RESEARCH COMMUNICATION

Indigofera Aspalathoides Protection Against 20-Methylcholanthrene-Induced Experimental Fibrosarcoma Growth after Transplantation in Rats - Role of Xenobiotic Drug Metabolizing Enzymes

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Abstract

A large number of active principles from traditional medicinal plants have been reported to have chemopreventive properties. In the present study, therapeutic efficacy of an aqueous extract of *Indigofera aspalathoides* against growth of transplanted experimental fibrosarcomas in Wistar strain male albino rats was tested. Tumors which appeared about six weeks after implantation were highly localized and were maintained by serial transplantation. Rats were divided into four groups. Group I served as normal control animals. Group II were fibrosarcoma bearing animals. Group III were animals with fibrosarcoma treated with *Indigofera aspalathoides* aqueous extracts at a dose of 250 mg/kg. b. w. per day for 30 days. Group IV animals were treated with aqueous extract of *Indigofera aspalathoides* alone. Reduction in tumor weight was noted in Group III as compared to II. The levels of cytochrome C in liver and kidney, the levels of cytochrome P450 and cytochrome b5 in liver microsomes, phase I biotransformation enzymes NADPH-cytochrome P450, NADPH-cytochrome b5, and aniline hydroxylase, and the phase II enzymes glutathione-S-transferase and UDP glucuronyl transferase indicated that their modulation played a role in the therapeutic efficacy of *Indigofera aspalathoides* against experimental fibrosarcoma.

Keywords: Fibrosarcoma growth - rat transplantation - *Indigofera aspalathoides* - chemoprevention

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Introduction

The etiology of most soft tissue sarcomas is highly complex and the recognized causes include various physical and chemical factors. The induction of cancer by chemical is complex, multistage disease process and each stage probably involves both genetic and epigenetic changes (Bishop, 1991). These observations have been substantiated experimentally by administration of carcinogens (Wattenberg, 1995). Metabolic activation of carcinogen is a free radical dependent reaction. DNA damage mediated by free radicals, play a critical role in carcinogenesis (Feig et al., 1994). 20-methylcholanthrene belongs to the group of polycyclic aromatic hydrocarbons (PAH). It is widely distributed in the environment (IARC Monographs, 1997) and has a broad range of target organs and generally produces cancers at the site of application. The specific type of cancer produced varies with the route of administration and induce tumors of the skin, breast and soft tissues.

The PAHs are metabolized by cytochrome P450 dependent mixed function oxidases to give electrophilic epoxides. The electrophilic epoxides either get detoxified or bind covalently with the DNA. The resulting abnormal DNA induces cell division and finally produce tumors. Many of the carcinogens appear to act solely by increasing proliferation. Molecular genetics of cancer reveal that cell division is essential in the complex process of genesis of human cancer. Cell division per second increases the rise of various kinds of cancer Ames (1990).Cell division is necessary for conversion of DNA adducts or damages to single stranded DNA, to gaps or mutations. The development of fully malignant tumor appears to involve the activation or altered expression of proto-oncogene to oncogene and the loss or inactivation of tumor suppression genes, the function of which is to control normal cellular activity Fearon et al., (1990).

Fibrosarcoma is a tumor composed of collagen fibres forming mesenchymal cells of fibroblasts and they arise from the subcutaneous fibrous tissue (Stout, 1948).

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Fibrosarcoma causes no characteristic symptoms and it is vary difficult to diagnose clinically. Most patients present with a solitary palpable mass ranging from 3-8 cm in greatest dimension. Previous radiation therapy is associated with an increased incidence of fibrosarcoma. Hence, the chemotherapeutic agents are sought to reduce the tumor burden for the patients.Chemoprevetion is a promising and novel strategy, for the prevention, inhibition, suppression and reversal of carcinogenesis through the use of natural plant products and synthetic agents. It has been suggested that compound that posses antimutagenic and anticarcinogenic effects on cell proliferation and antioxidant functions are considered to be good chemopreventive agents (Manoharan et al., 2009).

