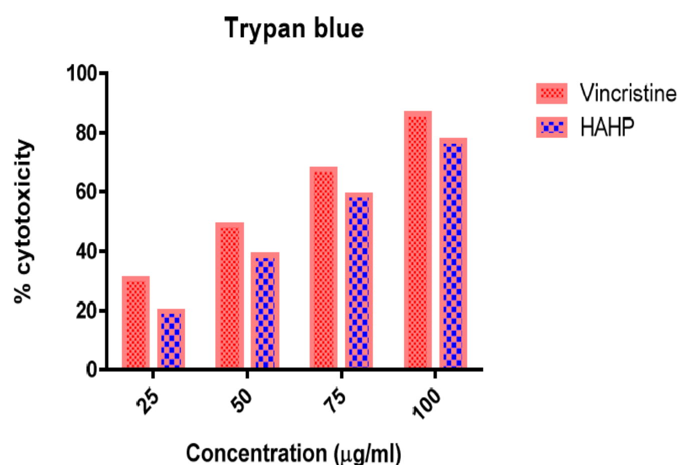

Figure 5: Evaluation of cytotoxicity by Trypan blue assay

HAHP- Hydroalcoholic extract of *Hopea ponga*

5. Discussion

Ethnopharmacology and drug discovery using natural products provides insight to many important hidden uses of plants. There are clear trends to show that the mainstream in pharmaceutical research is moving away from single molecule or single target approach to combinations and multiple target approaches.[11] This combined percentage (52% of all new chemical entities) suggests that natural products are important sources for new drugs and suitable for further modification during drug development.[12] The leaves of *H. ponga* were collected and hydroalcoholic extract was prepared. The percentage yield was found to be 6.92%. The physicochemical parameters like total ash, acid insoluble ash, water soluble ash, loss on drying, water and alcohol soluble extractives were studied.

The key to effective chemotherapy and hemoprevention is the identification of chemotherapeutic and chemopreventive agents that can effectively inhibit cancer development without toxic side effects. The measure of potency of an extract using the brine shrimp (*Artemia salina)* has become increasingly common since the early 1980's. Brine shrimp is much closer to humans than bacteria, in evolutionary and structural terms [13]. All compounds showing positive results in this assay, need not necessarily be cytotoxic[4]. An LC_{50} value of <1 mg/mL is considered to be significant, and lower the value the higher is the toxicity of the test sample.[14] Therefore the activity had to be confirmed by other cytotoxicity assays. The LC_{50} of hydroalcoholic extract by brine shrimp lethality assay was found to be 12.49 g/ml against the LC_{50} value of 2.48 g/ml by vincristine.

The colorimetric assay of MTT measures the reduction of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT that enters the cells and passes into mitochondria, gets reduced to an insoluble, coloured (purple) formazan product. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.[8] The MTT assay showed a concentration dependent increase in the growth inhibition of HeLa cells by the hydroalcoholic extract of *H. ponga* which is evident in the photographs provided. The viability of cells decreased with increasing concentration. Vincristine had an IC_{50} value of 42.75g/ml, whereas hydroalcoholic extract of *H. ponga* possessed an IC_{50} value of 95.06g/ml.

The trypan blue assay is used to assess the cell viability. It is based on the ability of the membrane of viable cells to exclude the dye, while nonviable cells are stained blue. Trypan blue as a dye cannot enter cells through an intact membrane and therefore stains only cells, which have punctured membranes. Since trypan blue has a macromolecular nature, the holes in the membrane must be pretty big to let the stain molecules pass inside the cell – in other words cell death, which is shown by membrane disintegration, must be fairly far progressed to be detectable by the trypan blue method. In trypan blue assay, a concentration dependent increase in % cytotoxicity was observed with the hydroalcoholic extract of *H. ponga* showing IC_{50} value of 64.18 g/ml and vincristine showing an IC_{50} value of 51.12g/ml.

6. Conclusion

Hopea ponga (Dennst.) Mabberly, an endangered species under the IUCN red list of threatened species was evaluated for their antiproliferative, anti-inflammatory and antioxidant properties. The present study indicated that the hydroalcoholic leaf extract consisted of important phytochemicals like flavonoids, saponins, tannins and phenolic compounds.

Flavonoids have been found to possess antimutagenic and antimalignant effects. Moreover it has protective effect against cancer by their effect on signal transduction in cell proliferation and angiogenesis. Therefore the antiproliferative property of *H. ponga* due to the presence of flavonoids. It is further suggested that in vivo studies and characterisation of fractionated extracts may be conducted in this extract, to establish its effect.

Hopea ponga (Dennst.) Mabberly, a subcanopy tree found in the Southern Western Ghats which has been used as a source of timbre for construction and agricultural implements shows its potential as being used as antiproliferative. So it can be concluded that this species should be conserved and explored further.

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