

Antioxidant and Hepato Protective Effect of *Myristica malabarica* Seed Aril Extracts on Carbon Tetrachloride Induced Hepatic Damage

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Abstract: The antioxidant activities of the ethanol extract, its benzene and chloroform fraction from seed aril of *Myristica malabarica* (Myristicaceae) were assessed in an effort to validate the hepatoprotective potency of this plant. The extract and the fractions showed scavenging of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibition of ABTS radical *in vitro*. Antioxidant activities of the extracts were also demonstrable *in vivo* by the inhibition of the carbon tetrachloride (CCl₄) - induced formation of lipid peroxides in the liver of rats by pretreatment with the extracts. CCl₄ - induced hepatotoxicity in rats, as judged by the raised serum enzymes, aspartate transaminase, alanine transaminase and alkaline phosphatases was prevented by pretreatment with the extracts/fractions, demonstrating the hepatoprotective action. Among the tested extracts benzene fraction recorded highest efficiency in protecting liver damage induced by toxic effect of CCl₄ followed by crude ethanolic extract and chloroform fraction. These findings were confirmed by histopathological study of the liver sections of the treated groups.

Key words: *Myristica malabarica* • Antioxidant activity • Hepatoprotective activity • Ethanol extract • Benzene fraction • Chloroform fraction

INTRODUCTION

The plant *Myristica malabarica* L. (Myristicaceae) is known for many medicinal properties such as indigestion, ulcers, wounds, aphrodisiac, as rejuvenator, in treating inflammation, cough, diarrhea, dropsy, liver disorders, paralysis, rheumatism, urinary calculi, vomiting [1], also used in bronchitis, fever, burning sensation, to relieve pain in muscles, sprains and sores [2]. The plant contains many active constituents viz. 7, 4-dimethoxy-5 hydroxyl isoflavone, biochanin A, prunetin, 1, 3-diarylpropanol and alpha-hydroxyldihydrochalcone [3], 2-acylresorcinol, diarylnonanooids, malabaricone C [4], malabaricone A [5], Malabaricones A-D, diarylnonanooids [2]. The plant is known for its antioxidant activity [4, 6]. In the present communication, the antioxidant and hepatoprotective potency of ethanol extract of seed aril and its benzene and chloroform fraction against CCl₄ induced hepatic damage is discussed.

MATERIALS AND METHODS

Collection of Plant Material and Extraction: Seed aril of *Myristica malabarica* were collected from the Sirsi range forest, Karnataka State, India, during December 2008. Taxonomic authenticity was confirmed by the first author and voucher specimens are deposited in the departmental herbaria, (BKM-133, BKM-134) as authentic specimen for future reference. The seed aril was shade dried, powdered mechanically (Sieve No. 10/44) and stored in airtight containers. About 250 g of the powdered material was subjected to soxhlation. It was first defatted with petroleum ether (Hi-Media, Bangalore) and then exhaustively extracted with 70 % ethanol (Hi-Media, Bangalore) for 48 hrs. The solvent was distilled off at low temperature under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland). The yield was 29.3 % w/w. The extract was subjected to preliminary phytochemical tests [7]. The extract was refluxed with

chloroform and Benzene separately. The yield was 10gm and 7gm respectively. The fractions were concentrated to dryness.

Free Radical Scavenging Activity by DPPH Method:

The ability of the extract to scavenge the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was evaluated as described by Sanchez-Moreno *et al.* [8] with minor modifications. A methanol DPPH solution (100 μ M) was mixed with serial dilutions (200 to 1000 μ g) of crude extract and fractions. Vitamin C is used as standard. After 10 min, the absorbance values (A) were recorded at 490 nm and converted into the percentage of scavenging capacity using the following equation:

$$\text{Scavenging capacity (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}})] \times 100$$

Free Radical Scavenging Activity by ABTS Method:

To determine ABTS radical scavenging assay, the method of Re *et al.* [9], with slight modification was adopted. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.703 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance (A) was taken at 734 nm after 10 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as ABTS radical scavenging activity (%) = $[(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}})] \times 100$

Where A_{control} is the absorbance of ABTS radical + methanol; A_{sample} is the absorbance of ABTS radical + extract / standard.

