Anticarcinogenic Effects of Indigofera Aspalathoides on 20-Methylcholanthrene Induced Fibrosarcoma in Rats

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ABSTRACT

Cancer is a ubiquitous disease. Multidisciplinary scientific investigation is making best efforts to combat this disease but the sure shot perfect cure is yet to be brought into the world of medicine. The complementary and alternative medicines such as Siddha and Ayurveda have been the basis of treatment and cure for various diseases and physiological abnormalities in traditional methods under practice. Indian folk medicine comprises of numerous prescriptions for therapeutic purposes which may be as varied as ulcer, leprosy, skin infections and cancer. The anticancer effects of the aqueous extract of Indigofera aspalathoides on 20-Methylcholanthrene induced fibrosarcoma were investigated in male albino rats. Fibrosarcoma was induced by subcutaneous implantation of Millipore filter disc impregnated with 5% suspension of 20-Methylcholanthrene, in paraffin oil and the tumors appeared in about six weeks. The fibrosarcoma was isolated and proved by pathological examinations. Intraperitoneous (i.p.) administration of 250 mg kg\(^{-1}\) b.wt. per day of aqueous extract of Indigofera aspalathoides for 30 days effectively suppressed the 20 Methylycholanthrene induced chemical carcinogenesis as revealed by the reduced incidence of fibrosarcoma in control and experimental animals. The levels of lipid peroxidation and non enzymatic antioxidants such as reduced glutathione, Vitamin C, Vitamin E and total thiols in liver and kidney of control animals were significantly altered. These findings clearly indicate that the antineoplastic efficacy of Indigofera aspalathoids on 20 Methylycholanthrene induced fibrosarcoma on male albino rats.

Key words: Fibrosarcoma, indigofera aspalathoides, chemoprevention, Vitamin C, Vitamin E

INTRODUCTION

A proliferating cell and its contact environment constitute a perfect balanced system in which each component influences the other. This interdependence is involved in all forms of cell division. The sensitive balance between pro oxidant and anti oxidant forces in the body appears to be crucial in determining the state of health and well being. Free radical initiated auto oxidation of cellular membrane lipids can lead to cellular necrosis and is now accepted to be important in connection with a variety of pathological condition (Liu et al., 2004). Living cell evolved defenses against free radicals and the reduction of these peroxyl radicals by anti-oxidant molecules is crucial to the protection of cells against the development of pro oxidant state (Premalatha and Sachdanandam, 1999). Anti-oxidants may protect against Reactive Oxygen Species (ROS) toxicity by prevention of ROS formation, by the interruption of ROS attack, by scavenging the reactive metabolites and
converting them to less reactive molecules and/or by enhancing the resistance of sensitive biological
target to ROS attacks, by facilitating the repair caused by the ROS and by providing co-factors for
the effective functioning of other antioxidants (Sen, 1995). Development of life threatening diseases
like cancer is linked to the availability of these antioxidants (Bakasso et al., 2008). Low levels of
anti-oxidants, which further increase the free radical activity, are clearly associated with cancer
conditions. Fibrosarcoma is the tumor composed of collagen fibers forming mesenchymal cells of
fibroblasts and they arise from subcutaneous tissues (Stout, 1948). The tumor may occur in any
one of the tissue site but is most common in 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 (Greager et al., 1994) including the nasal cavity, para nasal sinuses and
nasopharynx (Heffner and Gnepp, 1992). Rare examples of this tumor have also been reported in
the breast, heart, liver, lung and central nervous system (Jones et al., 1992). In many cases,
surgery and radio therapy fail to cure chiefly because the tumor must have disseminated. For this
reason, chemotherapeutic agents are sought to reduce the patient’s tumor burden so that a cure
may be possible. The major area of cancer chemoprevention that has been intensively studied in
recent years is biological modifiers of cancer cells. These agents are designed to retard the
proliferation of cancer cells and to reduce differentiation of these cells to quiescent, non-dividing
stage and/or to promote to cell death (Logrono et al., 1999). The effective chemoprevention of cancer
could have an important impact on cancer, cancer morbidity and mortality, such as that of
fibrosarcoma. Now a day, chemoprevention is gaining more attention. This approach aims to
decrease overall cancer mortality and morbidity 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. The plant
Indigofera aspalathoides Vahl., belongs to the family Papilionaceae, is a low under shrub with
copiously terete spreading branches. It is found in South India and Sri Lanka and is traditionally
used in treating various skin disorders and tumors (Kirtikar and Basu, 2000). It is found to be
active against transplantable tumors and inflammations (Rajkapor et al., 2005). The leaves,
flowers and tender shoots are said to be cooling and demulcent and are used to treat leprosy,
syphilis and skin disorders (Bhuvaneswari and Balasundaram, 2000). This is one of the important
ingredients of the specific oil for syphilitic and other skin diseases. The aqueous extract of
Indigofera aspalathoides contains mainly saponins, tannins, carbohydrates and steroids that have
the ability to counteract the adverse biological effects of carcinogens.