There is paucity of information about the chemopreventive efficacy of *Indigofera aspalathoides* on animal models. Therefore, it is of interest to investigate the anticarcinogenic efficacy of *Indigofera aspalathoides* n experimental fibrosarcoma in rats. The leaves, flowers and tender shoots are considered to have active ingredients (Kirtikar and Basu, 2000) which can be used in the cure of various diseases like skin disease, leprosy and syphilis (Nadkarni, 1982).

The aqueous extract of *Indigofera aspalathoides* mainly contains saponins, tannis, alkaloids, flavanoides, steroids and carbohydrates. It has the ability to counteract the adverse biological effects of carcinogens. Hence, the current study focused on the promising and novel antitumor potency of aqueous extract of *Indigofera aspalathoides* against 20-methylcholanthrene induced fibrosarcoma in 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.

1 kg. 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 hrs 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 under a 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 hrs of administration followed by the next 20 hrs). 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 LD_{50} 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 mgkg^{-1} 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.

Animals

Wister 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].

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 liver and kidney were homogenized in 0.1M 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 Wistar 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 $1x10^6$ cells in 0.5 ml of saline was injected subcutaneously, into the thigh. Transplanted tumors became palpable in 4-6 days time.

Histopathological Analysis

The liver and kidney of control and experimental animals were used for histopathological analysis by Dr. Vijayalakshmi, Professor of Pathology, Madras Medical College, Chennai, India.

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* intraperitonially at a dose of 250 mg/kg b. w. for 30 days and Group IV animals were administered with the aqueous extract of *Indigofera aspalathoides* alone, at a dose of 250 mg/kg b. w. for 30 days, serving as drug control animals.

Tumor Measurements

Tumor measurements were made using a vernier calipers and tumor diameter (Td) was calculated using the formula stated elsewhere.

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

Statistical Analysis

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

Biochemical estimation

Preparation of Liver Microsomes

The liver microsomes were separated according the method of Boyd and Burka, (1978) with slight modification of the method of Kamath and Narayan (1972). The tissue was homogenized and the tubes were covered with parafilm and mixed gently by inversion. The homogenate was centrifuged at 10,000 g for 30 min. The floating lipid layers and the top of supernatant were discarded. Calcium chloride as added and centrifuged at 15,000 g for 30 min. The pellet was suspended in buffer, homogenized and made up to a known volume. The microsomal suspension was used for the analysis of biotrasformation enzymes.

Estimation of Mirosomal Proteins

The protein content of the microsomes was estimated according to the method of Lowry et al (1951). The protein content was expressed as mg of microsomes/g wet tissue. Estimation of Cytochrome P_{450} was done by the method of Omura and Sato, (1964). This was expressed as n moles/mg protein. Estimation of Cytochrome b5 was estimated by the method of Omura and Sato (1964). The Cytochrome b5 content was expressed as n moles/ mg microsomal protein.

Estimation of Phase I enzymes

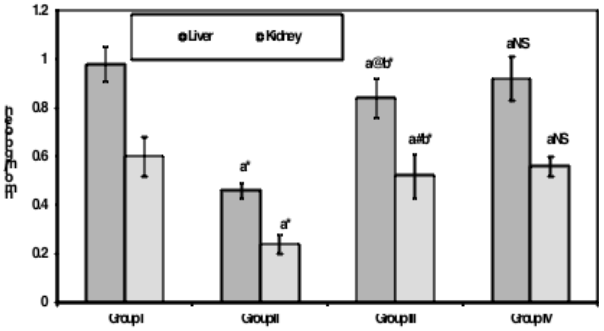
Glutathione oxide reductase was estimated by the method of Staal et al (1969). The enzyme activity was expressed as n moles of NDAPH oxidizesed/mg protein/ min. The aniline hydroxylase activity was assayed by the method of Imai et al (1966) as modified by Brien and Rahimtula (1978).

