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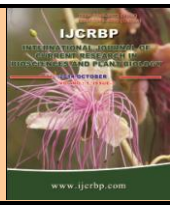

Original Research Article

In Vivo Antitumor and Antioxidant Activity of *Hugonia mystax* L. (Linaceae)

S. Anandakumar[1], M. Prakash[2*] and N. Karmegam[3]

[1] Department of Biotechnology, VMKV Engineering College, Periyaseeragapadi, Salem- 636 308, Tamil Nadu, India
[2]Department of Microbiology, Kanchi Shri Krishna College of Arts and Science, Kilambi- 631 551, Kancheepuram, Tamil Nadu, India
[3]Department of Botany, Government Arts College, Salem-636 007, Tamil Nadu, India

**Corresponding author.*

A b s t r a c t

Antitumor and antioxidant activities of methanolic extracts of leaf and stem bark of *Hugonia mystax* L. against Dalton's Lymphoma Ascites (DLA) bearing female Swiss albino mice was carried out. The effect of leaf and stem bark extracts of *H. mystax* on tumor growth and host's survival time was studied by the following parameters: average life span, percentage increase in lifespan, body weight analysis, packed cell volume and viable tumor cell count. Treatment with methanolic leaf and bark extracts at 250 and 500mg/kg b.w. increased the mean survival time of DLA tumor bearing mice. Decrease in tumor volume, packed cell volume, and viable cell count was observed in leaf and stem bark extract treated animals when compared to control group animals. Hematological studies reveal that the haemoglobin content was decreased in DLA treated animals, whereas restoration to near normal levels was observed in *H. mystax* methanolic extract treated animals. Treatment with methanolic leaf and stem bark extracts decreased the levels of catalase, superoxide dismutase and thiobarbituric acid reactive substances. The results suggest that the methanol extract of *H. mystax* leaf and stem bark exhibits significant antitumor and antioxidant effects in DLA tumor bearing mice.

K e y w o r d s

Anticancer activity
DLA
Hugonia mystax
Medicinal plants
Methanolic extract

Introduction

Traditional medicines are has been used throughout the world thousands of years. The conventional medicines mostly recovered from medicinal plants. Medicinal plants typically contain several chemical compounds that may act individually, additively or in synergy to improve health. Now a days, combating cancer is of dominant significance. Multidisciplinary scientific studies are making the best efforts to fight this disease, but the sure shot, perfect cure is yet to be brought into world medicine. An alternative solution to western medicine embodied with severe side effects is the

use of medicinal plant preparations to arrest the dangerous nature of the cancer. Approximately 62% of the commercially available anticancer drug before 1983 can be related to natural sources (Gordon et al., 2001).

Many medicinal plants have been assessed in clinical studies and are currently being investigated phytochemically to understand their tumoricidal actions against various cancers (Premalatha and Rajagopal, 2005). Medicinal plant extracts are increased the activity of all antioxidant enzymes. These enzymes are adapted in various diseases by free radical attack (Ames, 1998). Mammalian cells possess elaborate defense mechanisms for radical detoxification. Key metabolic steps are the superoxide dismutase (SOD) catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 into water and oxygen by catalase (CAT) and glutathione peroxidase (GPX), which destroys toxic peroxides. In addition to antioxidant enzymes, several small molecule antioxidants play important roles in the antioxidant defense systems (Halliwell and Gutteridge, 1998). The selected medicinal plant *Hugonia mystax* L. comes under small plant family Linaceae it consist of 34 species. *Hugonia* are found in the tropical regions of continental Africa, Madagascar and Mauritius. Some of these species, including *H. castaneifolia* Engl., are used as herbal remedies (Kokwaro, 1976 and Hutchings, 1996). Some *Hugonia* species yielded lignans related to the anti-tumor agent podophyllotoxin (Konuklugi, 1996) encouraged a few years ago to investigate the root bark of *H. castaneifolia* that occurs in East Africa, for cytotoxic and other constituents (Baraza et al., 2008).

Another plant *Linum usitatissimum* belonging to Family Linaceae has been shown to possess significant anti-arthritis activity (Singh et al., 2012). Earlier studies carried out in our laboratories have indicated the potent cytotoxic nature of *H. mystax* against several cancerous cell lines. Among the methanol extracts prepared from different parts of the plant, such as leaf and stem bark, the leaf extract was found to be more active with CTC_{50} (cytotoxic concentration to kill 50% cells) (Anandakumar and Karmegam, 2011). In the present study, the methanol extract of *H. mystax* leaf and stem bark was investigated for their *in vivo* antitumor properties against the DLA tumor model in mice.

