Antioxidant efficacy of endocarp with kernel of *Ziziphus mauritiana* Lam. in *p*-dimethylaminoazobenzene induced hepatocarcinoma in *Rattus norvigicus*

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Carcinogens, 0.06% of *p*-dimethylaminoazobenzene (*p*-DAB) with feed and 0.05% phenobarbital (PB) with water were orally administered to rats for 60 days. Powdered endocarps of *Ziziphus mauritiana* Lam. were extracted with two different solvents, ethanol and ethylacetate. Oral LD₅₀ of the two extracts were estimated to be 1200.24 mg/kg body weight. Preliminary phytochemical studies of the extracts revealed the presence of triterpenoids, reducing sugars, sugars, alkaloids, phenolics, catechins, flavonoids, saponins, tannins and amino acids. Experimental rats were orally co-fed with the ethanol extract (at two different doses of 300 and 150 mg/kg/bw) and carcinogens. Comparatively, other group was co-fed with ethylacetate extract (at two different doses of 300 and 150mg/kg/b.w) with carcinogens. Antioxidant biochemical studies revealed that ethanol extract was more effective than ethylacetate extract. Histological studies revealed a distinct hepatoprotective effect of *Z. mauritiana* endocarps (with kernel) at 300 mg/kg body weight of ethanol extract, which sustained the normal architecture of liver cells from *p*-Dimethylaminoazobenzene and phenobarbitol induced toxicity, whereas severe damages were evident in the hepatocarcinoma induced rats.

Keywords: *Ziziphus mauritiana*, Indian jujube, Ber, *p*-Dimethylaminoazobenzene, Phenobarbital, Endocarp, Antioxidants.

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Introduction

Liver plays an astonishing array of vital functions in the maintenance and performance of the body. Unfortunately, the liver is after abused by environmental toxins, poor eating habits, alcohol and over the counter drug use, which can damage and weaken the liver reactive oxygen species such as superoxide, hydroxyl radicals and hydrogen peroxide, which are released due to the induction by *p*-dimethylaminoazobenzene (*p*-DAB), which further stimulate the damage of liver by the metabolized products of lipid peroxidation, consequently leading to DNA aberrations. Hydroxyl radicals are the basis for the generation of lipid peroxide products such as malondialdehyde, which are considered to be a cause of carcino genesis. *p*-DAB is an azo dye which is widely used as an coloring agent in polishes, soaps, wax products and also as a food additive; included as a group-2 B carcinogen; teratogenicity and mutagenicity including its dose-response have been reported. Phenobarbital (PB) is carcinogenic to humans, mice and rats when administered orally.

Numerous plant products have been shown to have the anticancer property and the antioxidant vitamin, flavonoids and extensively reported as scavengers of free radicals and inhibitors of lipid peroxidation. Therefore, the present study was initiated, to investigate the anticancer and antioxidant efficacies of ethanol and ethylacetate extracts of the hard endocarp powder of *Ziziphus mauritiana* Lam. in *p*-DAB induced hepatocarcinoma rats through the observation and analysis of biochemical and histological changes.

Z. mauritiana

Materials and Methods

Identification of the plant

*Z. mauritiana*, a shrub or small tree, commonly known as Indian Jujube in English and Ber in Hindi. It is found growing wild or cultivated almost throughout India. Fruits are eaten fresh, dried like raisins, candied, stewed or smoked. Fresh fruits were collected from the forest around His Highness Rajah Thondaiman’s palace, Pudukkottai District, Tamil Nadu, India during the months of January to March
2006. The plant was taxonomically identified and authenticated (Voucher. No: AK001) by Dr. S. John Britto, The Director, The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph’s College, Thiruchirapalli, Tamil Nadu, India.

**Extraction from endocarp**

The fresh juicy pulp layer from the delicious fruits was removed. The hard endocarps along with cotyledon kernels were mechanically cracked, powdered and extracted by hot method with 80% ethanol\(^{11}\) and 80% ethyl acetate using soxhelet apparatus. The two extracts were concentrated to obtain a solid residue and stored in airtight container and kept in refrigerator until use. Preliminary phytochemical studies were performed by the method of Brindha\(^{12}\) et al (1977).

**Acute toxicity studies**

The acute toxicity studies were carried out based on the method described by Lork\(^{13}\). Extract was administered orally to rats and the dose that killed 50% of the animal population was estimated as the LD\(_{50}\).

**Experimental animals**

Healthy male albino *Rattus norvigicus* (wistar strain) weighing 150±10 g were used. Animals were acclimatized to the laboratory condition for 10-15 days before starting the experiments. The rats were fed with standard diet and provided with water *ad libidum*. All the experiments were performed according to the ethical norms approved (No.687/02/a CPCSEA, New Delhi) by Institutional Animal Ethical Committee (IAEC) guidelines.

