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Antiproliferative role of *Indigofera aspalathoides* on 20 methylcholanthrene induced fibrosarcoma in rats

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PEER REVIEW

Peer reviewer

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Comments

In general, the manuscript is well-written and the rationale for the work well described. The methods adopted are robust and the discussion is judicious in view of the results obtained. The work is certainly suitable for publication in *Asian Pacific Journal of Tropical Biomedicine*. I would suggest the following experiments in future after publishing this preliminary report:
1. Try to purify the crude extract and identify the antiproliferative compounds.
2. Do immunohistochemistry (IHC) to see the effect on Ki67 to understand the antiproliferative function of the extract.
3. Find the targets (genes and proteins) of the extract related to antiproliferative markers.
(Details on Page)

ABSTRACT

Objective: To find out the anticancer effect of *Indigofera aspalathoides* (*I. aspalathoides*) on 20-methylcholanthrene induced fibrosarcoma in rats. **Methods:** Fibrosarcoma was induced in Wistar strain male albino rats by 20-methylcholanthrene. Intraperitoneous (*i.p.*) administration of 250 mg/kg body weight/day of aqueous extract of *I. aspalathoides* for 30 d effectively suppressed chemically induced tumors. Parameters such as body weight, liver and kidney weight, tumor weight, mean survival time, behavioral changes, blood glucose, blood glycogen and marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), acid phosphatase (ACP) and 5´-nucleiotidase (5´-NT) in serum, liver and kidney and lipid profiles such as total cholesterol, phospholipids, free fatty acids in liver and kidney of control and experimental animals were studied. **Results:** Fibrosarcoma bearing animals were ferocious and anxious. The mean survival time was found to increase after the treatment. The body weights were significantly decreased ($P<0.001$) in group II fibrosarcoma animals which steadily increased after the treatment with *I. aspalathoides*. The liver and kidney weights were significantly increased whereas the tumor weights decreased as compared to the weights in untreated fibrosarcoma bearing rats. The blood glucose and the liver and kidney glycogen levels were found to decrease significantly ($P<0.001$) in group II animals. Elevated activities of marker enzymes were observed in serum, liver and kidney of fibrosarcoma bearing Group II animals which were normalize after *I. aspalathoides* treatment. In the liver and kidney of Group II animals the total cholesterol increased whereas the phospholipids and free fatty acid levels decreased ($P<0.001$) which were normalized after treatment. **Conclusions:** The treatment by *I. aspalathoides* on fibrosarcoma bearing rats has improved the levels of various parameters indicating its antiproliferative and anticancer activity.

KEYWORDS
Chemoprevention, Tumor weight, Mean survival time, Glucose, Glycogen, Marker enzymes

1. Introduction

In recent times focus on plant research has increased around the world and a large body of evidence has been collected to show the immense potential of medicinal plants used in various traditional systems of medicine.

The practice of native medicine is the synthesis of therapeutic experience of generations of practicing physicians. Herbal medicines have played a great role in the cure of many diseases in the history of mankind.

Herbal medicines play a vital role in the treatment of patients with soft tissue tumors[1]. Recent research revolves around the urgency to evolve suitable chemotherapy consistent with new discoveries for the treatment of cancer without any side effects.

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Chemotherapy being a major treatment modality used for the control of advanced stages of malignancy and as a prophylactic agent against possible metastasis, exhibits severe toxicity on normal tissues[2]. Plants have been used to maintain health and vitality, apart from being sources of medicines. More than 50% of all modern drugs in clinical use are of natural origin and many of them have the ability to control cancer[3].

Methylcholanthrene causes a wide range of tumors in all animal species. This compound belongs to polycyclic aromatic hydrocarbons (PAHs). PAHs are ubiquitous environmental agents commonly believed to significantly contribute to human as well as animal cancers. These chemicals are formed in the process of incomplete combustion of organic materials and, are found widely in the environment, for example, in engine exhaust, cigarette smoke, soil, water and food etc. and thus human exposure to PAHs is unavoidable. Like many other carcinogens, polycyclic aromatic hydrocarbons, like 20-methylcholanthrene are metabolized enzymatically to various metabolites, of which some are reactive. In the large group of enzymes involved in carcinogenic metabolism, 20-MCA has been used as an effective experimental model in the field of carcinogenesis and chemoprevention. In the present study 20-methylcholanthrene was used to induce fibrosarcoma in rats. Soft tissue sarcomas arise from primitive mesenchyma and its differentiative derivatives: fibrous and adipose connective tissues, blood and lymphatic vessels, smooth and striated muscles, facial and synovial structures. Soft tissue refer to the extraskeletal connective tissues of the body that convert, support and surround other anatomical structures such as muscles, tendons and supportive tissues. Soft tissue sarcomas are highly heterogeneous group of malignant neoplasms that can arise from mesenchymal elements anywhere in the body.