Materials and methods

Collection and identification

The leaves and stem barks of *H. mystax* L. (Fig. 1) were collected from Sirumalai Hills (Eastern Ghats), Dindigul District, Tamil Nadu, India. The identification was confirmed with Botanical Survey of India, Coimbatore, Tamil Nadu, India (Ref. No: BSI/SRC/5/23/10-11/Tech-1522).

Preparation of extract

The collected leaves and stem bark of *H. mystax* were shade dried and powdered with using mortor and pastel. Dried powder was filled in Soxhlet apparatus and methanol was used as solvent for extraction. The powdered plant material (100g) was taken in a thimble and kept the thimble on round bottom flask. Added 250ml of methanol in the round bottom flask, then the condenser was fixed on upper part of the thimble and heated about 50°C in the heating mantle. The Soxhlet apparatus was run for 24 h until the solvent in the siphon tube became colourless. The hexane was separated in the thimble, removed and discarded to recycled hexane. After evaporating the remaining solvent, the final crude extracts were collected, stored individually in air tight containers and kept under refrigerated condition for further investigation.

In vivo anticancer and antioxidant studies

(Dongre et al., 2007)

Ethical Clearance

Protocol used in this study for the use of mice as an animal model for cancer was approved by the Institutional Animal Ethical Committee (CPCSEA Approval Number: P.COI/51/2010/ IAEC/ VMCP).

Mice and tumor system

Female Swiss albino mice (20±2 g, 6–8 weeks old) were used for acute toxicity and anticancer study. Mice were housed in open top cages and maintained on food and water *ad labium*. Room temperature was maintained at 22±2°C with light and dark cycle of 14/10 h. Dalton's lymphoma ascites (DLA) were maintained in ascetic form by serial transplantation in Swiss albino mice or *in vitro* cell culture system by serial passage. Irrespective of whether the cells were obtained from *in vitro* culture or from ascetic fluid they exhibited typical phenotypic features.

Fig. 1: Plant species used in the study-*Hugonia mystax* Linn. (A) at flowering; (B) spiral hooks; (C) mature fruits prior to ripening; (D) a plant with fruits in different stages of growth.

Preparation of suspensions and solutions

The test drugs and standard 5-Fluorouracil (5-FU, procured from Ranbaxy Laboratories, New Delhi, India) were suspended in distilled water using sodium CMC (carboxy methyl cellulose) suspension (0.3%) and administered orally to the animals with the help of an intragastric catheter.

Evaluation of antitumor activity of test drugs in DLA-bearing mice model

The animals, total of 84 were divided into seven groups (twelve mice in each group) Group 1-Group 7. Under sterile condition, about 0.5 ml of DLA cell suspension (2×10^6cells/ml) was inoculated intraperitoneally to each mouse of Group 2 to Group 7 (total of 72) at day zero. After two days tumor inoculation the animals were treated as fallows,

Group 1: Normal control and received sodium CMC suspension (0.3%).

Group 2: Tumor control and received sodium CMC suspension (0.3%).

Group 3: Positive control was treated with 5-fluorouracil (20 mg/kg, p.o).

Group 4: Treated with methanol leaf extract (250 mg/kg, p.o) in sodium CMC suspension

Group 5: Treated with methanol leaf extract (500 mg/kg, p.o) in sodium CMC suspension

Group 6: Treated with methanol Stem bark extract (250 mg/kg, p.o) in sodium CMC suspension

Group 7: Treated with methanol Stem bark extract (500 mg/kg, p.o) in sodium CMC suspension

The treatment was continued for the next 10 days. On 11th day, i.e. after the last dose and 24 h fasting, six mice from each group were sacrificed for the study of antitumor, hematological and biochemical parameters. The rest of the animals were kept to check the average life span and change in the body weight. Blood was collected from the animals by retro-orbital puncture under slight anesthetic (diethyl ether) condition and the hematological parameters such as RBC, WBC, differential count and hemoglobin were studied. From the same animals liver and kidney were separated, serum was separated from the rest of the blood by keeping it at 37°C for 30 min and subjected for the biochemical estimations. The significance of the *in-vivo* data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. $p<0.05$ was considered as statistically significant.

Parameters analyzed

Average life Span: Average life span of the animals of all the groups was determined and noted.

