ANTI-TUMOR POTENCY AND TOXICOLOGY OF AN INDIAN AYURVEDIC PLANT, *HYGROPHILA SPINOSA*

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Summary

This study was designed to assess the potential anti-tumor effect of the administration of the hydroalcoholic extract of *Hygrophila spinosa* (HEHS), on 7,12 – Dimethylbenz(a)anthracene (DMBA) – induced mammary tumors in female rats and its sub-acute toxicity evaluation. In the sub-acute toxicity exposure of 28 days, HEHS showed no significant change in body weight, organ weight and serum biochemical parameters which established its low toxicity profile. Besides this the LD_{50} was also calculated and was found to be 3020mg/kg by graphical method. Tumor sizes were determined by palpation, comparing the volume of each tumor to that of preformed plasticine models. After 12 weeks of induction, the total weights of tumor were calculated. Only animals bearing a suitable tumor load (< 6g) subsequently classified as adenocarcinomas (from histopathological findings) were considered for data evaluation and treated with control, HEHS, tamoxifen for three weeks. The tumor reducing potency was assessed by calculating the reduction in tumor weight. This change was statistically significant in animals receiving 300mg/kg of HEHS. In addition, assessment of estrogen and progesterone receptor (ERα, PR) levels revealed a significant reduction in the percentage of ERα and PR positive tumors in extract treated animals when compared to controls.

Keywords: *Hygrophila Spinosa*; whole plant extract; female wistar rats; anti-tumor potency; acute and sub acute toxicity.

Introduction

The chemical carcinogen, 7,12- Dimethylbenz[a]anthracene (DMBA) induced mammary gland tumor in rodent has been widely used as an animal model for development of chemopreventive drugs for breast cancer in humans (1). Recently, a greater emphasis has been given towards the researches on complementary and alternative system of medicine that deals with cancer management. Several studies have been conducted on herbs under a multitude of ethno botanical ground (2).

Hygrophila spinosa (Family Acanthaceae), commonly known as 'Talmakhana'(Hindi) or 'Hydrophilia'(English), is a robust, erect, annual herb 60-120 cm in height with stems sub-quadrangular thickened at nodes, leaves 5-20 cm long, oblanceolate, with yellow spines in the axils, fruits 3cm long, oblong, glabrous capsules, 4-8 seeded with a diameter of 0.2-0.3cm and indigenous to India, Sri Lanka, Myanmar, Indo-china, Malaysia (3). Roots are used as diuretic and employed for Jaundice, dropsy, rheumatism, anasarea (4). The fruits of the plant are used for curing menorrhagea, while the roots for treating stomach tumors and snake bites by the tribal of Orissa, India (5). In view of the above information and folklore use of this plant as an anti-tumor agent, the present study was undertaken to evaluate the tumor reducing property of the whole plants of *Hygrophila spinosa* on chemical carcinogen (DMBA) induced mammary gland tumor model in female wistar rats. Its LD_{50} determination and sub-acute toxicological assessment were also evaluated to establish the low toxicity profile.

Material and Methods

Plant Material Collection and Preparation of Extracts

Fresh plants of *Hygrophila spinosa* were collected in the month of October 06 from the thick forest areas of Similipal biosphere reservoir, Mayurbhanj district of Orissa. Taxonomic identification was performed by Dr. N. K. Dhal, Scientist, Department of Natural products, Regional Research Laboratory (RRL), Bhubaneswar and the voucher specimen was deposited in the herbarium vide access no. 9997.

The whole plant parts were washed thoroughly with tap water and air dried in shade at room temperature. They were then mechanically powdered and sieved. 1000gm of powdered plant material was macerated in ethanol-water by soxhlation and dried in a rotary evaporator at 300C. The extractive yield was found to be 14.24% for hydroalcoholic extract of *Hygrophila spinosa* (HEHS).

Phytochemical Screening

A preliminary phytochemical screening was carried out for the extract employing the standard procedures to reveal the presence of alkaloids, steroids, terpenes, flavonoids, saponins, tannins, glycosides, carbohydrates, phytosterols and proteins (6, 7).

Animals Used

Fifty days old sexually matured wistar female rats (100 – 150gm) from M/s Ghosh Enterprises, Kolkata were housed in polypropylene cage with three animals in each cage and acclimatized for a period of 10 days, with 12h light –dark cycles, ambient temperature of 22 ±2 °C and relative humidity of 65% with free access to standard pellet diet and water *ad-libitum*. The experiment was carried out in between 10.00h to 17.00h. Approval for the study was obtained from the institutional animal ethical committee (Regd. No. 621/02/ac/CPCSEA).

