



International Journal of
Cancer Research

ISSN 1811-9727



Academic
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Chemopreventive Effects of *Indigofera aspalathoides* on 20-Methylcholanthrene Induced Fibrosarcoma in Rats

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ABSTRACT

To aim at providing an alternative, cheap and effective medicine for fibrosarcoma this study was undertaken. Indian folk medicine comprises numerous prescriptions for therapeutic purposes which may be as varied as ulcers, leprosy and cancer. The antineoplastic effect of the aqueous extracts of *Indigofera aspalathoides* on 20-methylcholanthrene (20-MCA) induced fibrosarcoma was investigated in the male albino rats. The levels of non-protein nitrogenous compounds such as urea, uric acid and creatinine, the levels of lysosomal enzymes such as N-acetyl β -D-glucuronidase and the levels of gluconeogenic enzymes such as glucose 6-phosphatase and fructose 1, 6-diphosphatase were used to monitor the antineoplastic potentials of *Indigofera aspalathoides*. Fibrosarcoma was induced in Wister strain of male albino rats by subcutaneous implantations of Millipore filter discs impregnated with 5% suspension of 20-MCA in paraffin oil and the tumors appeared in about 4 weeks. The fibrosarcoma was proved by pathological examinations. Intra-peritoneous (i.p.) administration of 250 mg kg⁻¹ b.wt. Aqueous extracts of *Indigofera aspalathoides* for 30 days effectively suppressed the 20-MCA induced chemical carcinogen, revealed by the reduced incidence of fibrosarcoma. The levels of non protein nitrogenous compounds such as urea, uric acid and creatinine, the levels of lysosomal enzymes and the levels of gluconeogenic enzymes in liver and kidney samples were significantly altered. The findings clearly indicate the antineoplastic potentials of *Indigofera aspalathoides* on 20-MCA induced fibrosarcoma in rats.

Key words: Fibrosarcoma, *Indigofera aspalathoides*, lysosomal enzymes, chemoprevention, gluconeogenic enzymes, antineoplastic, complementary medicine, siddha, aurveda

INTRODUCTION

Cancer remains a major public health problem in the world. The disease is responsible for several million deaths annually, mainly in the underdeveloped and developing countries. A large number of agents, including natural and synthetic compounds have been identified as having some potential cancer chemotherapeutic value (Kellof, 2000). A number of natural products have been studied for anti cancer activity on various experimental models (Ramakrishna *et al.*, 1984). Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure shot and perfect cure is yet to be brought into the world of medicine. The complimentary and alternative medicines have been the basis of treatment and cure for various diseases and physiological abnormalities, in traditional methods under practice, such as siddha and ayurveda (Kaufman *et al.*, 1999).

Chemoprevention is being tested as a major means of inhibition of cancer, suppression of neoplasia during recurrence and, at times, reversal of carcinogenic process. The failure of conventional chemotherapy to effect major reduction in the mortality indicates that new approaches are critically needed (Murthy *et al.*, 1990; Murray and Lopez, 1996). Plants are loaded with chemicals with chemopreventive activities and some of them are undergoing clinical trials. Most of the studies on chemoprevention is based on individual chemicals with defined mechanisms of action and have been reported to inhibit carcinogenesis in animal models (IARC, 1973).

Fibrosarcoma is the tumor of collagen fibres forming mesenchymal cells of fibroblasts and they arise from subcutaneous fibrous tissues (Stout, 1948). The tumor may occur in any one of the soft tissue sites but is most common in the deep soft tissue of the lower extremities and followed by the upper extremities and trunk.

There are numerous reports regarding fibrosarcoma in the head and neck (Bentz *et al.*, 2004) including the nasal cavity, paranasal sinuses and nasopharynx (Heffner and Gneep, 1992). Rare examples of this tumor have also been reported in the breast, heart, lung, liver and central nervous system (Jones *et al.*, 1992; Anand *et al.*, 1996). In many cases, surgery and radiotherapy fail to cure chiefly because the tumor is already disseminated and for this reason, chemotherapeutic agents are sought to reduce the patient's tumor burden so that a cure may be possible.

These agents are designed to retard the proliferation of cancer cells and to induce differentiation of these cells to a quiescent, non dividing stage and/or to promote cell death (Logrono *et al.*, 1999). Effective chemopreventive treatment for cancer could have an important impact on cancer morbidity and mortality, such as that of fibrosarcoma. Nowadays, chemoprevention is gaining more attention. This approach aims to decrease overall cancer morbidity and mortality by using substances that are capable of preventing cancer progression. Several classes of natural compounds have been evaluated for this purpose and each of these classes are plant derived compounds or extracts, which interact with the host to confer a preventive benefit by regulating cellular signaling of proliferation and death (Kirtikar and Basu, 2000).

