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Bacopa monniera extract inhibits tumor promotion in fibrosarcoma bearing rats

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Summary

Objective: To investigate the tumor inhibitory effect of the ethanolic extract of *Bacopa monniera* (*B. monniera*) in 3- Methylcholanthrene (3-MC) induced fibrosarcoma bearing rats. Methods: 3-MC induced fibrosarcoma in male Wistar rats was used to investigate the tumor inhibitory property of *B. monniera* (20mg/kg, sc, 30 days). Tumor inhibitory property was assessed by studying the mean survival time, body weight and tumor weight changes, activity of the tumor markers like gamma glutamyl transferase (GTT), cathepsin-D, ceruloplasmin, levels of circulating immune complexes (CIC) and histopathological changes. Results: Fibrosarcoma bearing rats exhibited decreased mean survival time (40.45 ± 4.5 days) and body weight (57.66± 4.5 gms) with increased tumor weight (12.36 ± 0.32 gms), $p<0.01$. Tumor markers like GTT (5.84± 1.13 IU/L, $p<0.01$) cathepsin – D (7.08 ± 1.29 nmoles of tyrosine liberated/min/mg protein, $p<0.01$), ceruloplasmin (2.95±0.41 mg/dl, $p<0.05$) and CIC (334.6 ± 4.63 OD x 10^3 , $p<0.05$) were increased. Histological assessment revealed fibrosarcoma tumor with a typical herring bone pattern. *B. monniera* treated rats exhibited enhanced mean survival time (54.5 ± 3.9 days), body weight (80 ± 6.2 gms), and decreased tumor weight (5.34± 0.22). GTT (3.13 ± 1.05, $p<0.01$), cathepsin-D (5.75 ± 0.28, $p<0.01$), ceruloplasmin (1.59 ± 0.20, $p<0.05$) and CIC (267 ± 12.98, $p<0.05$) were found to be lowered. Sections of tumor tissue revealed inhibited tumor progression. Conclusion: The ethanolic extract of *B. monniera* inhibits tumor progression in fibrosarcoma bearing rats.

Key Words: 3-Methylcholanthrene, Fibrosarcoma, *Bacopa monniera*, Circulating Immune Complexes (CIC).

1. Introduction

Better anti-tumor drugs with minimal disadvantages and maximum therapeutic benefits in cancer cure and prevention are still being researched for [1] and drugs resourced from plants have been successfully used in cancer therapy [2]. *Bacopa monniera* (Linn) syn. *Herpertis monniera* Linn (Scrophulariaceae) is a herbaceous plant traditionally used from time immemorial in Ayurvedic and folklore medications. Ayurvedic system of medicine describes the usage of this plant in the treatment of various conditions like neuralgia, epilepsy, insanity, tumors, ulcers, splenomegaly, inflammation, biliousness,

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amentia, ascites and asthma [3, 4]. *B. monniera* consists of saponins (Steroidal glycosides, Dammarene triterpenoid glycosides, Triterpenoid glycosides), alkaloids (Brahmine, Nicotine, Herpestine), flavanoids (Luteolin, Luteolin-7-glycoside) and phytosterols (ß-sitosterol, ß-stigmasterol) [5].

Experimental investigations report that the ethanolic extract of *B. monniera* exhibits antioxidant activity [6]. Anticancer activity of *B. monniera* extract has been evidenced in Walker carcinoma *in vivo* [7] and S-180 cells *in vitro* [8]. Bacoside - A in the ethanolic extract of *B. monniera* has been reported to exhibit maximum cytotoxic activity in Brine shrimp assay [9]. Prior studies from our lab point out that it enhances the antioxidant status in fibrosarcoma bearing rats [10]. Based on these reports the present study aims to investigate the tumor inhibitory property of *B. monniera*.

Experimentally induced fibrosarcoma serves as a convenient model to study the efficacy of plant drugs in preventing the progression of solid tumors and mimics human tumors. It is characterized by a rapid proliferation of immature fibroblasts with scanty collagen and abundant reticulum fibrils. Histologically, cells with a high mitotic activity and interlacing bundles in herring bone pattern are observed [11, 12]. Therefore the tumor inhibitory property of *B.monniera* was chosen to be studied using 3-MC induced fibrosarcoma in rats by studying the mean survival time, body weight and tumor weight along with the assessment of markers of tumor progression such as GTT, cathepsin-D, ceruloplasmin, CIC and histopathological changes.