Fibrosarcoma is a tumor composed of collagen fibers forming mesenchymal cells of fibroblast and they arise from subcutaneous fibrous tissue[4]. Chemical carcinogenesis is usually a multi stage disease process, comprising of different stages from the conversion of a normal somatic to tumor cell transformation and ultimately, after a long latency period, to a critically manifested malignant tumor. Chemicals cause cancer either directly or more often, after metabolic activation. The organic carcinogens require conversion to an ultimate, more reactive compound. This conversion is enzymatic and/or from conversion of pro carcinogens to their active forms.

Chemoprevention by plants is a promising and novel strategy for inhibition, suppression and reversal of carcinogenesis. It has been suggested that compounds that possess inhibitory effects on cell proliferation and as antioxidants, are considered to be good chemo preventive agents. A large number of active principles from traditional medicinal plants have been reported to have chemo preventive activities[5]. *Indigofera aspalathoides* (*I. aspalathoides*) treatment for rat arthritis is reported by Raj Kapoor *et al*[6]. *I. aspalathoides* was shown to have free radical scavenging activity by Philips *et al*[7]. Plants are loaded with chemicals with chemo preventive activity and some of them are undergoing clinical trials. Most of the studies on chemoprevention are based on individual chemicals with defined mechanism of action and were reported to inhibit carcinogenesis in animal models. As the carcinogenic process is multifactorial, individual component may not always be effective to inhibit the cancer at different stages.

These active principles have specific roles in treating diseases although they might have some undesirable side effects. Therefore the latest trend is to have a holistic approach of medication in the form of crude extract treatment rather than using the active principles. It is believed that the crude extracts contain active principles along with other minor components which give the required curative function without any side effects. It seems that the minor components of the extract might synergistically act along with the active principle, thus probably reducing the intensity of treatment, which otherwise could have caused toxicity.

Therefore, we have used the crude extract of the plant *I. aspalathoides* for the treatment of chemically induced fibrosarcoma in rats, although we had an analysis of the various principles present in the plant. Plant based drugs are used orally as a practice. In the process of digestion a part of the medicinal components might be lost, and also it is difficult to arrive at dose specificity. Therefore, in the present study we have treated the rats with intraperitonial injections of the crude extract.

In all the three Indian systems of medicine, Ayurveda, Siddha and Unani, the medicinal plant *I. aspalathoides* is widely used to treat various diseases, including tumors. The plant, *I. aspalathoides* Vahl., belongs to family Papilionaceae, is a low under shrub with copious terete branches. It is found in South India and Sri Lanka[8]. It is found to be active against transplantable tumors and inflammations[9]. The plant is one of important ingredient of the specific oil for syphilitic and other skin diseases.

The aqueous extracts of *I. aspalathoides* contain mainly, flavonoides, alkaloids, terpenoids, steroids, tannins, carbohydrates and reducing sugars, which have the ability to counteract the adverse biological effects of carcinogens. Hence, it is of interest to investigate the chemopreventive efficacy of the crude aqueous extract of *I. aspalathoides* on 20-methylcholanthrene induced fibrosarcoma in rats. In continuance of our earlier work, the present study concentrated on the parameters such as body weight, liver and kidney weight, tumor weight, mean survival time, behavioral changes, blood glucose, blood glycogen and marker enzymes such as Alanine aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaine Phosphatase (ALP), Acid Phosphatase (ACP) and 5′ Nucleiotidase (5′-NT) in serum, liver and kidney and lipid profiles such as total cholesterol, phospholipids, free fatty acids in liver and kidney of control and experimental animals[10-13].

2. Materials and methods

2.1. Plant materials

Fresh aerial parts (leaves, stems and seeds) of the plant *I. aspalathoides* were obtained and authenticated by the Chief Botanist, Tamil Nadu Aromatic and Medicinal Plants Corporation Limited (TAMPCOL) at Government Siddha Medical College Campus, Arumbakkam, Chennai, India.

2.2. Preparation of plant extract

One kilogram of the shade dried and coarsely powdered aerial parts of the plant *Indigofera aspalathoides* was put in an aspiration bottle and allowed to soak in double distilled

water for 24 h at room temperature. The extract was filtered and concentrated on a water bath. The inorganic material was precipitated and filtered off. The filtrate was again concentrated in a China dish and dried in vacuum. The yield of the extract was 100 mg/g of the powdered aqueous extract. This was stored in refrigerator for further and future use.

2.3. Acute toxicity studies

Acute toxicity study of aqueous extract of *I. aspalathoides* (AEIA) was done as per OECD guideline 425, using albino male rats. The animals were kept fasting overnight providing only water, after which the extract was administered orally for one animal at the limit dose of 2 500 mg/kg and observed for 14 d (special attention was given for the first 4 h of administration followed by the next 20 h). In case of the death, the limit test was terminated and main test was conducted. If the animal survived, four additional animals were dosed sequentially so that five animals could be tested. However, if three animals died, the limit test was terminated and the main test performed. The LD_{50} is greater than 2500 mg/kg if three or more animals survived. If an animal died unexpectedly late in the study and there were other survivors, it was appropriate to stop dosing and observing all animals to see if other animals also die during a similar observation period.