The parameters, i.e., the levels of lipid peroxidation and nonenzymatic antioxidants were
chosen to find out the drug’s efficacy in controlling them, which have a direct bearing of
fibrosarcoma condition. The results clearly have shown in the current study about the promising
antitumor potency of aqueous extract of Indigofera aspalathoides on 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.
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 chemo-preventive 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) No. 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 1×10⁶ cells in 0.5 mL of saline was injected subcutaneously, into the thigh. The transplanted tumor became palpable in 2 weeks.

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.
Biochemical estimations: Lipid Peroxidation was performed by the method of Devasagayam and Tarachand (1987). The level of Ascorbic Acid was estimated by Omaye et al. (1979). The level of α-Tocopherol was estimated by Desai (1984). Reduced glutathione was estimated by the method of Moron et al. (1979) and non-protein thiols were assayed by the method of Sedlak and Lindsay (1968).

Histopathological analysis: In Group I, the liver sections of control animals showed the normal architecture of the hepatic cells with well preserved cytoplasm. In Group II, the liver sections of fibrosarcoma bearing animals showed mild congestion of sinusoids with central dilatation.

In Group III, the liver sections of fibrosarcoma bearing animals treated with Indigofera aspalathoides showed mild congestion of the sinusoids. In Group IV, the liver sections of drug control animals treated with Indigofera aspalathoides showed near normal architecture of the tissue.

In Group I, the kidney sections of controlled animals showed the normal architecture of the renal cells with regular histology. In Group II, the kidney sections of fibrosarcoma bearing animals showed tubular dilatation with congested vessels. In Group III, the kidney sections of fibrosarcoma bearing animals treated with Indigofera aspalathoides showed near normal histological architecture. In Group IV, the kidney sections of drug control animals treated with Indigofera aspalathoides showed near normal architecture.

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 (Group III) animals were fibrosarcoma bearing animals treated with aqueous extract of Indigofera aspalathoides intraperitonially at a dose of 250 mg kg⁻¹ b.wt. day⁻¹ 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.

Statistical analysis: The results of all the assays were repeated as Mean±SD for groups of 6 rats. Statistically significant differences between the groups were calculated using one way Analysis of Variance (ANOVA) and the Student’s Newmann and Keul test.

RESULTS

Table 1 and 2 show the activities of lipid peroxidation in liver and kidney of control and experimental animals. In both liver and kidney, the basal, hydrogen peroxide, ascorbate and ferrous sulphate induced lipid peroxidation was significantly increased in fibrosarcoma bearing group II animals as compared to group I control animals. The tumor bearing Indigofera aspalathoides treated group III animals showed a significant normalization in the levels of lipid peroxidation as compared to the cancerous animals.

No marked alteration of LPO in group IV drug control animals were observed as compared to the normal animals. This shows that the aqueous extract of Indigogera aspalathoides does not have any side effects.
Table 1: The levels of lipid peroxidation in the liver of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters (μmol of TBARS)</th>
<th>Group I (Control)</th>
<th>Group II (Fibrosarcoma)</th>
<th>Group III (Fibrosarcoma+L. aspalathoides)</th>
<th>Group IV (Indigofera aspalathoides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formed/mg protein/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.54±0.12</td>
<td>1.70±0.11</td>
<td>0.83±0.12</td>
<td>0.57±0.11</td>
</tr>
<tr>
<td>H₂O₂ induced</td>
<td>0.86±0.12</td>
<td>2.12±0.19</td>
<td>1.02±0.14</td>
<td>0.90±0.16</td>
</tr>
<tr>
<td>Ascorbic acid (Induced)</td>
<td>0.63±0.07</td>
<td>1.98±0.15</td>
<td>0.98±0.16</td>
<td>0.62±0.06</td>
</tr>
<tr>
<td>FeSO₄ (Induced)</td>
<td>1.10±0.20</td>
<td>2.06±0.34</td>
<td>1.19±0.22</td>
<td>1.12±0.20</td>
</tr>
</tbody>
</table>