2. Materials and methods

3-MC was purchased from Sigma Chemicals Co. (St. Louis, USA). All other chemicals used were of analytical grade.

2.1 Animals

The experimental animals were purchased from the Tamil Nadu University of Veterinary and Animal Sciences (TANUVAS) and housed under standard laboratory conditions. The animals were maintained with a pellet diet (Hindustan Lever Limited) and water *ad libitum*. The experiments were performed on male Wistar strain albino rats weighing 80-110 g. Four groups of animals, each group comprising of six animals, were taken for the study. Group I consisted of normal rats as control. Group II consisted of fibrosarcoma bearing rats. Group III was *B.monniera* administered normal rats serving as drug control (20mg/kg body weight, sc). All the animal experiments adhered to the guidelines of the Institutional Animals Ethics Committee.

2.2 Preparation of B. monniera extract

The whole plant of *B. monniera* was collected from the areas around Chennai in the month of February-March. A voucher specimen of the plant has been deposited in the Department of Botany Drug Research, Captain Srinivasa Murthi Research Institute for Ayurveda, Chennai. The shade dried, coarsely powdered plant material (1 kg) was soaked in 90% ethanol (5L) in cold (48 hours). The extract was stirred, filtered, and distilled on a water bath to a syrupy mass, and the last traces of the solvent were removed under vaccum (yield 50 gms). The extracted material was subjected to a similar treatment twice and the filtrates were mixed together and lyophilized. The lyophilized extract suspended in physiological saline was administered to the experimental animals.

2.3 Dosage fixation

Dose–dependent effects of the ethanol extract of *B.monniera* (5-160mg/kg body weight), administered subcutaneously, were analyzed for the effective dosage. At a period of 30 days, a dosage of 20 mg/kg body weight exhibited

effective results while a dosage beyond 80 mg/kg body weight produced no mortality but revealed weakness, weight loss and lethargy which were reversible with the withdrawal of the extract. From the preliminary study a minimum effective dosage of 20mg/kg body weight, for a period of 30 days, subcutaneous administration, was fixed for further experiments.

2.4 Induction of fibrosarcoma

Fibrosarcoma was induced experimentally in rats weighing 80-110g by implanting subcutaneously, Millipore filters (150mm^2) soaked with 5% 3-MC in paraffin oil, according to the method of Nagarajan *et al*. [13]. A series of tumor transplanted animals were maintained by subcutaneous injections of 2 x 10^6 cells/ml tumor tissue in the right flank using a hypodermal syringe. A palpable tumor was detected from about the 7th – 10th day of transplantation and the treatment schedule for the experiments commenced from the 10th day.

2.5 Experimental study and methods

After the experimental period of 30 days the animals were ether anaesthetized and blood was collected from the external jugular vein. Serum was separated by centrifugation at 4°C and used for the biochemical studies.

2.6 Assessment of mean survival time, body weight and tumor weight

The survival period in the group II and group IV animals and the body weight changes in all the four groups of animals were recorded. Growth of subcutaneous tumor was followed by caliper measurements of two perpendicular diameters and the tumor mass was assumed to approximate a prolate ellipsoid with the volume equal to length (mm) x width (mm)^2 / 2 [14]. Tumor weights obtained from vernier caliper measurement and the actual weight measurements were found to be nearly the same and hence tumor weight was taken in the present study.

2.7 Assay of tumor markers

Sera was used for the assay of markers like GTT, cathepsin-D, ceruloplasmin and the levels of CIC using the methods of Rosalki & Tau (1972) [15], Sapolsky *et al*., (1973) [16], Ravin (1961) [17], and Digeon *et al*., (1973) [18] respectively.

2.8 Histopathological study

Sections of tissues (5µm) from the group I and group III control animals, tumor tissue from Group II and Group IV *B.monniera* treated fibrosarcoma bearing animals in 10% buffered formalin was taken for the histopathological studies.

2.9 Statistical analysis

Statistical significance was analyzed using one way ANOVA followed by Bonferroni multiple comparison method. P values<0.05 and <0.01 were considered statistically significant.