2.4. Acute toxicity test

The AEIA has not shown any mortality at the limit dose of 2 500 mg/kg body weight. AEIA was found to be safe even at a higher concentration and based on this, the dose for the chemo-preventive activity was chosen.

2.5. Animals

Wistar strain male albino rats weighing 100 to 120 g, were obtained from TANUVAS-LAMU, Madhavaram, Chennai, India. The animals were fed with normal pellet diet (rat chew) and water *ad libitum*. The study protocol, approved by the Ministry of Social Justice and Empowerment, Government of India, was followed[Institutional Animals Ethics Committee (IAEC) number 07/15/02.

2.6. Sample collection

The animals were sacrificed by cervical decapitation at the end of the experimental period and blood was collected to separate serum for biochemical analysis. The liver and kidney were dissected out and known weight of both liver and kidney were homogenized in 0.1 mol/L Tris-HCl buffer (pH 7.4). Animals were starved overnight before sacrifice.

2.7. Chemicals

All the chemicals and reagents used were purchased from M/s. Sigma Chemicals, USA.

2.8. Induction of fibrosarcoma

Fibrosarcoma was induced in Wistar strain of male albino rats by subcutaneous implantation of Millipore filter disc, impregnated with 5% suspension of 20 MCA in paraffin oil[14]. Tumors which appeared in about 4 weeks after implantation were, highly localized and were maintained by serial transplantation. The tumor was minced and suspended in normal saline. A suspension of about 1×10^6 cells in 0.5 mL of saline was injected subcutaneously, into the thigh. The transplanted tumor became palpable in 4-6 d.

2.9. Histopathological analysis

The liver and kidney of control and experimental animals were used for histopathological analysis.

2.10. Experimental design

The rats were divided into four different groups each group consisting of six animals. Group I animals served as normal control, Group II animals were fibrosarcoma bearing animals after the incubation period, Group III animals were fibrosarcoma bearing animals treated with aqueous extract of *I. aspalathoides* intraperiotonially at a dose of 250 mg/kg body weight for 30 d and Group IV animals were administered with the aqueous extract of *I. aspalathoides* alone, at a dose of 250 mg/kg bodyweight for 30 d, served as drug control animals.

2.11. Tumor measurements

Tumor measurements were made using a Verniar calipers and tumor diameter (Td) was calculated using the formula stated elsewhere. The experiments were repeated twice.

$$\text{Td (cm)} = \frac{\text{Length of tumor (cm)} + \text{Width of tumor (cm)}}{2}$$

2.12. Statistical analysis

One-way ANOVA, using SPSS 7.5 student version was used for statistical significance between groups.

2.13. Biochemical estimation

The body weight, liver and kidney weight, tumor weight and mean survival time was analyzed by the standard procedure followed regularly. The levels of blood glucose was estimated by the method Sasaki *et al*[15] and the levels of glycogen in liver and kidney were estimated by the method of Morales *et al*[16]. The levels of serum alkaline phosphatase (ALP) and acid phosphatase (ACP) were studied by the method of Balasubramanian *et al*[17]. The serum levels of aspartate amino transferase (AST) were studied by the method of King[18] and those of 5′-Nucleotidase was analyzed by the method of Luly *et al*[19]. Lipids were extracted by the method of Folsh *et al*[20]. The tissues of liver and kidney were washed with normal saline and dried with filter paper. A weighed amount of the tissue was homogenized with 7 mL of methanol in a Potter Elvehjem homoginizer and filtered through a Whatman No.1 filter paper into a conical flask. The residue was once again scrapped from the filter paper and homogenized with 7 mL of chloroform: methanol mixture (2:1 v/v) and the resulting filtrate evaporated to dryness. The dried lipid residue, after evaporation was dissolved in 5 mL of chloroform: methanol mixture. The redissolved lipid extract was mixed with 1 mL of 0.1 mol/L potassium chloride and the contents were shaken well. The upper aqueous

phase contained gangliosides. The lower layer was again washed 3 times with 2 mL of Folch's reagent and the upper aqueous phase was aspirated. The lower chloroform phase was made up to a known volume for the analysis of total cholesterol, phospholipids and free fatty acids.

2.15. Estimation of total cholesterol

This was done by the method of Parekh and Jung[21]. One milliliter of the aliquot was taken and was evaporated to dryness. The dried extract and standards were made up to 3 mL with ferric chloride-uranyl acetate reagent. Than 2 mL of sulphuric acid-ferrous sulphate reagent was added to all the tubes and the contents were mixed well. After 20 min. the color developed and was read at 540 nm using spectrophotometer. The total cholesterol was expressed as mg/g of wet weight tissue for liver and kidney.