Evaluation of Hepatoprotective Activity

Drug Formulation: Oral suspensions containing 100 mg/ml of the hydro-alcohol extract and 50 mg/ml of benzene and chloroform fractions were prepared in 1% W/V gum tragacanth.

Animals: Fifty male Wistar albino rats, weighing 150-200 g/rat were procured from the National College of Pharmacy, Shivamogga and were maintained at standard housing conditions. The animals were fed with commercial diet and water *ad libitum* during the experiment. The study was permitted by the Institutional Animal Ethical Committee with Reg. No. 144/1999/CPCSEA/SMG.

Acute Toxicity Studies: Acute toxicity study was conducted for the extracts by stair case method [10]. The extract was found to be non toxic up to the dose of 2000mg per kg b.w.

Grouping of Animals: The animals were divided into six groups of six rats each. The animals in group I served as control and received the vehicle (1ml/kg/day of 1% w/v gum tragacanth p.o./ per os/orally) for 14 days. All the animals of group II to VI received 0.1 ml/kg/day of carbon tetrachloride intra peritoneally (Hi-Media, Bangalore) for 14 days. Group III animals received the standard drug Silymarin (Ranbaxy Lab. Dewas) in the dose of 100 mg/kg/day/rat for 14 days. Ethanol extract was administered to the animals of group IV in the dose of 100 mg/kg/day/ rat for 14 days. Chloroform and Benzene fraction were administered to the animals of group V and VI in the dose of 50 mg/kg/day/ rat, respectively for 14 days. The carbon tetrachloride, Silymarin and the extracts were administered concomitantly to the respective groups of animals.

The animals of all the groups were sacrificed on 14th day under light ether anesthesia. The blood sample of each animal was collected separately by carotid bleeding into sterilized dry centrifuge tubes and allowed to coagulate for 30 min at 37°C. The clear serum was separated at 2500 rpm for 10 min and was subjected to biochemical investigation *viz.* total bilirubin [11], total protein [12], serum alanine transaminase, aspartate transaminase [13] and alkaline phosphatase [14]. Results of biochemical estimations were recorded as mean \pm SE of six animals in each group. The data was subjected to one way ANOVA followed by Dunnett's test. p values ≤ 0.001 was considered as statistically significant.

Histopathology: The liver samples were excised from the experimental animals of each group and washed with the normal saline. Initially the materials were fixed in 10% buffered neutral formalin for 48 h and then with bovine solution for 6 h. They were processed for paraffin embedding. The sections were taken at 5 μ thickness using microtome, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin [15]. The sections were examined microscopically for the evaluation of histopathological changes.

RESULTS

Results of DPPH scavenging and ABTS scavenging studies are depicted in Tables 1 and 2 and figures 1 and 2, respectively. The anti oxidant studies revealed that both crude extract and benzene fraction are good antioxidants.

Table 1: Percentage free radical scavenging activity of Crude extract and Benzene fraction and ascorbic acid in DPPH method

Concentration ($\mu\text{g/ml}$)	Crude extract (M \pm SE)	Benzene fraction (M \pm SE)	Ascorbic acid (Standard) (M \pm SE)
10	8.75 \pm 0.07	25.35 \pm 0.11	20.63 \pm 0.12
20	31.25 \pm 0.16	35.21 \pm 0.09	39.38 \pm 0.16
40	46.88 \pm 0.20	51.25 \pm 0.14	65.00 \pm 0.24
60	63.75 \pm 0.18	67.61 \pm 0.16	85.00 \pm 0.22
80	73.13 \pm 0.20	86.6 \pm 0.22	96.25 \pm 0.18

Table 2: Percentage free radical scavenging activity of Crude extract and Benzene fraction and ascorbic acid in ABTS method

Concentration ($\mu\text{g/ml}$)	Crude extract(M \pm SE)	Benzene fraction (M \pm SE)	Ascorbic acid (Standard) (M \pm SE)
10	29.50 \pm 0.15	19.95 \pm 0.12	15.65 \pm 0.15
20	46.58 \pm 0.09	34.00 \pm 0.21	37.41 \pm 0.24
40	60.23 \pm 0.13	50.57 \pm 0.13	61.16 \pm 0.16
60	67.01 \pm 0.24	63.78 \pm 0.15	82.93 \pm 0.45
80	78.41 \pm 0.21	79.66 \pm 0.22	99.50 \pm 0.26