3. Results

Fig.1 shows the effect of the ethanolic extract of *B.monniera* on the mean survival period monitored for 60 days. No mortality was observed in the control group I animals while the fibrosarcoma induced group II animals showed reduced survival time (40.5 ± 4.5 days, $p<0.01$). The group III *B. monniera* administered animals exhibited non significant changes (60 days ± 4.6, NS) whereas the group IV *B. Monniera* treated fibrosarcoma bearing animals exhibited a marked increase in survival period (54.5 ± 3.9 days, $p<0.01$). Fig.2 represents the body weight of the experimental group of animals on the 0th and 30th day of the treatment regime. The control group I animals recorded a normal weight gain pattern at the end of the 20th day (121 ± 9.8g). The group II fibrosarcoma bearing animals recorded only 57.66 ± 4.5 g ($p<0.01$) but the group IV *B. monniera* treated fibrosarcoma bearing animals showed 80 ± 4.6 g at the 30th day ($p<0.01$).

The group III *B.monniera* administered animals showed non significant changes from the control group I animals (119.25 ± 3.9g). The tumor weight differences on the 0th and 30th day of the treatment period are presented in Fig3. The group II fibrosarcoma bearing animals had a tumor mass weighing around 12.36 ± 0.45 ($p<0.01$) while the *B.monneira* treated fibrosarcoma bearing animals exhibited a mass of 5.12 ± 0.23 g on the 30th day ($p<0.01$).

A significant increase in the activities of GTT (5.84 ± 1.13 IU/L, $p<0.01$), cathepsin-D (7.08 ± 1.29 nmol of tyrosine liberated/min/mg protein, $p<0.01$), ceruloplasmin (2.95 ± 0.41 mg/dl, $p<0.01$) and CIC (334.6 ± 4.63 OD x 10^3, $p<0.05$) in the sera of fibrosarcoma bearing animals of group II was observed , as shown in Table 1. The *B. monniera* extract treated group IV animals showed a reduction in GTT (3.13 ± 1.05, $p<0.01$), cathepsin-D (5.75 ± 0.28, $p<0.01$), ceruloplasmin (1.59 ± 0.20, $p<0.05$) and CIC (267 ± 12.98, $p<0.05$) markers. No significant change was observed in the group III *B. monneira* treated animals when compared to the control group I animals. The histological sections taken from the right flank of the group I and group III normal animals (A) and (B), tumor tissue from the group II (C) and *B. monniera* treated group IV fibrosarcoma animals (D) are shown in Fig 4. Sections from the group II animals (B) revealed well differentiated fibrosarcoma with a typical herring bone pattern

Table 1. Active constituents of *B. monniera*

Saponins	Steroidal glycosides
	Dammarene triterpenoid glycosides
	Triterpenoid glycosides.
Alkaloids	Brahmine
	Nicotine
	Herpestine
Flavanoids	Luteolin
	Luteolin - 7 - glycoside
Phytosterols	β-Sitosterol
	β-Stigmasterol

Table 2. Effect of *B. monniera* on the tumor markers

Parameters	I	II	III	IV	F value
GTT (IU/I)	2.26 ± 0.38	5.84 ± 1.13 [a]*	3.36 ± 0.69	3.13 ± 1.05[@]*	17.53
CATHEPSIN - D (n moles of tyrosine liberated/min/mg protein)	5.15 ± 0.31	7.08 ± 1.29 [a]*	6.3 ± 0.28	5.75 ± 0.28[@]*	88.02
CERULOPLASMIN (mg/dl)	1.48 ± 1.11	2.95 ± 0.41[a]*	1.67 ± 0.60	1.59 ± 0.20[a]*	6.35
CIC (ODX10^3)	218 ± 7.92	334.6 ± 4.63[a]*	221.16 ± 11.21	267.12.98[a]*	36.29

Significance was assessed using One-Way ANOVA followed by Bonferroni multiple comparison test between groups I Vs II and groups II Vs IV. Values are expressed as mean ± SD. $P<0.05$ is denoted as a* and $p<0.01$ is denoted as @*.

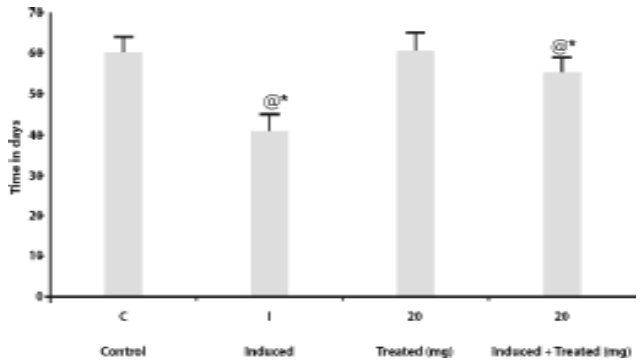

Fig. 1: Effect of *B. monniera* on the Mean Survival Time in control and experimental animals

Values are mean ± SD with six animals. Significance assessed by One way ANOVA followed by Bonferroni multiple comparison test. $p<0.01$ is denoted as @*.