Chemoprevention is a promising and novel strategy for the prevention, inhibition, suppression and reversal of carcinogenesis through the use of natural plant products. It has been suggested that compounds that possess antimutagenic, anticarcinogenic and inhibitory effects on cell proliferation and antioxidant functions are considered to be good chemopreventive agents (Manoharan *et al.*, 2009). It was reported that the plant extract of *Bacopa monnieri* has modulating effects on antioxidant and marker enzyme status in fibrosarcoma bearing rats (Rohini *et al.*, 2004). The relatively lower incidence of the adverse reactions to plant preparations compared to modern conventional pharmaceuticals, coupled with their reduced cost, is encouraging for both the consuming public and national health care institutions to consider plant medicines as alternative to synthetic drugs. *Indigofera aspalathoides* Vahl., a plant belonging to the family Papilionaceae, is a low under shrub with copiously spreading terete branching. It is found in South India and Sri Lanka and is traditionally used for treating various skin diseases and tumors. It is found to be active against transplantable tumors and inflammations (Raj Kapoor *et al.*, 2005).

In continuance of our previous study on this plant, this study attempts to evaluate the chemopreventive effect of aqueous extracts of *Indigofera aspalathoides* against 20-methylcholanthrene induced fibrosarcoma in male albino rats.

MATERIALS AND METHODS

Plant materials: Fresh aerial parts (leaves, stems and seeds) of the plant *Indigofera 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 in 2001. The study continued till 2005.

Preparation of plant extract: One kilogram of the shade dried and coarsely powdered aerial parts of the plant *Indigofera aspalathoides* was charged in an aspiration bottle and allowed to soak in double distilled water for 48 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 10% w/w of the powdered aqueous extract. This was stored in refrigerator for further and future use.

Acute toxicity studies: Acute toxicity study of 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 2500 mg kg⁻¹ and observed for 14 days (special attention 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 was performed. The LD₅₀ is greater than 2500 mg kg⁻¹ 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.

Acute toxicity test: The AEIA has not shown any mortality at the limit dose of 2500 mg kg⁻¹ b.wt. AEIA was found to be safe even at a higher concentration and based on this, the dose for the chemopreventive activity was chosen.

Animals: Wister strain male albino rats weighing 100-120 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).

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

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

Induction of fibrosarcoma: Fibrosarcoma was induced in Wister strain of male albino rats by subcutaneous implantation of Millipore filter disc, impregnated with 5% suspension of 20 MCA in paraffin oil (Nagarajan and Sankaran, 1973). 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 days time.

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 *Indigofera aspalathoides* intraperitoneally at a dose of 250 mg kg⁻¹ b.wt. for 30 days and Group IV animals were administered with the aqueous extract of *Indigofera aspalathoides* alone, at a dose of 250 mg kg⁻¹ b.wt. for 30 days, served as drug control animals.

Tumor estimation: Tumor measurements were made using a vernier calipers and Tumor Diameter (Td) was calculated using the formula stated elsewhere. The experiments were repeated twice:

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

Bio chemical estimation: The urea was assayed by the method as described by Natelson *et al.* (1951). The levels of uric acid were determined using the method of Caraway (1963). The activity of creatinine was assayed by the method of Owen *et al.* (1954). N-acetyl β -D-glucosaminidase was estimated by the method of Marhun (1976) and β -D-glucuronidase was assayed by the method of Kawai and Anno (1971). The gluconeogenic enzymes, i.e., glucose-6 phosphatase was assayed by the method of King (1965) and fructose-1-6-diphosphatase was analysed by the method of Gancedo and Gancedo (1971). The tissue total protein was estimated by the method of Lowry *et al.* (1951) using BSA as standard.

Statistical analysis: One-way Analysis of Variance (ANOVA), using SPSS 7.5 student version was used for statistical significance between groups.

RESULTS AND DISCUSSION

Table 1 shows the levels of non-protein nitrogenous compounds such as urea, uric acid and creatinine in control and experimental animals. In group II fibrosarcoma bearing animals, the levels of urea, uric acid and creatinine were increased as compare to group I normal control animals. The levels of urea, uric acid and creatinine were reversed to near normal levels in fibrosarcoma bearing *Indigofera aspalathoides* treated group III animals. No significant changes were observed in *Indigofera aspalathoides* treated animals without fibrosarcoma in group IV animals.