Estimation of Phase II enzymes

The NADPH-Cytochrome C reductase was estimated by the method of Philips and Langdon (1962). The enzyme activity was expressed as n moles Cytochrome C Oxidised/mg protein/min. Glutathione-S-transferase was essayed by the method of Habig et al., (1974). The enzyme activity was expressed as µ moles of CDNB conjugated/ mg protein/ min. and UDP-glucuronyl transferase was estimated by the method of Isselbacher et al., (1962) modified by Hollman and Touster (1962).

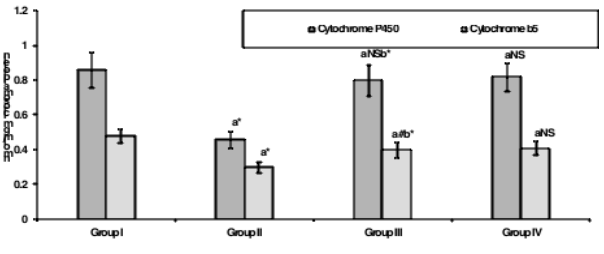
Results

Figure 1 depicts the cytochrome$_{450}$ content in liver and kidney of control and experimental animals. The Cytochrome$_{450}$ content decreased in these tissues of Group II animals but reverted back to near normal ($p<0.001$) in Group III animals when compared to cancerous Group II animals. The drug control animals Group IV did not show any significant changes in Cytochrome$_{450}$ content when compared to control animals. Figure 2 shows the levels of Cytochrome$_{450}$ and Cytochrome b5 in liver microsomes of control and experimental animals. Levels were found to decrease ($p<0.001$) in fibrosarcoma bearing Group II animals when compared to normal control animals. Tumor bearing *Indigofera aspalathoides* treated Group III animals brought back these hemo-proteins to near normal levels as compared with the fibrosarcoma bearing Group II animals. Although the drug has some inducing action on these proteins in drug control Group IV animals when compared with Group I animals, the changes were not significant. Table 1 shows the activities of Phase I and Phase II enzymes in liver microsomes of control and experimental animals. The activities of Phase I enzymes viz. NADPH- cytochrome$_{450}$, NADH-cytochrome b_5 reductase, aniline hydroxylase were found to be inhibited ($p<0.001$) in fibrosarcoma bearing animals. These enzyme activities were resumed significantly to near normal levels in tumor bearing *Indigofera aspalathoides* treated animals.



a - Group II, III and IV compared with Group I
b - Group III compared with Group II
Values are mean ± S.D.; n=6; *p<0.001; #p<0.01; @p<0.05; NS-Not signifiant .

Figure 1. Levels of Cytochrome P450 in Liver and Kidney



- Group II, III and IV compared with Group I,
b - Group III compared with Group II
Values are mean ± S.D.; n=6, *p<0.001; #p<0.01; @p<0.05; NS-Not signifiant .

Figure 2. Levels of Cytochrome b5 in Liver Microsomes

Table 1. Activities of Phase I and Phase II Biotransformation Enzymes in Liver Microsomes of Control and Experimental Animals

Parameters (mg./g. wet weight)	Group I (Control)	Group II (Fibrosarcoma)	Group III(Fibrosarcoma+ (I.aspalathoides)	Group IV (I.aspalathoides)
Phase I Enzymes				
NADPH Cytochrome P450 (n mol/mg. microsomal Proteins)	10.7±0.80	5.10±0.43^{a*}	8.80±0.84$^{a\# b*}$	11.6±0.88$^{a@}$
NADPH-Cytochrome b5 Reductase (Units/mg microsomal Proteins)	0.66±0.05	0.41±0.03^{a*}	0.60±0.01$^{aNS b*}$	0.69±0.06^{aNS}
Aniline hydroxylase (n mol of p-aminophenol formed/mg. microsomal protein/min.)	0.80±0.06	0.39±0.03$^{a@}$	0.79±0.07$^{a@ b*}$	0.87±0.07^{aNS}
Phase II Enzymes				
Glutathione-s-transferase (n mol CDNB utilized/ mg.micros Proteins/min.)	860.1±63.10	488.5±50.6^{a*}	790.1±61.4$^{aNS b*}$	896.3±76.2^{aNS}
UDP-glucuronyl transferase (n mol/mg. microsomal Proteins/min.)	33.51±2.84	20.63±2.13^{a*}	28.90±2.36$^{a@ b*}$	34.4±3.09^{aNS}

The plant drug alone treated animals got stimulating action on Phase I biotransformation enzymes when compared to normal control animals but the differences were not significant.