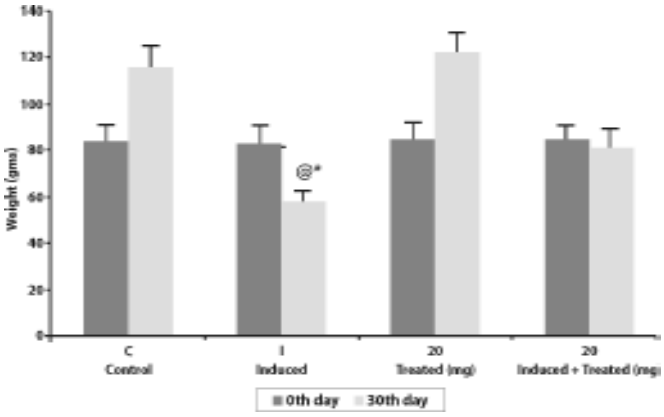

Fig. 2: Effect of *B. monniera* on body weight changes in control and experimental animals

Values are mean ± SD with six animals. Significance assessed by One way ANOVA followed by Bonferroni multiple comparison test. $p<0.01$ is denoted as @*.

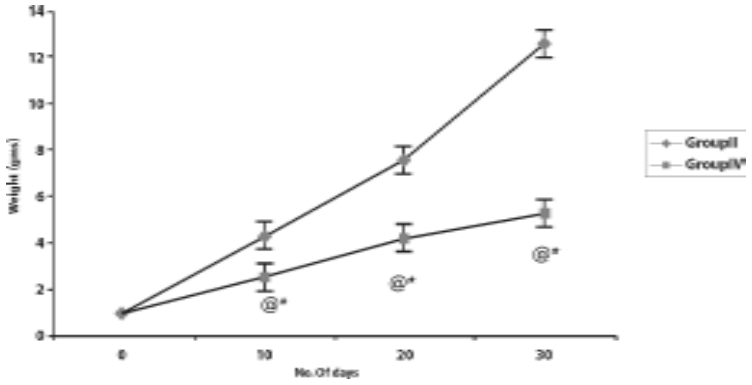

Fig. 3: Tumor Weight in *B. monniera* treated fibrosarcoma bearing rats

Values are mean ± SD with six animals. Significance assessed by One way ANOVA followed by Bonferroni multiple comparison test. $p<0.01$ is denoted as @*.