Drug Formulations

For oral administration, suspensions of 300mg/kg b.wt. of the hydroalcoholic extract was prepared by triturating the accurately weighed quantity of the extract with

0.3% w/v sodium Carboxy methyl cellulose (Na CMC) in a mortar, with the gradual addition of water for injection, to make up the required volume. The drug formulations were prepared every 3rd day during drug therapy. The drugs were administered orally by using a feeding tube.

Acute Toxicity Study

The acute toxicity of HEHS was performed as described by Graphical method (8). Different doses of 2, 2.5, 3, 3.5 and 4g/kg were administered orally to the animals of five groups, each containing four animals. The toxicological effect was assessed on the basis of mortality after 24h, which was expressed as an LD_{50} value. The percentage of mortality was converted to probits and the values were plotted against log dose. The LD_{50} was the dose intersected by probit 5.

Sub-acute Toxicity Evaluation

To determine the preliminary toxicity, 3 groups of wistar female albino rats (n=6) were taken. Group I, II & III were administered orally, 0(control), 300 and 600mg/kg of HEHS respectively, in every 24h for 4 weeks. The control group received 0.3% w/v Na carboxy methyl cellulose in an identical manner. During the period of administration, the animals were weighed; food and water intake were monitored. The body weight changes were recorded on day 0, 10, 20 and day 28 with simultaneous observation of toxic manifestation and mortality. At the end of 28-day period, the animals were sacrificed by decapitation. The vital organs like heart, liver, lung, kidney, and spleen were carefully dissected out and weighed. Portion of each fixed in 10% neutral formalin for histopathological investigations.

Biochemical Parameters Analysis in Serum, Plasma and Liver

Glutamate pyruvata transaminage, glutamate oxaloacetate transaminase (9), alkaline phosphatase (10) assays were carried out in serum and liver tissue homogenate. Plasma and tissue lipid profiles: cholesterol (11), phospholipids (12) and triglyceride (13) were also determined.

DMBA Induced Breast Carcinogenesis

Wistar female rats were orally intubated with a single dose of 50mg/kg body weight of DMBA (Sigma chemicals, St louis, MO), suspended in olive oil using a feeding tube. The first mammary tumors were detected 20 – 30 days later. Animals were palpated weekly and the locations of tumors were noticed. Tumors sizes were determined by palpation, comparing the volume of each tumor to that of preformed plasticine models. Tumor sizes were calculated using the formula: $\pi/6 \times$ length (cm) $\times$ width^2 (cm) (14, 15). Then, the total weight of the tumors were calculated (assuming a density of 1gm/ml) after 12 weeks of induction. The animals having suitable tumors (<6 g wt.) were randomly divided into three groups consisting of ten animals per group. Group 1 received vehicle (0.3% Na CMC); Groups 2 and 3 received HEHS 300mg/kg and Tamoxifen (50μg/day) respectively for a period of four weeks. During the treatment period the animals were subjected for calculation of tumor weight in each week. At the end of 4th week of treatment period, the animals were sacrificed for ethical reasons. At the time of necropsy all tumors were removed, fixed in 10% buffered formal saline and subsequently

dehydrated and blocked in paraffin. The paraffin block was cut into 5µm sections, fixed on slides and processed for light microscopy (stained with hematoxylin and eosin) or immunohistochemistry.

Immunohistochemical Analysis

Cells expressing progesterone and estrogen receptors were identified after 1h incubation at room temperature by using the rabbit polyclonal antibody PR at 1:50 dilution and the rabbit polyclonal antibody ERα at 1:80 dilution, respectively. Tissue slices were then incubated for 30min with the anti-rabbit mouse/human adsorbed Biotin conjugated antibody at 1:300 dilution, treated with the avidin-peroxidase complex and the product of the reaction was revealed by incubation with 3-amino-9-ethylcarbazole. Positive cells from control and treated samples were identified in a count from five randomly selected fields each containing at least 300 histologically identified neoplastic cells. Immunoreactivity was determined by two independent observers according to a simplified scoring system: cases were rated negative if none of the cells within the lesions were stained. Evaluation was performed on 13 tumors from both control and treated animals (16, 17).

Statistical Methods

Statistical analysis was carried out using One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. *P* <0.05 was considered to be statistically significant.