The activities of phase II enzymes, Viz. Glutathione-S-transferase (GST) and UDP-glucuronyl transferase (UDPGT) were found to be inhibited ($p<0.001$) in fibrosarcoma bearing animals. These enzymes levels were reverted back to near normal levels after the treatment of the plant extract. The Group IV drug alone treated animals got stimulating action on Phase II enzymes but not significantly.

Discussion

Cancer is basically a disease of cells characterized by a shift in the control mechanism that governs cell proliferation and differentiation. A simple universally effective therapy for all forms of cancer is unlikely in the near future, but several more specific, less toxic therapies are a reasonable expectation. The recent success of many drugs, often administered singly or in combination, and frequently included at least one form of plant sources, has in the past decade has given greatest new hope of recovery from this dreaded disease.

The Cytochrome$_{450}$ content decreased sharply in the tissues of fibrosarcoma bearing animals. Their contents were reversed to near normal in *Indigofera aspalathoides* treatment. The decrease in this hemo protein was assumed to be caused by lipid peroxidation induced by the carcinogen. It has been noted that lipid peroxidation caused the degradation of cytochrome$_{450}$, (Mori et al., 1992). Reduced hepatic concentrations of cytochrome450 was also reported in cancer condition (Gregus et al., 1982). The mechanism of cytochrome$_{450}$ driven reaction involves the formation of oxy- and subsequently peroxy-intermediates.

Break down of those intermediates yield ROS-Cytochrome$_{450}$ has functional multiplicity and also act as peroxidase in which peroxides are used as Oxygen donors. The suicidal inactivation process of Cytochrome$_{450}$ involves in the generation of OH ion as an intermediate (Karuzina and Archakov, 1984). This increased lipid peroxidation leads to further decrease of cytochrome$_{450}$. Due to potent free radical scavenging action of the plant extract the abnormal lipid peroxidation reaction was arrested in Group III drug treated animals. This might be the reason for the recoupment of Cytochrome$_{450}$ content in this group of animals. Cytochrome$_{450}$ and Cytochrome b_5 are the principle components of the mixed function oxidation (MFO) of the Phase I biotrasformation enzyme systems. Most of the components of mixed function Oxidase enzyme system, including Cytochrome$_{450}$ and Cytochrome b_5, were found to be severely impaired in the tumor bearing Group II animals. These observations are in accordance with Brown et al., (1971) who reported a decrease in these components in rats bearing mammary gland tumors. The decrease may be due to the alterations in the activities of key enzymes involved in the regulation of Hapatin, Heme and Hemoprotein synthesis and degradation, evidence of which is recently accumulating (Dogra et al., 1985).

The decreased Cytochrome$_{450}$ content may also be due to the increased lipid peroxidation. Wills (1969) hypothesized that the system involved in the lipid peroxidation partially resemble the drug hydroxylating system and appropriate parallelism was observed between the formation of malondialdehyde and loss of microsomal enzymes and cytochrome$_{450}$. Enhanced Cytochrome$_{450}$ catalytic activity may result in increased first pass clearance of Xenobiotics. Hepatic microsomal Cytochrome$_{450}$ and Cytochrome b_5 contents were increased in drug treatment which shows its ability to metabolize carcinogen and sparing effect. This detoxification and clearance of target tissues could account for the observed anti tumorigenic activity of the *Indigofera aspalathoides* extract. The carcinogenicity of many xenobiotics dependson the balance between the activities of Phase I and Phase II enzymes. The activities of hepatic microsomal Cytochrome$_{450}$ dependent enzymes were found to be depressed in 20-MCA induced fibrosarcoma bearing animals because the carcinogen causes damages not only to the Cytochrome$_{450}$, UDPG T and GST but also to other Cytochrome$_{450}$ associated enzyme systems and this may lead to the accumulation of the carcinogen. Activities of both oxidative (Phase I) and conjugative