2.16. Estimation of phospholipids

This was performed by the method of Rouser *et al*[22].The dried lipid extract was dissolved in 0.1 mL of perchloric acid and kept in sand bath till the solution becomes colorless. After cooling, the solution was made up to 5 mL with distilled water. To all the tubes, 0.5 mL of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 min. The blue color developed was read at 710 nm using spectrophotometer. The phospholipid content was expressed as mg/g of wet weight tissue of liver and kidney.

2.17. Estimation of free fatty acid

This was performed by the method of Hron and Menahan[23]. A total of 0.2 mL of lipid extract was mixed with 6 mL of chloroform: heptane: methanol solvent (200:150:7 v/v/v) and was shaken vigorously. Two hundred milligram of activated sialic acid was added, shaken and left aside for 30 min. The solution was than centrifuged and the supernatant was transferred to a tube containing copper nitrate-triethylamine reagent (Cu-TEA). The contents were agitated using a mechanical shaker for 20 min. The mixture was separated into 2 phases by centrifugation. Upper layer (2 mL) was mixed with 1 mL of the color reagent and shaken well. The yellow color developed was read at 430 nm in a spectrophotometer. The free fatty acid level was expressed as mg/g of wet tissue weight of liver and kidney.

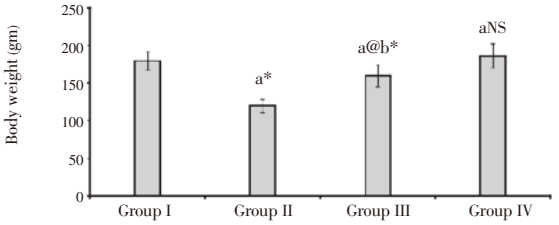
3. Results

During the entire period of our study no difference in food and water intake was observed among the groups of animals. Mean food and water intake per day were (9.1±2.4) and (17.7±2.6) mL/100 g, for the control and trials respectively.

3.1. Behavioral changes

The fibrosarcoma bearing animals Group II animals showed marked difference in their behavior as compared to their normal control counterparts in being more ferocious and anxious. This could be due to the pain and psychological stress on them due to the disease. But, during the course of treatment with *I. aspalathoides* the behavior became slowly normal as cure to the disease was progressing.

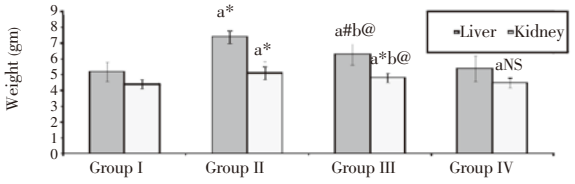
Figure 1 represents the body weight changes in both control and experimental animals. Body weights were significantly decreased ($P<0.001$) in group II fibrosarcoma bearing animals. Body weight slowly and steadily increased after the treatment with *I. aspalathoides* group III animals. Drug control animals did not show any significant variation in the body weights.



a-Group II, III and IV comparedwith Group I
b-Group III compared with Group II
Valuse are mean±SD; n=6; *P<0.001; #P<0.01; @P<0.05; NS-Not significant

Figure 1. Effect of *I. astalathoides* on body weight of control and experimental animals.

Figure 2 shows liver and kidney weights changes in both control and experimental animals. The liver and kidney weights were significantly increased ($P<0.001$) in Group II fibrosarcoma bearing animals as compared with normal control Group I animals. But after the treatment with aqueous extract of *I. aspalathoides* the organ weights reached near normal levels.

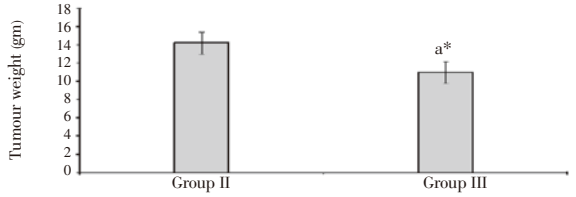


a-Group II, III and IV comparedwith Group I
b-Group III compared with Group II
Valuse are mean±SD; n=6; *P<0.001; #P<0.01; @P<0.05; NS-Not significant

Figure 2. Effect of *I. astalathoides* on liver and kidney weight of control and experimental animals.