**Experimental group segregation**

The experimental animals were divided into six groups. A group contains six animals each, (where, n=6). Group 1 served as normal control; Group 2 received carcinogens (0.06% p-DAB + 0.05% PB); Group 3 received ethanol extract (300 mg/kg/day) + carcinogens; Group 4 received ethanol extract (150 mg/kg/day) + carcinogens; Group 5 received ethyl acetate extract (300 mg/kg/day) + carcinogens; Group 6 received ethyl acetate extract (150 mg/kg/day) + carcinogens. All the experimental rats were maintained for 60 days in the laboratory.

**Biochemical Analysis**

*p*-Dimethylaminoazobenzene was purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade. Induction of hepatocarcinoma was performed with 0.06% p-DAB, mixed with feed\(^{14,15}\). An oral dose of 0.06 mL of 0.05% of aqueous solution of PB daily was fed as a hepatocarcinoma promoter with a fine pipette\(^{16}\). Biochemical analysis was carried out after fasting the animals for 12 hours. Animals were sacrificed by cervical decapitation and the blood was collected in two test tubes. Serum and plasma were separated as per the standard procedures. Liver samples were removed and the weight was measured. Homogenization was prepared, using 10% saline. The homogenate was centrifuged at 15000 × g for 30 min at 4ºC and the supernatant was used for the analysis of biochemical parameters. The tissue homogenate was placed at -20ºC. Reduced Glutathione (GSH), Glutathione-S-transferase (GST), Superoxide dismutase (SOD) and Catalase (CAT) activity assays were determined by the methods Moron et al\(^{17}\), Habig et al\(^{18}\), Kakkar et al\(^{19}\) and Sinha\(^{20}\), respectively. Vitamin E and vitamin C contents were determined in the plasma as per the methods of Baker et al\(^{21}\) and Omaye et al\(^{22}\). Lipid peroxidation was evaluated by measuring the hepatic content of thiobarbituric acid reacting substances (TBARS)\(^{23}\).

**Histological analysis**

Small pieces of liver, fixed in 10% neutral buffered formalin were processed for embedding in paraffin. Sections of 5-6 µm were cut and stained with haematoxylin and eosin for histological analysis as per approved procedure\(^{24}\).

**Statistical Analysis**

All the data were subjected to statistical analysis using one-way analysis of variance (ANOVA) and mean values were compared using the Duncan’s multiple range test (DMRT) at 5.0% level of significance. All the data were represented as Mean ± Standard Error of Mean (SEM).

**Results**

Extraction with ethanol and ethyl acetate yielded 10 g/100 g representing 10% of plant material. Phytochemical screening of the two extracts revealed the presence of triterpenoids, reducing sugars, sugars, alkaloids, phenolics, catechins, flavonoids, saponins, tannins and amino acids. The LD\(_{50}\) of the ethanol and ethyl acetate extracts were estimated to be 1200.24 mg/kg in rats. Above this dose the animals exhibited toxic signs, which indicated that the
extracts might not be relatively safe. In p-DAB+PB intoxicated rats, a marked decrease in the activities of Reduced Glutathione (GSH), Glutathione-S-transferase (GST), Superoxide dismutase (SOD), Catalase (CAT) and the concentration of vitamin C and E was observed than control rats (Table 1). A significant increase in lipid peroxides (LPO) was observed in p-DAB+PB intoxicated rats. Oral administration of ethanol and ethyl acetate extracts at a dose of 150 and 300 mg/kg b w/day in p-DAB+PB intoxicated rats showed an increase in the activities of Reduced Glutathione (GSH), Glutathione-S-transferase (GST), Superoxide dismutase (SOD), Catalase (CAT) and contents of Vitamin C and Vitamin E than carcinogen intoxicated rats. Oral administration of ethanol extract in p-DAB+PB intoxicated rats at a dose of 300 mg/kg b w/day showed a remarkable increase in the activities of GSH, GST, SOD, CAT and in the contents of vitamin C and vitamin E 73.39, 89.92, 89.51, 90.92, 88.29 and 96.22%, respectively, but the administration of 300 mg/kg b w/day of ethyl acetate extract in p-DAB+PB intoxicated rats at a dose of 300 mg/kg b w/day resulted in a mere increase in the activities of GSH, GST, SOD, CAT and in the contents vitamin C and vitamin E 68.42, 74.62, 86.39, 88.51, 83.98 and 92.77%, respectively.