Results

Phytochemical Screening

Phytochemical screening of the hydroalcoholic extract of *Hygrophila spinosa* revealed the presence of compounds like flavonoids, phenolics, carbohydrates, phytosterols, tannins, fixed oils and proteins.

Acute Toxicity Evaluation

From the acute toxicity study data it was found that at the dose level of 2000mg/kg there was no mortality and at 3000mg/kg all the animals were dead. The LD_{50} was also determined from the acute toxicity data and was found to be 3019.95mg/kg (Table 1, Fig.1).

Table.1. Determination of LD_{50} values for the hydroalcoholic extract of *Hygrophila spinosa*

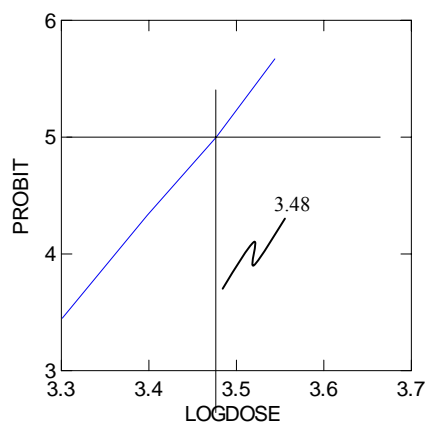| Sl. No.	Dose (mg/kg body wt.)	Log dose	Percent mortality (after 24 h)	Corrected mortality (%)	Probit
1	2000	3.301	0	6.25	3.45
2	2500	3.398	25	25	4.33
3	3000	3.477	50	50	5.00
4	3500	3.544	75	75	5.67
5	3000	3.602	100	93.75	6.55

Fig.1. Determination of LD_{50} value for the hydroalcoholic extract of *Hygrophila spinosa* administered to rats for 24h, using a graphical method. LD_{50} = log dose 3.48 = 3019.95mg/kg ~ 3020mg/kg.

Sub-acute Toxicity Evaluation

In sub-acute toxicity studies, the results as illustrated in fig. 2 showed non significant ($p > 0.05$) change in body weight gain when compared with group I (control). Gross necropsy findings did not show any adverse effects in any organ. No statistically significant difference in organ weights were present in any of the female rats receiving the extracts at the therapeutic dose (300mg/kg) and a dose double than that (600mg/kg). More ever, no lethality was recorded for any dose up to the maximum of 600mg/kg body wt of the extracts during 28 days of treatment. No target organs were identified by gross pathological examination in animals of the high-dose group and histopathological examination was therefore performed on animals in the control group and high dose group only. No histopathological change was noted in the high-dose groups as compared to control. Table 3 depicts the activity of marker enzymes (GPT, GOT, alkaline phosphatase) in serum and liver tissue. No significant changes were observed in the enzymes' activity in all groups tested. Table 4 represents the level of plasma and tissue lipid profiles in experimental and control animals. There is non significant changes in cholesterol, phospholipids and triglyceride levels of plasma and liver when compared with group I.
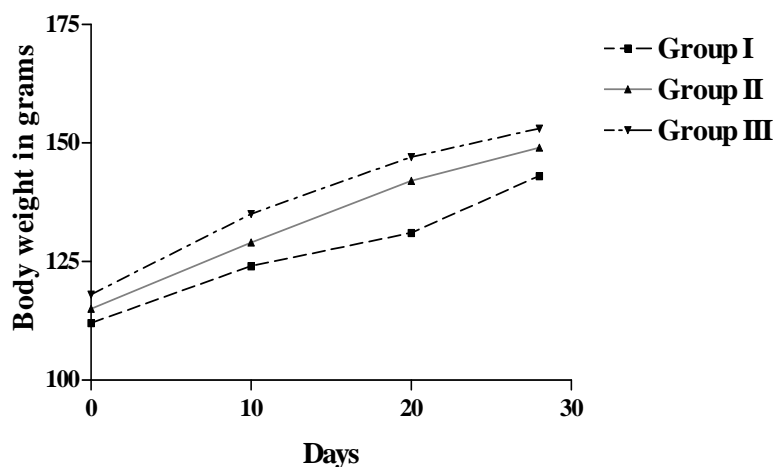
Fig. 2. Effect of *Hygrophila spinosa* on body weight changes in female rats. Group I – Control, Group II – 300mg/kg HEHS treated and Group III – 600mg/kg HEHS treated.