Percentage increase in life span (% ILS): The effect of MML and ARL on tumor growth was monitored by recording the mortality daily for a period of 6 weeks and percentage increase in life span (% ILS) was calculated, by using following formula,

$$\%\ \text{ILS} = \left(\frac{\text{Mean survival of treated group}}{\text{Mean survival of control group}} - 1\right) \times 100$$

Body weight analysis: Body weights of the experimental mice were recorded both in the treated and control group at the beginning of the experiment (day 0) and sequentially on every day during the treatment period. Percent increase in body weight (%IBW) was calculated on day 11 of the experiment using the following formula.

$$\%\ \text{IBW} = \left(\frac{\text{BWA on } 11^{th} \text{ day - BWA on } 0^{th} \text{ day}}{\text{Body wt. of animal on } 0^{th} \text{ day}}\right) \times 100$$

Where, BWA= body weight of animals;

Packed cell volume: Peritoneal fluid from the each animal was collected on 11th day and 5 ml of fluid was transferred to sterile centrifuge tubes and centrifuged at 2000 rpm for 10 min. Cell sedimentation levels were measured and expressed in ml.

Viable tumor cell count: Peritoneal fluid from the each animal was collected on 11th day and cell viability was measured by using trypan blue dye exclusion method.

Hematological Parameters

The blood collected was immediately used for the estimation Hemoglobin (Hb) content, red blood cell count (RBC) and white blood cell count (WBC) using haemocytometer.

Biochemical parameters

The antioxidant parameters, catalase (Beers and Sizer, 1952), superoxide dismutase (Misra and Fridovich, 1972) and thiobarbituric reactive

substances (Ohkawa et al., 1979) were estimated in blood serum, liver and kidney homogenates. The results were statistically analysed for the level of significant difference at $p<0.05$.

Results

Effect of *Hugonia mystax* L. leaf and stem bark extracts on antitumor parameters of DLA bearing mice

The treatment with methanolic extract of *H. mystax* at 250 mg/kg and 500 mg/kg significantly and dose dependently increased the average life span of DLA bearing mice from 18.0 ± 0.22 to 26.78 ± 0.45 and 32.00 ± 1.05 days, respectively and methanolic extract of stem bark at same dosage, the life span of DLA bearing mice increased 23.26 ± 0.56 and 29.15 ± 1.84 when compared with the DLA tumor control group ($p<0.001$). Hence, the extract treatment at 500 mg/kg body weight was found to be closely near the standard drug with a percentage increase in life span of 77.77. The potent anticancer nature of both the doses of extract treatment was also confirmed by the significant and dose dependent reduction in percentage increase in body weight, packed cell volume and viable tumor cell count when compared with the DLA control ($p<0.001$) (Table 1). The standard 5-Fluorouracil (0.3%) gave more or less similar.

Table 1. Effect of *Hugonia mystax* L. leaf and stem bark extracts on antitumor parameters of DLA bearing mice.

Parameters	Normal	DLA control (1×10^6 cells /ml / mice)	DLA + 5-FU	DLA + LME (250 mg/kg)	DLA + LME (500 mg/kg)	DLA + BME (250 mg/kg)	DLA + BME (500 mg/kg)
Average life span, Days	---	18.0 ± 0.22	34.50 ± 0.85^{f}	26.78 ± 0.45^{f}	32.00 ± 1.05^{f}	23.26 ± 0.56^{f}	29.15 ± 1.84^{f}
Increase in life span, %	---	---	91.66	48.77	77.77	29.22	61.94
Increase in body wt, %	---	39.56 ± 0.78	14.65 ± 1.00^{f}	30.47 ± 2.10^{f}	23.00 ± 0.70^{f}	31.76 ± 1.06^{f}	25.58 ± 0.50^{f}
Packed cell volume, ml	---	0.95 ± 0.05	0.51 ± 0.01^{e}	0.79 ± 0.01^{e}	0.66 ± 0.01^{f}	0.82 ± 0.01^{e}	0.71 ± 0.01^{f}
Viable tumor cell count ($\times 10^7$ cells/ml)	---	546.00 ±11.45	306.00 ± 8.21^{f}	495.00 ± 3.78^{d}	379.33 ± 7.65^{f}	510.15 ± 5.00^{d}	390.66 ± 5.50^{f}
Total WBC (10^3/mm^3)	9.81 ± 0.75	19.65 ± 0.43^{c}	9.95 ± 1.34^{f}	15.83 ± 0.72^{e}	12.13 ± 0.60^{f}	17.00 ± 0.75^{e}	12.38 ± 0.50^{f}
RBC (1×10^6/mm^3)	11.38 ±0.65	5.98 ± 0.71^{c}	9.78 ± 0.56^{f}	8.76 ± 0.36^{f}	10.33 ± 0.10^{f}	7.95 ± 0.65^{f}	9.45 ± 0.29^{f}
Hgb (g/dl)	14.92 ±0.38	7.96 ± 0.58^{b}	13.69 ± 0.85^{d}	9.15 ± 1.00	13.50 ± 1.35	9.34 ± 0.90	12.75 ± 0.70