Comparatively, oral administration of ethanol extracts of endocarp with seeds of Z. mauritiana excelled over the ethyl acetate extracts in antioxidant enzyme activities and vitamin contents. Histological studies revealed that, in normal control rats the hepatic portal vein and hepatic cells were very compact and well distributed with junction complexes (Plate 1b). In p-DAB + PB orally administered rats, the normal architecture of hepatoma was completely lost, the hepatic veins were highly damaged (Plate 1a). The livers of rats treated with Z. mauritiana ethanol extract at a dose of 300 mg/kg (Plate 1d) showed a significant recovery from p-DAB + PB intoxicated liver damage as apparently evident from normal well defined nuclei. Vacuolization were remarkably prevented by the treatment with extracts. The rat’s liver treated with 150 mg/kg of ethanol extract (Plate 1c) also showed recovery but nucleus was damaged and infiltration of numerous blood cells was visible inside the hepatic portal vein. Histology of the liver tissues revealed that a dose dependent response; in carcinogens intoxicated rats (Plate 1a) the hepatic veins were highly damaged,
blood cells and hepatic cells were disintegrated, nucleus were enlarged, whereas in control (Plate 1b) the hepatic portal veins and the hepatic cells were normal and there was no accumulation of blood cells inside the hepatic portal vein. In the rats treated with carcinogen and *Z. mauritiana* endocarp (with kernel) ethanol extract (150 mg/kg/day) (Plate 1c), the hepatic portal veins were not damaged much that of the carcinogens alone fed rats. But hepatic portal vein was largely accumulated with blood cells. Hepatic cells were distinct and were not much damaged or disintegrated. In the rats treated with carcinogen and *Z. mauritiana* endocarp (with kernel) ethanol extract (300 mg/kg/day) (Plate 1d), the hepatic portal veins were less damaged, accumulation of blood cells were lesser, hepatocytes and nucleus appeared normal.

**Discussion**

Abalaka *et al.*^25^ detected some of the organic compounds in the ethanolic extracts of *Z. mauritiana* leaves and reported tannins, saponins, resins, polyphenols and cardiac glycosides. Similarly *Z. mauritiana* endocarp extractions indicated the presence of triterpenoids, reducing sugars, sugars, alkaloids, phenolics, catechins, flavonoids, saponins, tannins and amino acids. Li *et al.*^26^ identified *Z. mauritiana* varieties for soluble sugars (fructose, glucose, rhamnose, sorbitol and sucrose). They found high amount of fructose, glucose, potassium, phosphorus, calcium and vitamin C including soluble and insoluble dietary fiber. Similarly, the phytochemical analysis of the two extracts from *Z. mauritiana* indicated the presence of sugars and reducing sugars. Li *et al.*^27^ revealed the antioxidant properties in *Z. mauritiana* with different contents of phenolic and other phytochemicals like ascorbic acid, tocopherol and pigments in different varieties. Similarly phenolics are present in the two extractions from *Z. mauritiana* Lam. Mahajan and Chopda^28^ (2009) reported that alkaloids are distributed in all parts of *Z. jujuba* Mill. Stem bark of *Ziziphus* species contain alkaloids^29^. The seeds of *Z. jujuba* var. *spinosa* also contain cyclic peptide alkaloids sanjoinenine, franguloine and amphibine-D.

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Plate 1 – Photograph showing histology of hepatoprotective and antioxidant efficacy of *Ziziphus mauritiana* endocarp and kernel extract in p-DAB+ PB intoxicated rats (haematoxylin and eosin x 40 view)—a. p-DAB+ PB intoxicated; b. normal control; c. *Z. mauritiana* endocarp and kernel ethanol extract (150 mg/kg body wt/day); d. *Z. mauritiana* endocarp and kernel ethanol extract (300 mg/kg body wt/day)
and four peptide alkaloids; sanjoinine-B-D-F and -G2. The cyclopeptide alkaloid, mauritine J, was isolated from the root bark of Z. mauritiana. For the first time Tschesche reported six cyclopeptide alkaloids isolated from the stem bark of Z. jujuba. Similarly the phytochemical analysis revealed the presence of alkaloids in present study. Pawlowska et al. reported phenolic compounds from the fruit of Z. jujuba. Similarly the present study reports the presence of phenolics in the two extractions. Polyphenolic compounds have been shown to protect various cell types from oxidative stress mediated cell injury. Flavonoids such as Swertish and spinosin were isolated and reported by Gong et al. from fruit and seeds of Z. jujuba. Ten flavonoids were reported by Pawlowska et al. Phytochemical analysis indicated the presence of flavonoids in extracts used in this study. Flavonoids have been reported to inhibit peroxidation of polyunsaturated fatty acids in cell membranes. These substances could react with the deeper membrane domains and intracellular structures and protect the cells from oxidant injury. The saponins isolated from the seeds of Z. jujuba. Kurihara et al. extracted the saponin, ziziphin from the dried leaves of Z. jujuba. Saponins are widely researched for cancer prevention as mentioned by Ogihara et al. Similarly, present phytochemical analysis indicated the presence of saponins. Comprehensive and an exhaustive account on 70 antioxidant Korean medicinal plants have been reported by Seong et al. and they confirmed antioxidant effect of Z. jujuba (in vitro) as reported by Na et al. Dahiru and Obidao reported that phytochemical compounds present in the aqueous extract of Z. mauritiana leaf with possible antioxidant activity are tannins, phenolic compounds and flavonoids. Tannins are known to exert antihepatotoxic action. Similarly, we also found presence of tannins. The basis for the hepatoprotective action of tannins has been attributed to the formation of an impervious polyphenol-protein and/or polysaccharide layer under which the natural healing processes can occur. Therefore, it is apparent that a consortium of combating activities of all these phytochemicals is involved in the antioxidant mechanism.