Figure 3 illustrates the tumor weights in fibrosarcoma bearing group III animals and fibrosarcoma bearing plant extract treated animals. Tumor weight measurements are commonly used to study the antitumor nature of desired drug. Echitamine chloride, a plant alkaloid has been reported to inhibit tumor growth as compared to untreated

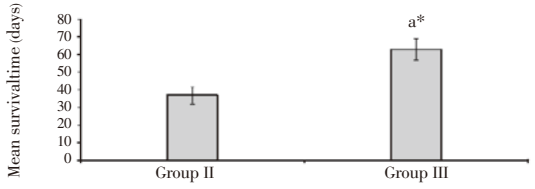
tumor[24]. During the experimental period the treatment with aqueous extract of *I. aspalathoides* the tumor weights were found to decrease significantly when compared to the weights in untreated fibrosarcoma bearing rats. Hence the observed reduction in the tumor weight in plant extract treated fibrosarcoma bearing animals, clearly show the tumor growth inhibitory effects of the plant extract.



b–Group III compared with Group II
Valuse are mean±SD; n=6; *P<0.001; #P<0.01; @P<0.05; NS–Not significant

Figure 3. Effect of *I. aspalathoides* on tumour weight in untreated and treated fibrosarcoma bearing animals.

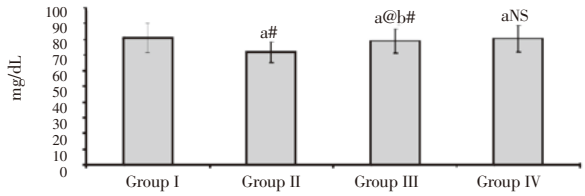
Figure 4 represents mean survival time of the fibrosarcoma bearing and *I. aspalathoides* treated animals. A total of two animals died before the end of study, two from Group II animals. None of animals from any other groups died during the experiment period. The mean survival time was found to increase in all the treated animals. The aqueous extract of *I. aspalathoides* treatment significantly enhanced the mean survival time as compared to both control and *I. aspalathoides* alone treated animals.



b–Group III compared with Group II
Valuse are mean±SD; n=6; *P<0.001; #P<0.01; @P<0.05; NS–Not significant

Figure 4. Effect of *I. aspalathoides* on the mean survival time of untreated and treated fibrosarcoma animals.

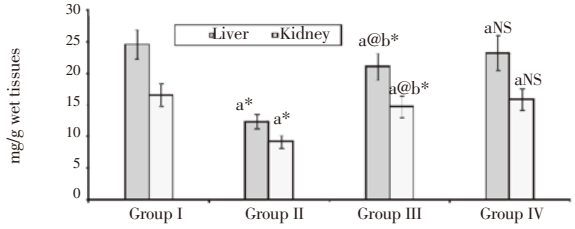
Figure 5 depicts the levels of blood glucose in control and experimental animals. Group I and Group IV animals did not show any significant changes. The blood glucose was found to decrease significantly in fibrosarcoma bearing Group II animals. After the treatment with *I. aspalathoides* aqueous extracts the blood glucose levels slowly reversed to near normal in Group III animals. This result evidently indicated the anticancer potency of the plant.



a–Group II, III and IV comparedwith Group I
b–Group III compared with Group II
Valuse are mean±SD; n=6; *P<0.001; #P<0.01; @P<0.05; NS–Not significant

Figure 5. Comparative blood glucose levels in control and treated animals.

Figure 6 represents the levels of glycogen in liver and kidney of control and experimental animals. The liver and kidney glycogen levels were found to decrease significantly (P<0.001) in Group II animals. However, the maximum beneficial effect of the plant extract was observed in Group III animals in the form of increased levels of blood glycogen in them. The drug alone treated animals did not show any marked variation as compared to normal control animals.



a–Group II, III and IV comparedwith Group I
b–Group III compared with Group II
Valuse are mean±SD; n=6; *P<0.001; #P<0.01; @P<0.05; NS–Not significant

Figure 6. Comparative levels of glycogen in liver and kidney of control and experimental animals.

Table 1 shows the levels of lipids, such as total cholesterol, phospholipids and free fatty acids in liver and kidney of control and experimental animals. In the liver and kidney of Group II fibrosarcoma bearing animals the total cholesterol increased whereas the phospholipids and free fatty acid levels were significantly decreased (P<0.001) when compared to normal Group I animals. But the levels of all the three parameters reached near normal states after treatment of

Table 1
The levels of lipids in liver and kidney of control and experimental animals.

Parameters (mg/g wet tissue)		Group I (Control)	Group II (Fibrosarcoma)	Group III (Fibrosarcoma+ *I. aspalathoides*)	Group IV (*I. aspalathoides*)
Liver	Total cholesterol	3.68±0.40	7.88±0.87^{a*}	4.82±0.52$^{a@b*}$	4.01±0.40^{aNS}
	Phospholipids	13.70±1.32	6.47±0.72^{a*}	12.86±1.32$^{a@b*}$	13.72±1.32^{aNS}
	Free fatty acids	7.96±0.71	4.46±0.50^{a*}	7.14±0.68^{aNSb*}	7.82±0.71^{aNS}
Kidney	Total cholesterol	2.11±0.25	3.96±0.40^{a*}	2.74±0.20$^{a\#b*}$	2.29±0.22^{aNS}
	Phospholipids	11.52±1.13	8.75±0.70^{a*}	10.92±0.86$^{a\#b*}$	11.64±1.05^{aNS}
	Free fatty acids	6.48±0.67	5.23±0.54^{a*}	6.46±0.61^{aNSb*}	6.34±0.63^{aNS}

^{a}: Group II, III and IV compared with Group I, ^{b}: Group III compared with Group II. Values are mean±SD, n=6.
*: P<0.001, $^{\#}$: P<0.01, $^{@}$: P<0.05, ^{NS}: Not Significant.