Table 2 shows the activities of lysosomal enzymes such as N-acetyl- β -D-glucosaminidase and D-glucuronidase in liver and kidney of control and experimental animals. The activities of

Table 1: The levels of nonprotein nitrogenous and compounds and experimental animals

Parameters	Group I	Group II	Group III	Group IV
Blood urea (mg dL ⁻¹)	17.4±1.31	18.80±2.10 ^{aNS}	20.14±1.90 ^{a#bNS}	17.6±1.10 ^{aNS}
Serum uric acid (mg dL ⁻¹)	1.85±0.20	1.09±0.16 ^{a*}	1.76±0.02 ^{aNSb*}	1.84±0.08 ^{aNS}
Serum creatinine (mg dL ⁻¹)	0.40±0.07	0.42±0.07 ^{aNS}	0.56±0.06 ^{a#b@}	0.39±0.04 ^{aNS}

^aGroup II, III and IV compared with group I, ^bGroup III compared with group II. Values are Mean±SD: N = 6, *p<0.001, #p<0.01, @p<0.05, NS: Not significant

Table 2: The levels of lysosomal enzymes in liver and kidney of control and experimental animals

Parameters (μmol of phenol liberated mg^{-1} protein h^{-1})	Group I	Group II	Group III	Group IV
Liver				
N-acetyl- β -D-glucosaminidase	1.600 \pm 0.150	4.720 \pm 0.50 ^{a*}	2.270 \pm 0.24 ^{a*b*}	1.880 \pm 0.160 ^{aNS}
β -D-glucuronidase	1.830 \pm 0.160	7.720 \pm 0.84 ^{a*}	3.460 \pm 0.45 ^{a*b*}	2.150 \pm 0.190 ^{aNS}
Kidney				
N-acetyl- β -D-glucosaminidase	1.860 \pm 0.220	1.200 \pm 0.22 ^{a*}	1.460 \pm 0.210 ^{ab@}	1.770 \pm 0.170 ^{aNS}
β -D-glucuronidase	0.028 \pm 0.005	0.046 \pm 0.005 ^{a*}	0.029 \pm 0.003 ^{aNSb@}	0.027 \pm 0.002 ^{aNS}

^aGroup II, III and IV compared with group I, ^bGroup III compared with group II. Values are Mean \pm SD: N = 6, *p<0.001, ^{*}p<0.01, [@]p<0.05, NS: Not significant

Table 3: The Levels of gluconeogenic enzymes in liver and kidney of control and experimental animals

Parameters (μmol of phenol liberated mg^{-1} protein h^{-1})	Group I	Group II	Group III	Group IV
Liver				
Glucose-6 phosphatase	13.82 \pm 0.31	9.48 \pm 1.12 ^{a*}	12.60 \pm 1.11 ^{aNSb*}	13.31 \pm 1.04 ^{aNS}
Fructose-1-6 diphosphatase	17.60 \pm 1.95	12.39 \pm 1.28 ^{a*}	16.39 \pm 1.83 ^{aNSb*}	17.88 \pm 1.74 ^{aNS}
Kidney				
Glucose-6 phosphatase	1.500 \pm 0.21	0.76 \pm 0.10 ^{a*}	10.39 \pm 0.25 ^{aNSb*}	1.49 \pm 0.26 ^{aNS}
Fructose-1-6 phosphatase	1.580 \pm 0.30	0.61 \pm 0.17 ^{a*}	1.52 \pm 0.22 ^{aNSb*}	1.53 \pm 0.24 ^{aNS}

^aGroup II, III and IV compared with group I, ^bGroup III compared with group II. Values are Mean \pm SD: N = 6, *p<0.001, ^{*}p<0.01, [@]p<0.05, NS: Not significant

in both liver and kidney were significantly increased (p<0.001) in fibrosarcoma bearing (group II) animals when compared to control (group I) animals. All the lysosomal enzyme levels were reversed back to near normal in (group III) animals and no significant variations was found in (group IV) drug control animals.

Table 3 shows the activities of gluconeogenic enzymes such as glucose 6-phosphatase and fructose 1, 6-diphosphatase in liver and kidney of control and experiment animals.

The activities of gluconeogenic enzymes in both liver and kidney were decreased significantly (p<0.001) in fibrosarcoma included (group II) animals. All these gluconeogenic enzyme levels were reverted back to near normal in (group III) animals. There are no significant variations found in (group IV) drug control animals.

DISCUSSION

In the present study the anti cancer effects of *Indigofera aspalathoides* on 20-MCA induced fibrosarcoma in rats was investigated. Medicinal plants are the oldest known sources of pharmacologically active compounds and provided virtually the only source of medicinally useful compounds for centuries (Cordell, 1981).