In the present study, the administration of (0.06%) \(p\)-DAB+ (0.05%) PB significantly reduced the activity of GSH, GST, SOD, CAT and the level of vitamin C and E and MDA concentration was found to be increased with significance. The decreasing trends in the \(p\)-DAB+PB administered rats might be due to the carcinogenicity of the chemicals. Similarly, carcinogenesis may be considered as a form of toxicity in which the cell achieves a different stage from the normal and oxidative damage and it has been postulated to play a major role in the mechanism of carcinogenicity in mammalian species. Oxidative stress has been suggested to play a key role in some physiological conditions and in many disease processes, including cancer prevention as mentioned by Na et al. Dahiru et al. reported that the ethanol extract of Z. mauritiana leaves (200 and 300 mg/kg bw) significantly restored the levels of glutathione and vitamin E compared to group treated with \(CCl_4\) alone, who further reported that, treatment with 300 mg/kg bw of the extract had better lowering effect on the enzyme markers and other parameters of liver tissue damage compared to group pretreated with 200 mg/kg bw of the extract. GSH metabolism is one of the most essential antioxidative defense mechanisms. GST plays a major physiological role in initiating the detoxification of electrophilic ultimate carcinogens. Chemicals like \(CCl_4\) and \(p\)-DAB alter the hepatic GST activity. In this present study, GSH has shown a significant alteration in activity in carcinogen alone intoxicated rats. In the carcinogen intoxicated rats when treated with ethanol extract of Z. mauritiana (300 mg/kg bw) the GSH level was restored almost close to normal control rats. It might be due to the detoxification activity of the active principles present in the plant seed extract which may enhance the protective activity of the liver. GST catalyzes the reaction between GSH and either hydrophobic or electrophilic compounds depletion in GSH is also associated with the activity of glutathione reductase (GR) and GSH directly reduces the radicals that are crucial to tumour enhancement, who further stated that GST catalyzes the reaction between
GSH and either hydrophobic or electrophilic compounds. The extract treated rats shown a remarkable alteration with much significance in the SOD activity in the carcinogen fed rats and the SOD activity was retained almost close to normal, in rats treated with the ethanol extract at a dose of 300 mg/kg bw. The most important enzyme in antioxidant defense is SOD, whose main function is to remove $\text{O}_2^-$ radicals$^{69}$. The significant decrease in the activity of CAT might be due to the toxicity and utilization of catalase enzymes in detoxification process in the carcinogen treated rats. CAT is a hemoprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals$^{60}$. In the present study, the retention of CAT activity was evident in the carcinogen intoxicated rats treated with the ethanol extract at a dose of 300 mg/kg bw. It might be due to the protective efficacy of the phytochemicals present in the seed and endocarp extracts. The elevated levels of MDA were observed in carcinogens fed animals, which lead to the hepatic damage indicating over production of free radicals and/or the inability of the antioxidant defense system. Similarly, Simmons$^{61}$ also suggested that, elevated levels of MDA in $p$-DAB induced group clearly reflect the over production of free radicals and/or the inability of antioxidant defense system. An elevation to retain normal levels was evident in the carcinogen intoxicated rats treated with the ethanol extract at a dose of 300 mg/kg bw.

Conclusion

Thus, Z. mauritiana Lam. ethanol extract of endocarp along with seeds is rich in antioxidant principles and properties and it is confirmed by a remarkable anti-hepatotoxic and liver amelioration activity. Moreover, clinical studies are required to evaluate the real therapeutic value of this natural endocarp along with seed extract to contribute to the healthcare industries across the globe.

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