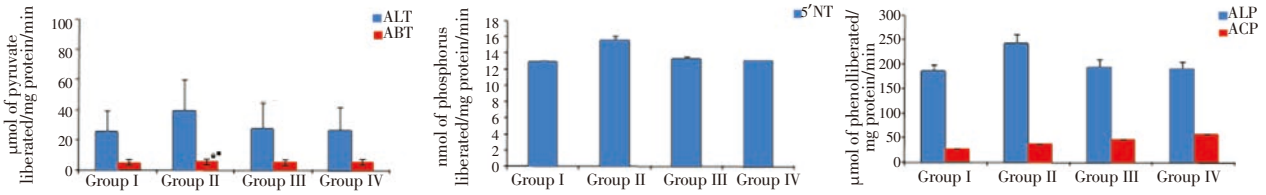


Figure 7. The activitles of marker enzymes in serum of control and experimental anlmals.

Table 2
The activities of marker enzymes in liver of control and experimental animals.

Parameters	Group I (Control)	Group II (Fibrosarcoma)	Group III (Fibrosarcoma+*I. aspalathoides*)	Group IV (*I. aspalathoides*)
ALT (µmol of pyurvate operated/ mg protein/min)	10.02±0.44	17.20±0.22^{a*}	15.20±0.22$^{a*\ b*}$	10.09±0.30^{aNS}
AST (µmol of pyurvate operated/ mg protein/min)	4.35±0.44	6.89±0.44^{a*}	4.97±0.46$^{a*\ b*}$	4.32±0.22^{aNS}
ALP (µmol of phenol liberated/mg protein/min)	9.02±0.35	11.45±0.22^{a*}	9.76±0.26$^{a*\ b*}$	9.08±0.22^{aNS}
ACP (µmol of phenol liberated/mg protein/min)	4.43±0.16	6.53±0.19^{a*}	5.48±0.24$^{a*\ b*}$	4.61±0.61^{aNS}
5′-NT (nmol of phosphate liberated/mg protein/min)	1.66±0.88	2.65±0.07	2.18±0.06$^{a*\ b*}$	1.67±0.07^{aNS}

^{a}: Group II, III and IV compared with Group I, ^{b}: Group III compared with Group II, Values are mean±SD; n= 6 ,*: P<0.001, $^{\#}$: P<0.01, $^{@}$: P<0.05, ^{NS}: Not significant.

Table 3
The activities of marker enzymes in kidney of control and experimental animals.

Parameters	Group I (Control)	Group II (Fibrosarcoma)	Group III (Fibrosarcoma+*I. aspalathoides*)	Group IV (*I. aspalathoides*)
ALT (µmol of pyurvate operated/ mg protein/min)	11.71±0.77	18.70±0.44^{a*}	16.01±0.33$^{a*\ b*}$	11.08±0.44^{aNS}
AST (µmol of pyurvate operated/ mg protein/min)	3.08±0.08	5.72±0.09^{a*}	3.78±0.08$^{a*\ b*}$	3.12±0.07^{aNS}
ALP (µmol of phenol liberated/mg protein/min)	7.17±0.33	11.83±0.66^{a*}	9.75±0.19$^{a*\ b*}$	7.68±0.69^{aNS}
ACP (µmol of phenol liberated/mg protein/min)	5.31±0.19	7.25±0.22^{a*}	6.35±0.26$^{a*\ b*}$	5.34±0.26^{aNS}
5′-NT (nmol of phosphate liberated/mg protein/min)	1.48±0.17	2.55±0.10^{a*}	2.12±0.06$^{a*\ b*}$	1.51±0.16^{aNS}

^{a}: Group II, III and IV compared with Group I, ^{b}: Group III compared with Group II, Values are mean±SD; n= 6 ,*: P<0.001, $^{\#}$: P<0.01, $^{@}$: P<0.05, ^{NS}: Not significant.

I. aspalathoides in Group III animals. Group IV drug control animals did not show any marked alterations in these lipid levels indicating that the plant extract does not have any toxic side effects.