Values are expressed as mean ± STDEV for six animals in each group $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ between normal and tumor group values. $^{d}p<0.05$, $^{e}p<0.01$ and $^{f}p<0.001$ between tumor control and treated groups; LME – methanolic leaf extract; BME: methanolic stem bark extract; 5-FU: 5-Fluorouracil;

Hematological parameters

Inoculation of DLA cells resulted in a significant increase in the level of total WBC ($p<0.001$) and a significant increase in the levels of RBC ($p<0.001$) and hemoglobin ($p<0.001$) when compared with normal animals. The extract at both doses and the standard treatment reversed these changes towards normal values. A significant and dose dependent decrease in the levels of WBC and increase in the levels of RBC and hemoglobin when compared with DLA control animals was observed (Table 1). The inoculation of DLA cells to tumor control animals resulted in a significant decrease in the level of CAT ($p<0.05$) and SOD ($p<0.05$) and increase in the level of TBARS ($p< 0.05$) in serum (Table 2).

Table 2. Effect of *H. mystax* extracts on antioxidant parameters of DLA bearing mice

Groups	Blood serum			Liver homogenate			Kidney homogenate		
	CAT (IU/min/ml)	SOD (IU/min/ml)	TBARS (MDA ng/ml)	CAT (µ/mg)	SOD (µ/mg)	TBARS (MDA ng/mg)	CAT (µ/mg)	SOD (µ/mg)	TBARS (MDA ng/mg)
Normal	0.80 ± 0.03	1.35 ± 0.15	2.75 ± 0.25	0.68 ± 0.05	1.10 ± 0.08	3.95 ± 0.25	0.76 ± 0.02	0.64 ± 0.04	6.00 ± 0.25
DLA control (1×10^6 cells/ml/ mice)	0.35 ± 0.06[c]	0.37 ± 0.01[c]	5.78 ± 0.50[c]	0.40 ± 0.01[c]	0.26 ± 0.05[c]	7.85 ± 0.45[c]	0.31 ± 0.05[c]	0.39 ± 0.08	9.15 ± 0.26[b]
DLA + 5-FU	0.69 ± 0.01[e]	1.15 ± 0.01[e]	3.00 ± 0.20[e]	0.59 ± 0.01[e]	0.95 ± 0.05[e]	4.23 ± 0.01[e]	0.68 ± 0.02[e]	0.56 ± 0.05[e]	5.75 ± 0.15[e]
DLA + LME (250 mg/kg)	0.47 ± 0.01	0.79 ± 0.04	3.68 ± 0.15	0.46 ± 0.15	0.68 ± 0.05	5.80 ± 0.35	0.40 ± 0.03	0.48 ± 0.10	8.00 ± 0.14
DLA + LME (500 mg/kg)	0.66 ± 0.03[e]	1.00 ± 0.01[e]	2.90 ± 0.23[e]	0.53 ± 0.01[e]	1.00 ± 0.01[e]	4.40 ± 0.25	0.59 ± 0.05	0.55 ± 0.06	6.75 ± 0.15[e]
DLA + BME (250 mg/kg)	0.41 ± 0.04	0.57 ± 0.01	4.35 ± 0.45	0.46 ± 0.02	0.48 ± 0.05	6.78 ± 0.35	0.33 ± 0.02	0.42 ± 0.05	8.75 ± 0.25
DLA + BME (500 mg/kg)	0.59 ± 0.01[d]	0.89 ± 0.03[e]	3.56 ± 0.25[d]	0.49 ± 0.01[d]	0.75 ± 0.10[d]	5.38 ± 0.20	0.48 ± 0.01	0.50 ± 0.01[d]	7.00 ± 0.30[d]

Values are expressed as mean ± SE for six animals in each group [a]$p<0.01$ and [b]$p<0.001$ between normal and tumor control, [c]$p<0.05$, [d]$p<0.01$ and [e]$p< 0.001$ between tumor control and treated groups. LME – methanolic leaf extract; BME: methanolic stem bark extract;