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; TBARS: Thiobarbituric acid reactive substances

Table 2: The levels of lipid peroxidation in the kidney of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters (μmol of TBARS)</th>
<th>Group I (Control)</th>
<th>Group II (Fibrosarcoma)</th>
<th>Group III (Fibrosarcoma+L. aspalathoides)</th>
<th>Group IV (Indigofera aspalathoides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formed/mg protein/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.78±0.14</td>
<td>0.88±0.12</td>
<td>0.87±0.10</td>
<td>0.82±0.19</td>
</tr>
<tr>
<td>H₂O₂ induced</td>
<td>1.02±0.15</td>
<td>2.06±0.17</td>
<td>0.98±0.10</td>
<td>0.90±0.25</td>
</tr>
<tr>
<td>Ascorbic acid (Induced)</td>
<td>1.64±0.21</td>
<td>2.60±0.19</td>
<td>1.75±0.10</td>
<td>1.66±0.34</td>
</tr>
<tr>
<td>FeSO₄ (Induced)</td>
<td>1.56±0.33</td>
<td>2.62±0.19</td>
<td>1.40±0.18</td>
<td>1.56±0.22</td>
</tr>
</tbody>
</table>

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; TBARS: Thiobarbituric acid reactive substances

Table 3: The levels of non-enzymatic antioxidants in the liver of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced glutathione (μg of GSH mg⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control animals</td>
<td>2.98±0.27</td>
<td>2.58±0.29</td>
<td>3.82±0.38</td>
<td>4.19±0.03</td>
</tr>
<tr>
<td>Fibrosarcoma-bearing animals</td>
<td>2.91±0.19</td>
<td>1.42±0.16</td>
<td>2.60±0.29</td>
<td>2.90±0.15</td>
</tr>
<tr>
<td>Reduced glutathione (μg of GSH mg⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control animals</td>
<td>23.70±2.90</td>
<td>14.01±2.70</td>
<td>23.50±1.70</td>
<td>24.06±1.40</td>
</tr>
<tr>
<td>Fibrosarcoma-bearing animals</td>
<td>15.20±0.76</td>
<td>10.24±0.72</td>
<td>14.26±0.66</td>
<td>15.60±0.68</td>
</tr>
</tbody>
</table>

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 4: The levels of non-enzymatic antioxidants in the kidney of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced glutathione (μg of GSH mg⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control animals</td>
<td>4.00±0.27</td>
<td>1.92±0.17</td>
<td>2.84±0.24</td>
<td>2.96±0.31</td>
</tr>
<tr>
<td>Fibrosarcoma-bearing animals</td>
<td>1.48±0.15</td>
<td>0.64±0.16</td>
<td>1.46±0.32</td>
<td>1.52±0.28</td>
</tr>
<tr>
<td>Reduced glutathione (μg of GSH mg⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control animals</td>
<td>18.62±1.44</td>
<td>9.54±1.68</td>
<td>16.10±0.65</td>
<td>18.64±0.90</td>
</tr>
<tr>
<td>Fibrosarcoma-bearing animals</td>
<td>15.20±0.76</td>
<td>5.30±0.28</td>
<td>7.92±0.36</td>
<td>8.16±0.38</td>
</tr>
</tbody>
</table>

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 and 4 show the activities of non enzymatic antioxidants on liver and kidney of control and experimental animals. Reduction in the levels of the antioxidants were observed in the liver and kidney of cancerous animals as compared to control group I.
Interestingly, the reduced levels of non enzymatic antioxidants increased significantly to near normal levels in cancer bearing Indigofera aspalathoides treated animals when compared to group II cancer bearing animals. There was no significant alteration of these antioxidants in plant extract alone treated group IV animals as compared to normal animals. The antioxidant status had improved during the chemotherapy of Indigofera aspalathoides extracts.