3.2. Results of marker enzymes

Figure 7, Table 2 and Table 3, illustrate the activities of marker enzymes namely, ALT, AST, ALP, ACP and 5′ Nucleiotidase (5′-NT) in serum, liver and kidney of control and experimental animals, respectively. Elevated activities of marker enzymes were observed in serum, liver and kidney of fibrosarcoma bearing Group II animals. The activities of these enzymes were found to normalize in *I. aspalathoides* treated fibrosarcoma bearing Group III animals. No significant variations in the levels of these marker enzymes were observed in drug alone treated Group IV animals as compared with Group I normal control animals.

4. Discussion

These findings suggest that the plant extract does not have any toxic side effects. In the present study the therapeutic efficacy of *I. aspalathoides* on 20-methylcholanthrene induced experimental fibrosarcoma in male albino rats was observed. Chemical carcinogenesis is usually a multistage disease process, comprising of different stages of conversion from a normal somatic cell into a tumor cell and ultimately, after a long latency period, to a clinically manifested malignant tumor. Chemicals cause cancer, either directly or more often after metabolic activation. In addition to a number of organic compounds metalssuch as nickel, lead, cadmium, cobalt and barium are also included in the category of carcinogens.

Organic carcinogens acquire conversion to an ultimate more reactive compound. This conversion is enzymatic and or converted from procarcinogenes to their active forms. A large proportion of the world population, especially in developing countries depend on traditional system of medicine for a variety of diseases including cancer. Several hundred genera are used medicinally mainly as herbal preparations in the indigenous system of medicine in different countries and were the source of potent and powerful drugs which have stood the test of time and modern chemistry has not been able to replace most of them. Many pharmaceuticals that we use today are of botanical origin and are based on herbal remedies from the folk medicine of native people. Important drugs of the past 50 years or so were first isolated from plants and used ethnomedically. In fact

74% of the 119 biologically active plant derived compounds at present used were discovered as a result of research first identified on ethnobotanical survey.

In the present study it is observed that the food and water intake in different experimental groups did not get altered. This feature is of paramount importance because nutritional depreciation causing body weight loss. Thus the observed inhibitory effect of *I. aspalathoides* on tumor appearance and growth is likely to be mediated through the impairment of the nutritional status of the experimental animals.

The aqueous extract of *I. aspalathoides* showed more significant tumor regression suggesting its anticancer effects. Visible observations itself indicated the tumor regression during treatment with this extract. The reduction of tumor growth may be due to a cytotoxic compound or compounds in the plant extract. The survival time of the animals was found to be high in *I. aspalathoides* treated animals. These results indicate the positive nature of the extract as an anticancer agent which acts against tumors.

Malignancies may engender complex metabolic disturbances in both humans and experimental animals resulting in rapid loss of body weight and resulting in tissue wasting. The body weight progressively declined in tumor hosts and by contrast it increased regularly in controls. Body weights steadily increased after the treatment of *I. aspalathoides* extracts rather than the fibrosarcoma bearing animals. It was shown that tumor growth elicited marked loss of body weight in growing ascetis hepatoma bearing animals[25]. This may be due to the decreased food intake and/or absorption, which contribute to muscle wasting in tumor Cachexia[26]. Tumor may act as a nitrogen trap and the cells are more efficient in utilizing amino acids for gluconeogenesis[27]. Also from one mole of glucose, tumor produces two moles of ATP and lactic acid for its use. This lactic acid is converted by the host liver to glucose which the tumor uses, consuming six ATP moles. This results in a loss to the host of eight moles of ATP per one glucose mole.

These moles of glucose used by the tumor is a loss to the host which might have been used by the liver to generate 36 ATP moles in Kreb's cycle[28]. The host responds to increased tumor load by decreased muscle protein synthesis and muscle breakdown. Amino acids resulting from breakdown of proteins are subsequently used by the liver, further increasing the host's metabolic burden[29]. The liver and kidney weights in fibrosarcoma conditions may largely be due to protein degradation during tumor growth.

Protein metabolism perturbations in the host although causing tissue waste may themselves favor the growth of tumor itself. The steadily increasing body weights in treatment of aqueous extract of *I. aspalathoides* may be due to its anticancer potency. The extract of this plant causes disappearance of some symptoms by giving subjective and objective improvements. Absence of significant variation in weights of body, liver and kidney in drug control animals reveals the non toxic nature of the plant drug.

Natural products from plants and animals have been isolated as biologically active Pharmophores[30]. Approximately one third of the top selling drugs in the world are natural products or their derivatives often with ethnopharmacological background[31-34]. According to Chatterjee *et al.*[35], by tribal healers in most of the countries, the ethnomedical treatment is frequently used to treat skin infection, swelling, mental illness, ageing, diabetes, jaundice, cancer, scabbies, snake bite and venereal diseases.