The treatment with methanolic extracts of *H. mystax* at 500 mg/kg body weight caused variation in the levels of CAT ($p<0.05$) and SOD ($p<0.05$) and significant decrease in the level of TBARS ($p<0.001$) when compared with the tumor control animals. Similar significant ($p < 0.05$) results were observed in the case of SOD for 250 mg/kg body weight. In the liver and kidney tissues the inoculation of DLA cells showed a significant ($p < 0.05$) increase in the levels of TBARS and a significant decrease in the levels of CAT ($p<0.05$) and SOD ($p<0.05$) when compared with normal animals. The treatment with the methanolic extracts of *H. mystax* at both doses and the standard 5-FU exhibited a reversal of all these parameters towards the normal group. Most of the readings were found to be significant.

Discussion

The results of the present study clearly revealed that the tumor inhibitory activity of methanolic leaf and stem bark crude extract against DLA strain. Multidisciplinary scientific investigations are making the best efforts to combat diseases, but the sure-shot, perfect cure is yet to be brought into world medicine. An alternative solution to western medicine embodied with severe side effects is the use of medicinal plant preparations to arrest the insidious nature of the disease. Ascitic fluid is the straight nutritional basis for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional necessity of tumor cells (Prasad and Giri, 1994). Plants have served as a good source of antitumor agents. In this study we have observed the average life span of DLA induced tumor control 18.0±0.22 days. However mice treated with extracts showed significant increases in the life span of the mice indicating the anticancer activity of the extract, and this could be due to free radical scavenging and antioxidant properties of the extract. In this study, increased life span of the mice was observed, but it was not statistically significant. The packed cell volume was found to be significantly less than the tumor control animals, which is also considered to be the indication of the anticancer nature of the extract.

The percent increase in viable tumor cell was found to be significant in control group animals. The reversible of total WBC count by the extract treatment indicates the protective action of hepatopoitic system. However, the elevation in the

level of WBC may be due to its adverse effects on hematopoitic systems (Bansal et al., 2005). On treatment with *H. mystax* extract on reversal effect of RBC count and Hgb content towards the near normal groups indicate the protective action on the hematopoitic system which could be due to free radical scavenging and membrane stabilizing properties of the extract.

Mammalian cells possess elaborate defense mechanisms for radical detoxification. The key metabolic steps are the superoxide dismutase catalysis of the dismutation of super oxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 into water and oxygen by catalase and glutathione peroxidase, which destroys toxic peroxides. In addition to antioxidant enzymes, several small-molecule antioxidants play important roles in the antioxidant defense systems. These can be divided into compounds made *in vivo*, and compounds obtained from diet. In the present study also it was observed that treating mice with total extracts of *H. mystax* increased the activity of all antioxidant enzymes examined, including SOD and CAT. These enzymes are modulated in various diseases by free radical attack (Ames, 1998). Thus, maintaining the balance between the rate of radical generation and the rate of radical scavenging is an essential part of biological homeostasis. Among the plant extract used in this study, extracts of *H. mystax* were found to activate CAT to a greater extent than SOD, which suggests that the H_2O_2 formed by SOD can be effectively scavenged. These results suggest that the antioxidant activity of extracts of *H. mystax* which may be due to the degradation of H_2O_2 and other peroxides.

Numerous plant constituents have proven to possess free radical scavenging or antioxidant activity (Aruoma and Cuppett, 1997). Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines, etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Formica and Regelson, 1995). Reactive oxygen species (ROS) have been implicated in many human degenerative diseases including aging, cancer and neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases (Cheeseman and Slater, 1993). Free radicals have important roles in source lipid peroxidation and DNA damage in cells. Recently, considerable effort has been directed towards identifying naturally occurring substances that can protect oxidative stress. Natural antioxidants contain a broad range of biological actions, as well as inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential. Free radicals are known to cause injury of the liver, and many antioxidants are shown to shield the liver against hepatotoxicants (Badami et al., 2005; Chu et al., 2000). The elevation in the lipid peroxidation is associated with cancer thiobarbutric acid (TBARS) is an indicator of oxidative stress, reported to be higher in cancer condition than the normal levels. TBARS levels in the circulations in tumor control mice found to be higher than the normal control mice. Treatment with methanolic extract of leaf and stem bark showed significant reduction in TBARS levels in serum and tissues. These indicate the reduction in free radical yield and subsequent decrease in harm and damage to cell membrane and TBARS formation. The study reveals that the methanolic leaf extract of *H. mystax* could be used as antioxidant and antitumor drug.

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