Table. 2. Relative weight of vital organs of female rats with respect to body weight after 28-days oral exposure to *Hygrophila spinosa*

Parameters(g/100g of body weights)	Group I (Control)	Group II (300mg/kg HEHS)	Group III (600mg/kg HEHS)
Heart	0.343 ± 0.003	0.357 ± 0.003^{ns}	0.354 ± 0.004^{ns}
Kidney	0.813 ± 0.17	0.81 ± 0.13^{ns}	$0.78 \pm 0.24^{*}$
Lung	0.421 ± 0.03	0.42 ± 0.002^{ns}	0.433 ± 0.07^{ns}
Liver	3.981 ± 0.91	$4.033 \pm 0.008^{*}$	$4.094 \pm 0.45^{*}$
Spleen	0.34 ± 0.05	0.33 ± 0.04^{ns}	0.33 ± 0.001^{ns}
Mortality (%)	Nil	Nil	Nil

Values are expressed as mean ± S.D. for six rats. Comparisons were made between group I with II and III. $^{*}p<0.05$, ns = not significant.

Table. 3. Effect of *Hygrophila spinosa* on serum, liver enzymes activity in control and experimental groups

Parameters	Group I (Control)	Group II (300mg/kg HEHS)	Group III (600mg/kg HEHS)
Serum (units/ml)			
GPT	26.54±5.4	27.23±4.8*	26.67±4.6^{ns}
GOT	34.28±2.98	34.21±6.04^{ns}	34.16±4.41^{ns}
Alkaline phosphatase	0.015±0.017	0.018±0.011^{ns}	0.019±0.003^{ns}
Liver (units/mg protein)			
GPT	171.6±19.2	168.43±13.6**	164.12±12.3**
GOT	36.02±5.34	37.89±4.80*	39.91±6.31**
Alkaline phosphatase	0.022±0.005	0.021±0.004^{ns}	0.017±0.003*

values are expressed as mean ± S.D. for six rats. Comparisons were made between group I with II and III. $^{*}p<0.05$, $^{**}p<0.01$, ns = not significant.

Table. 4. Effect of *Hygrophila spinosa* on plasma and liver lipids level in control and experimental groups

Parameters	Group I (Control)	Group II (300mg/kg HEHS)	Group III (600mg/kg HEHS)
Plasma (mg/dl)			
Cholesterol	59.08±5.47	54.18±6.24[**]	53.22±5.21[**]
Phospholipids	106.20±2.98	102.91±8.06[*]	104.08±7.01[*]
Triglycerides	6.32±0.61	5.52±1.42[**]	5.36±1.87[**]
Liver (mg/g wet tissue)			
Cholesterol	3.45±0.89	3.11±0.47[**]	3.02±0.69[**]
Phospholipids	3.54±0.78	3.19±0.91[**]	3.21±1.21[**]
Triglycerides	6.22±0.28	5.99±1.09[*]	5.93±0.65[*]

Values are expressed as mean ± S.D. for six rats. Comparisons were made between group I with II and III. [*]$p<0.05$, [**]$p<0.01$.

DMBA Induced Breast Carcinogenesis

Fig. 3 presents the results of the antitumor activity for the chemical induced breast carcinoma of the extract revealed promising activity by reducing the tumor size significantly (*p* value between 0.001 to 0.05) on day 14, 21 and day 28 when compared to untreated group I. The activity is comparable with the standard drug tamoxifen, when it is compared after two weeks. The most widely used prototype drug for breast cancer showed significant ($p < 0.01$) activity after one week of the treatment period. The histological classification of mammary adenocarcinomas did not reveal any differences among the groups on the distribution of various tumor subtypes (data not shown). On contrary, when considering the degree of tumor differentiation, a reduction in the percent of poorly differentiated tumors, with a concominant increase of the well-differentiated ones, was observed in treated animals (extract and tamoxifen).
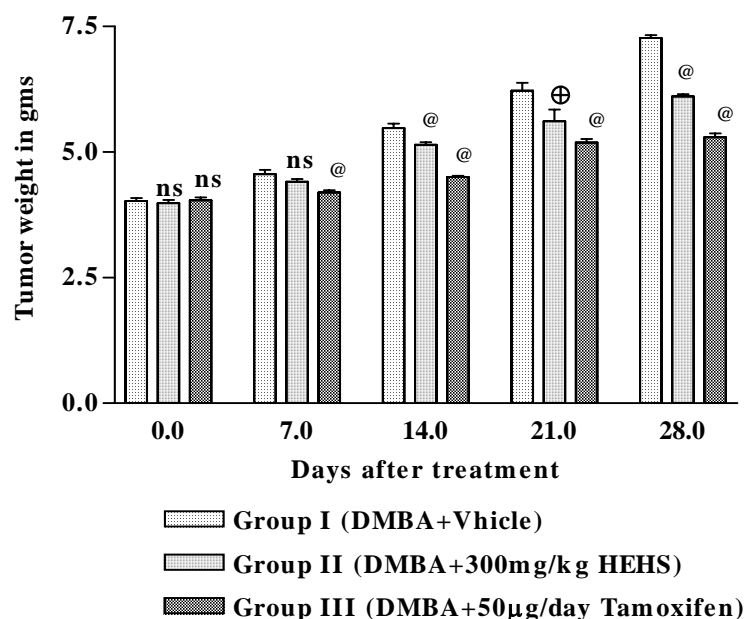
Fig. 3. *Hygrophila spinosa* hydroalcoholic extract protects the growth of breast tumor in Carcinogen(DMBA)-induced rats, treated for a period of four weeks after the suitable growth of tumors (< 6g wt). Columns are the mean of each group of ten rats; bars, S.E.M. Comparisons were made in each week up to the end of fourth week between Group I (induced control) with Group II (HEHS treated) and Group III (Tamoxifen treated) ; ns: non significant difference, [@]: $p < 0.01$, [⊕] : $p < 0.05$.