Although, a number of workers have investigated the biochemical evaluation of anti-tumor effect of various medicinal plants (Palani *et al.*, 1999; Prashar and Kumar, 1995; Soudamini and Kuttan, 1989; Talalay and Fahey, 2001) but there is a paucity of information regarding the biochemical evaluation of anticancer effect of natural products on fibrosarcoma. Hence, the present investigation was undertaken to study the anticancer effect of *Indigofera aspalathoides* on 20 MCA induced fibrosarcoma in rats.

The blood urea and creatinine levels were not significantly changed in group II fibrosarcoma bearing animals. On the other hand uric acid level was found to be significantly decreased in group II animals. In the present study urea and creatinine levels were found to be significantly increased

in group III fibrosarcoma bearing animals treated with aqueous extract of *Indigofera aspalathoides*. Elevation of urea concentration is considered to be an indicator of nephrotoxicity in fibrosarcoma bearing animals treated with aqueous extracts of *Indigofera aspalathoides*. The amount of urea excreted depends upon the glomerular filtration rate and when this excretion fails to balance the production, the plasma level rises.

Serum creatinine is an index for renal function. It is produced endogenously by tissue creatinine breakdown and an increase in serum creatinine may be due to the tissue damage. The amount of creatinine excreted depends on the glomerular filtration rate, when this excretion fails to balance the production, serum creatinine rises (Nosaka *et al.*, 1992).

When, the animals were given *Indigofera aspalathoides* treatment, the elevated levels of blood urea and serum creatinine were almost brought back to near normal levels, thus proving its beneficial role on cancer chemotherapy.

Uric acid is the metabolic end product of purine reacting with hydroxyl radicals and hypochlorous acid (Hasugawa and Kuroda, 1989). The reduced levels of uric acid in fibrosarcoma animals may be due to increased utilization of uric acid against lipid peroxidation, which is a characteristic feature of cancer condition.

Increased uric acid level observed after the treatment of *Indigofera aspalathoides* may be due to the decreased tumor burden. Lysosomal enzymes have been implicated as having a role in tissue injury repair and diseases like cancer, arthritis and so on. Increased activities of enzymes in liver and kidney of the tumor tissue may be due to abnormal fragility of lysosomes in sarcoma conditions, the elevated levels should reflect increased synthesis and secretion of enzymes by the tumor (Goren *et al.*, 1986). Increased production of free radicals in cancer led to destruction of membrane which resulted in the leakage of enclosed enzymes from the lysosomal sacs (Geetha, 1993). Increased expressions of lysosomal enzymes were observed in various tumors with increased activity of acid phosphatase, which may be due to the lysosomal imbalance resulting in the destruction of the intact membrane (Kalra *et al.*, 1988).

β -D-glucuronidase is a sensitive marker of lysosomal integrity and it is released due to the presence of oxygen free radicals (Sharma *et al.*, 1995). β -D-glucuronidase is a cellular hydrolase able to degrade cell organelles and digest cell materials, has been used as marker for tubular damage by many nephrotoxic agents (Fishman and Bernfield, 1955). N-acetyl- β -D-glucosaminidase (NAG) is a high molecular mass lysosomal enzyme found in the proximal and distal renal tubules the recouping of lysosomal enzymes upon plant drug treatment to fibrosarcoma bearing animals may be due to the stabilizing property of *Indigofera aspalathoides* on lysosomal membrane which may protect the rapid leakage of enzymes and obstruct the rise in enzymes activity.

Malignant tumors are known to have high rates of glycolytic activity leading to increased production of lactic acid, utilization of lactate and amino acids for glucose synthesis leads to increased, uncontrolled gluconeogenesis from the precursors and this was found in tumor bearing hosts.

Glucose 6-phosphate (G-6-P) and fructose 1,6-diphosphate (FDP) which are key enzymes that regulate gluconeogenesis, it is natural that with the increase in tumor growth rate, lactate production from glucose rises, whereas glucose production from pyruvate decreases, the progressive failure of gluconeogenesis, manifested most extensively in the rapidly growing tumors is explained partly by marked decrease or complete absence of glucose-6 phosphatase and fructose 1,6-diphosphatase activities in cancer conditions.

In conclusion, the present findings demonstrated the chemopreventive efficacy of aqueous extract of *Indigofera aspalathoides* in the experimental rat model and further work needs to be done to isolate and purify the active principles involved in the antineoplastic activity of this plant. These results support our earlier findings of the anticancer efficacy of *Indigofera aspalathoides* extracts on rats.

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