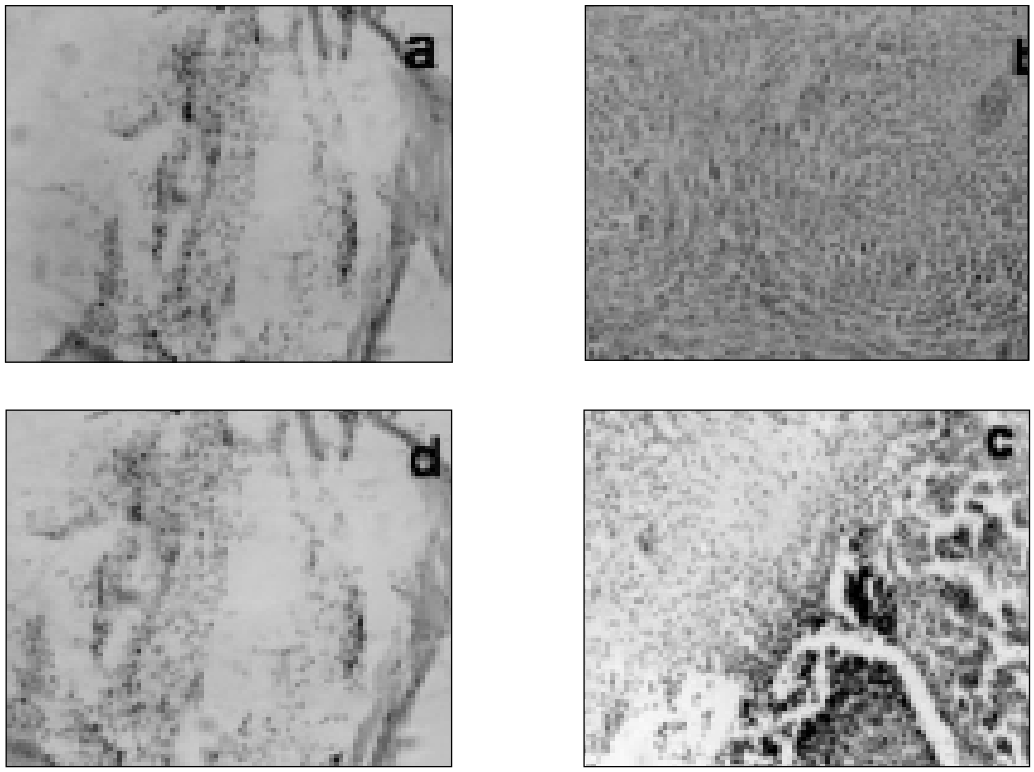

Fig. 4: Histopathological Changes in the Tumor Tissue
a, d - Normal architecture of control Group I and BM administered Group III animals
b - Section of fibrosarcoma in Group II animals
c - Section of fibrosarcoma in Group IV BM treated animals

while those from *B. monniera* treated group IV animals (C) presented a small cluster of tumor cells with the presence of fibroblasts and connective tissue. *B. monniera* extract administered group III animals showed normal architecture as group I control animals. (A).

4. Discussion

Reduced survival time, increased tumor weight and decreased body weight are all indices of cancer cachexia [19]. Hence significant improvements in the mean survival time, and body weight with a reduction of tumor weight indicate positive prognosis in cancer treatment. Enhancement in the survival time and body weight with decreased tumor weight in the *B. monniera* treated fibrosarcoma bearing rats of group IV of the present study indicate that *B. monniera* may inhibit tumor progression. The nontoxic nature of *B. monniera* has already been established in different studies through oral and intraperitoneal routes [20]. The present study is the first to investigate the effective dosage and toxicity of *B. monniera* upon subcutaneous administration. The lack of significant changes in the mean survival time and the body weight of group III *B.monniera* administered animals indicate nontoxicity.

Gamma glutamyl Transferase (GTT), a key enzyme involved in the cellular glutathione (GSH) homeostasis, is frequently increased in tumor conditions and is over expressed in tumor cells resistant to therapeutic drugs [21, 22]. Hypercupremia is also commonly observed in malignancy and is associated with increase in the levels of copper and the activity of ceruloplasmin [23]. Correlatively, increased activity of GTT and ceruloplasmin are observed in the fibrosarcoma bearing Group II animals of the present study. Cathepsin - D, an acid protease is used to assess the tumor burden [24] and is related to the metastatic status of the tumors. Supportively, results from the present study also exhibit increased cathepsin-D activity in the fibrosarcoma bearing animals of Group II. The *B. monniera* treated group IV animals show a significant and concomitant decrease in the activity of GTT, ceruloplasmin and cathepsin-D. The evidenced decrease in the activity of these enzymes may be due to the active components of *B. monniera*. Several saponins like triterpenoid glycosides and dammarene triterpeniod glycosides exhibit anti-oxidant and antitumor property. Such components have also been identified in the ethanolic fraction of the extract of *B. monniera* [25, 26]. Among its various cytotoxic constituents, Bacoside-A is maximally cytotoxic [10]. The anti tumorigenic nature of the saponin components are attributed to their biological properties such as membrane permeabilisation, selective cytotoxicity and cell aggregation [27, 28]. Therefore, it may be suggested that the anticarcinogenic action of the saponin components of *B. monniera* and the cumulative activation of phytosterols [29], flavanoids [30] and saponins may attribute to the decreased activity of tumor marker enzymes thereby leading to a reduction in tumor weight, increased mean survival time and body weight.

Immunological changes take place in cancer conditions and the levels of circulating immune complexes are found to be elevated in conditions like tumor growth [32]. Similarly, an elevation is also observed in the group II fibrosarcoma bearing animals of this study. Chemotherapy with drugs like cisplatin is observed to bring down the levels of CIC in head and neck cancer patients [33]. A decrease in the levels of CIC in the *B. monniera* extract treated group IV animals in the recent study demonstrates a positive role for the extract in tumor inhibition. Saponins are also known to play a role in immuno stimulation and modulation [34]. Hence, it can be put forward that the components of *B. monniera* may also modulate immune responses in cancer conditions. Histological observations in the treated group IV fibrosarcoma bearing animals also reveal the tumor inhibitory property of *B. monniera*. Further detailed investigations on Bacoside-A, the active component of *B. monniera* as well as the other constituents, will identify the potent antitumor component.

The anticancer components in *B. monniera* inhibit tumor progression in fibrosarcoma bearing rats.

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