Immunohistochemical analysis

Results from immunohistochemical analysis revealed that 10/13 (76.9%) of control masses, 7/13(53.8%) of HEHS and 3/13(23.1%) of 50μg/day tamoxifen treated tumors were ERα positive, while 5/13 (38.56%) control, 2/13(15.4%) of extract treated and 1/13(7.7%) tamoxifen treated tumors were PR positive. The pattern of immunohistochemical staining for ERα is demonstrated in Fig.4.
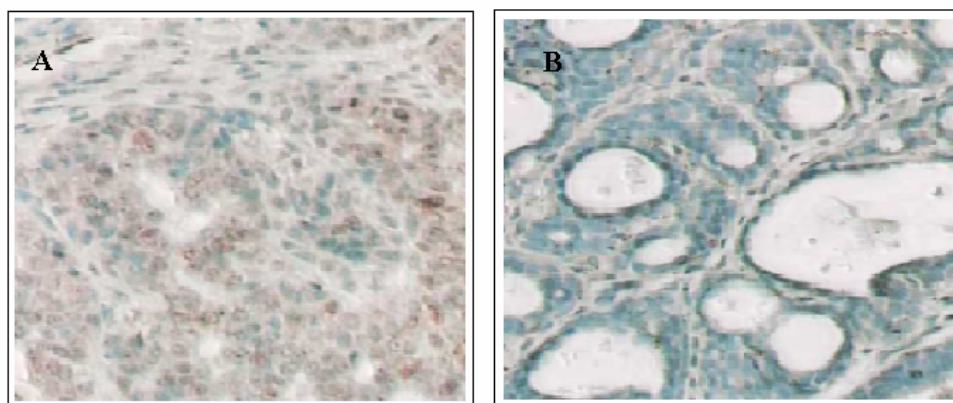

Fig.4. Immunohistochemical staining for ERα showing (A) numerous positively stained (brown) cells in a tumor from a control animal and (B) absence of stained cells in a tumor from a HEHS (400mg/kg) treated animal.

Discussion

DMBA-induced mammary gland tumor in rodent has been widely used as an animal model for development of chemo-preventive drugs for breast cancer in humans (1, 15). Data of the present experiment indicate that daily oral intake of *Hygrophila spinosa* extract could prevent or delay the development of breast cancer in the rats. The effect is significant as it decreases the size of tumors induced by the carcinogen. We also found that the percentage of ERα positive tumors was significantly lower in animals receiving HEHS with respect to control. HEHS may affect cancer development and growth via various mechanisms. A possible mechanism is that it selectively reduces the pre-cancerous cells formation due to the presence of specific phenolics. The presence of flavonoids (from preliminary phytochemical screening) also potentate the activity against breast cancer as flavonoids have important effects on cancer chemoprevention and therapy, the data were previously proved in vitro, in vivo, epidemiological investigation and human clinical trial. Flavonoids may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen detoxifying systems (18, 19, 20). The results suggest that the hydroalcoholic extract of *Hygrophila spinosa* which posses low toxicity could be used in neoplasm particularly when many anticancer drugs, currently in use, have limited application due to their inherent high toxicity.

Acknowledgements

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