(Phase II) enzymes were decreased in many tumors, Rao et al., (1987), Roy and Lieher (1988). These changes may have significant effect on the pharmacokinetics of specific agents in vivo and may contribute to alterations in the endocrine status of the host, Mouelhi et al., (1987).

The role of drug metabolism is more closely linked to NADPH- Cytochrome450 reductase than to the amount of Cytochrome450 present Testa and Jenner (1976). This inhibition of the enzyme could also result from binding of the carcinogen, either to the reductase or Cytochrome$_{450}$ to itself. O_2- stays tightly bound to reductase when this enzyme is still located in the microsaomal membrane Koster and Slee (1980). Feuer (1988) has reported a 40% reduction in the enzyme activity in fibroarcoma. NADH is unable to replace NADPH efficiently in many microsomal mixed function oxidase reaction but in the presence of NADPH, a synergistic effect on NADH on metabolic process is apparent which cannot be explained simple summation activity Testa and Jenner (1976). The decreased activity of NADPH-Cytochrome450 reductase in cancer bearing animals may lead to the inhibition of NADPH- Cytochrome b_5 reductase.Anilin hydroxylase activity is also linked to the amount of Cytochrome$_{450}$ present and to the activity of NADPH- Cytochrome$_{450}$ reductase , Testa and Jenner (1976). The enzyme activity was reported to be depressed in AFB treated rats, Raisuddin et al., (1994) and on administration of carcinogens like 3-Methylcholanthrene Matsubara et al., (1976). The present investigation also showed similar results.

Hepatic microsomal Phase II enzymes such as UDPGT and GST are known to be important preneoplastic and neoplastic markers. In this study these enzyme levels were reduced significantly. Activities of UDPG T and GST were found to be decreased also in lung cancer bearing rats Dogra et al., (1985).

UDPGT is intimately associated with the structure of the membranes to which it is tightly bound. The formation of Glucuromide conjugates of drugs occurs predominantly in the hepatocytes, Testa and Jenner (1976). UDPGT is constrained to phospholipids of the microsaomal membranes Erickson et al., (1978) and hence, the observed decrease in group II fibrosarcoma bearing animals may be due to proooxidation damage to the microsomal lipids in cancer conditions. The depression of conjugative enzymes have been reported in primary liver cance, Kamdem et al., (1982).

The enhancement of these Phase I and Phase II enzymes in *Indigofera aspalathoides* treated animals show the reduction in tumerigenesis. In addition to the impairment of drug metabolism, the decrease in the activities of these enzymes and the content of GSH could impair the overall biotransformation process. The *Indigofera aspalathoides* extract is found to act as a biofunctional inducer because it induces both Phase I and Phase II enzymes. Such induction may inhibit the formation of covalently bound complexes of 20- Methylcholanthrene with DNA, RNA and protein and this in turn might cause inhibition of tumor process. The aqueous extract of *Indigofera aspalathoides* is also found to be better inducer of Phase II enzymes and hence it is also acts as a potential protective agent against 20-Methylcholanthrene induced fibrosarcoma. Due to the increased activity of GST, the microsome mediated 20-MCA binding to DNA may be reduced in the presence of *Indigofera aspalathoides*. The UDPGT was buried deeply behind a permeability barrier and the enzyme was constrained by same membrane components in a conformation not optional for catalytic activity and this enzyme might have been released from this constraint by the chemotherapy. This might be the reason for the increased activity of UDPG T in *Indigofera aspalathoides* treated animals. The anticancer activity of the plant may be due to its ability in enhancing the activity of Phase I and II enzymes.

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