DISCUSSION

Free radicals and non radicals oxidizing species are regularly being produced in animals treated with carcinogens and also in human tissues (Sun, 1990), which are capable of damaging DNA, proteins and lipids. The increased levels of lipid peroxides in vitro, i.e., basal and also in vivo, i.e., in the presence of inducers (Hyrogen peroxide, Vitamin C, Vitamin E and Ferrous Sulphate) were observed in cancer bearing animals. The formation of Malondialdehyde (MDA) is considered as an index of lipid peroxidation that causes cell injury. Elevation in lipid peroxidation as indication by increased MDA was observed in fibrosarcoma bearing animals. The naturally occurring free radical scavenger Indigofera aspalathoides lowered the MDA levels suggesting reduced lipid peroxidation, due to its free radical scavenging property. Indigofera aspalathoides helps to improve the antioxidant defense system and prevent the damage induced by free radicals. Induction of 20 Methylcholanthrene generated lipid peroxidation products. In general, significant increases in lipid peroxidation in carcinogenic process may be due to abnormal levels of Reactive Oxygen Species (ROS). ROS production in excess of cellular antioxidant capacity may result in damage to lipid, protein, RNA and DNA as well as other negative effects (Ceutti, 1985; Breimer, 1990). The non enzymatic antioxidants, such as, glutathione, Vitamin C, Vitamin E and total thiols was significantly reduced in fibrosarcoma bearing animals. The anti oxidant status has improved during the chemotherapy of aqueous extract of Indigofera aspalathoides.

The activity of non enzymatic anti oxidants is presumably essential for the removal of radicals (Allen, 1991). Decreased levels of antioxidants, which further increase the free radical activity, are clearly associated with cancer conditions (Barber and Harris, 1994).

Free radicals being formed by reaction of superoxide radicals with Hydrogen peroxide (H₂O₂) produces, hydroxyl radical, a highly destructive radical species that can react with practically any molecule present within the cells. Enzymatic antioxidants are inactivated by these radicals (Wiseman and Halliwell, 1993; Roussyn et al., 1996; Marnett, 2000). GSH, acting through enzymes utilizing it as a substrate or cofactor, is thought to be involved in an important defense mechanism. It conjugates with a variety of endogenous and exogenous compounds and deoxyzifies super oxide and hydroxyl radicals following formation of oxidized glutathione (GSSG). GSH also appears to impart acute cellular effects of oxidation injury induced by hydrogen peroxide.

The water soluble Vitamin C plays several important roles in vivo (Levine, 1986). It is a good scavenger of most reactive oxygen species (Halliwell, 1990) and protects lipid and plasma membrane (Frei et al., 1989) thereby preventing degenerative diseases including cancer (Block and Mankes, 1989). It demonstrates a synergistic interaction with tocopherol radical resulting in the regeneration of α-tocopherol. Ascorbate imparts its protection by undergoing oxidation ultimately forming dehydro ascorbate (Grimble and Huges, 1967). GSH is required for the reduction of dehydroascorbate back to ascorbate.

Vitamin E acts as a biological antioxidant. As a free radical quencher, Vitamin E accounts for much of the lipid soluble chain breaking capacities of the human blood plasma ad erythrocyte membrane (Sies et al., 1992). It is the most important free radical scavenger with membrane
lipoproteins. The free radical clearing ability of this fat soluble Vitamin is due to the delocalization of an unpaired electron in its conjugated double bond system. In bio membranes, Vitamin E has been found to have potent antioxidant activity due to its ability to penetrate to a precise site into the membrane which may be the important feature of protection against highly reactive radicals (Packer et al., 1979).

Thiols are water soluble antioxidants associated with membrane proteins and are important for the antioxidant system. Thiols, which are the main components of intracellular non proteins sulphhydryl groups and they participate in many cellular functions including metabolism of drugs and detoxification of free radicals (Lai et al., 1991).

Total thiols were decreased in 20 methylcholanthrene induced fibrosarcoma conditions. LPO and decreased membrane fluidity in cancer condition decrease the reactivity of thiol groups (Oyashiki et al., 1994).

Administration of Indigofera aspalathoides reveals its effectiveness in affording protection to cell membrane by maintaining the non enzymatic antioxidants, namely GSH, Ascorbic acid, \alpha-tocopherol and thiols. The observation made in this study highlights the antioxidant property of Indigofera aspalathoides in fibrosarcoma bearing animals.

The results of the parameters are a part of the total Ph.D. work. Since there many different parameters were chosen to know the effect of this plant extract on fibrosarcoma, all the results which were published or communicated for publication have proved clearly the anticancer effect of this drug. The other parameters mentioned elsewhere by us a part of the total work to prove this plant’s efficacy as a cancer drug.

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