In the present study, there was an observed elevated level of total cholesterol with decrease in phospholipids and free fatty acids in fibrosarcoma bearing Group III animals. It was sighted that the elevated cholesterol level preceedes the observed changes in DNA protein contents suggesting a link between cholesterol and DNA synthetic pathway[36,37]. In *I. aspalathoides* treated animals the above lipid profiles were reverted to near normal level on a time dependent manner. The protective effect of *I. aspalathoides* seems to be through its antioxidant properties.

The marker enzymes are more unique and change in their activities reflect the effect on proliferation of cells with growth potential and its metabolic turnover is dramatically different from normal cells.

The altered activities of ALT and AST in the serum of fibrosarcoma bearing animals were brought to near normal levels after treatment with the plant extract. In course of our study it was observed that the levels of enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase were restored to near normal states after the treatment with this plant extract on fibrosarcoma bearing rats. A study on non enzymatic antioxidants such as reduced glutathione, Vitamin C, Vitamin E and total thiols by us also indicated that after the treatment with the aqueous extract of *I. aspalothoides* the levels of these antioxidants in liver and kidney increased to near normal levels as compared to the levels in fibrosarcoma bearing animals. ALP is membrane bound and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites. ALP is abundant in kidney and serves as a marker enzyme for the renal proximal tubular injury.

Elevated level of ALP was noticed in serum of fibrosarcoma bearing Group II animals. Patel *et al.*[38] observed that ALP level was raised in the serum of cervical carcinoma patients. *I. aspalathoides* treatment decreased the enzyme levels as was observed in the Group III animals which was evident by the histopathological patterns in liver and kidney of fibrosarcoma bearing animals treated with the plant extract. In the present study, fibrosarcoma bearing animals showed increased activity of the enzyme ACP, which were brought

to near normal levels after treating with *I. aspalathoides* in group III animals. Reversals of these enzyme levels correlate well with tumor response to therapy. 5′-nucleotidase has been reported to be altered in sera of the patients with solid tumors[39]. In human lymphoid system, 5′-nucleotidase is anchored to the plasma membrane and has been described as an important marker for differentiation of B lymphocytes[40]. In the present study 5′-nucleotidase has increased significantly in control and experimental animals. Schwartz and Bodansky have demonstrated elevated activities of 5′-nucleotidase in liver carcinoma, gastrointestinal tract and pancreatic cancer[41]. During *I. aspalathoides* therapy, this enzyme activity decreased significantly in fibrosarcoma bearing animals. Recoupment of this marker enzyme on treatment with *I. asplathoides* gives some protective mechanism against abnormal cell growth by changing the permeability of membrane or affecting cellular growth.

The altered activities of ALT and AST in the serum, liver and kidney of fibrosarcoma bearing animals were brought back to near normal levels in Group III drug treated animals.

The present study has tried to highlight the importance of using *I. aspalathoides* as a treatment module against cancer, since it is available in plenty, cost effective and did not show any perceptible side effects. This was evident from the results that the anticancer effect of *I. aspalathoides* may also be attributed to its antioxidant properties.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The research is a descriptive study of the effect of *I. aspalathoides* (a very important medicinal plant in Ayurveda treatment) on fibrosercoma. Fibrosarcoma (fibroblastic sarcoma) is a malignant tumor derived from fibrous connective tissue. It is very common in middle aged and older adults irrespective of sex. Such type of malignant cancer commonly involves deep tissue extremities, trunk, neck and head. The exact cause of fibrosercoma and other soft tissue tumor is not completely understood. Some studies have indicated that genetic alterations could be involved. Moreover, no proper treatment has been developed to completely cure the disease. The interesting aspect of the present work is the therapeutic aspects of this medicinal plant to prevent the tumor growth without any toxic effect on the animal.

Research frontiers

Due to tremendous drug resistant of cancer cells it is usually very hard to treat various type cancers. Herbal medicine is very traditional and ancient to treat various types of diseases. This study would certainly help in future to through light on the cure of malignant cancer.

Related reports

There are not much reports of this plant extract on fibrosercoma as well as other types of cancer. But, the authors presented very well the medicinal value of the plants with citing the related reports on these aspects.

Innovations and breakthroughs

Antiproliferative function of the plant extract showing the reduction of the tumor growth is very interesting and innovative without any toxic effect in the body of the animal.

Applications

If the authors could able to know the exact compound(s) in the crude extract then it may be very useful to use as medicine to control tumor growth and suppress metastasis.

Peer review

In general, the manuscript is well-written and the rationale for the work well described. The methods adopted are robust and the discussion is judicious in view of the results obtained. The work is certainly suitable for publication in *Asian Pacific Journal of Tropical Biomedicine*. I would suggest the following experiments in future after publishing this preliminary report:

1. Try to purify the crud extract and identify the antiproliferative compounds.
2. Do immunohistochemistry (IHC) to see the effect on Ki67 to understand the antiproliferative function of the extract.
3. Find the targets (genes and proteins) of the extract related to antiproliferative markers.

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