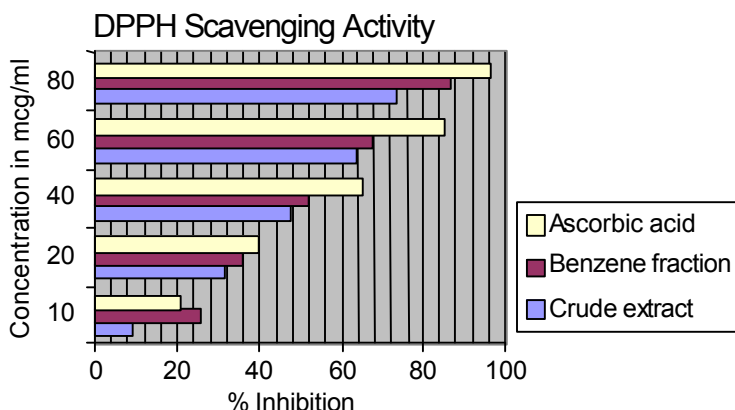


Fig. 1: Graph showing percentage free radical scavenging activity of crude extract and Benzene fraction and ascorbic acid in DPPH method

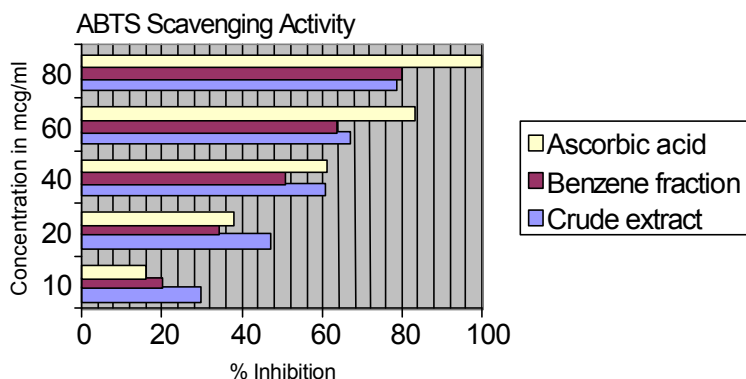


Fig. 2: Graph showing percentage free radical scavenging activity of crude extract and Benzene fraction and ascorbic acid in ABTS method

Fig. 1 shows the dose response chart of DPPH radical scavenging activity of crude extract and benzene fraction compared with ascorbic acid. Among the two samples tested, benzene fraction showed significant activity and this activity was comparable to ascorbic acid. But at 10 $\mu\text{g/ml}$, the activity of benzene fraction is more than the

ascorbic acid. Fig. 2 shows the dose response chart of ABTS scavenging activity of crude extract and benzene fraction compared with ascorbic acid. In this, the crude extract showed significant activity than the benzene fraction and it is significantly higher than the activity of ascorbic acid at 20 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$.

Table 3: Effect of benzene seed aril extracts of *Myristica malabarica* on carbon tetrachloride induced hepatotoxicity in rats

Group (N)	Total Bilirubin (mg/dl)	Total Protein (gm %)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control (1% w/v gum tragacanth p.o.)	0.462±0.02	9.843±0.113	53.04±0.611	151.8±0.323	184.6±2.134
Carbon tetrachloride	2.683±0.11	5.813±0.003	1490.7±0.115	2148.3±0.134	433.15±1.144
Carbon tetrachloride + Silymarin	0.521±0.001	9.314±0.012	205.12±0.132	212.3±0.145	180.24±1.004
Carbon tetrachloride + ethanol extract	0.583±0.003	9.012±0.001	215.7±0.143	232.14±0.131	231.35±1.024
Carbon tetrachloride + chloroform fraction	0.883±0.21	8.913±0.13	290.7±0.02	248.1±0.24	283.15±0.14
Carbon tetrachloride + Benzene fraction	0.543±0.004	9.104±0.020	206.31±0.1	215.13±0.143	185.13±1.025
ANOVAF	9.86	11.19	1018.41	120.25	11.295
df	4,25	4,25	4,25	4,25	4,25
P	<0.001	<0.001	<0.001	<0.001	<0.001

N=six animals in each group. Values are expressed as mean ±SE.

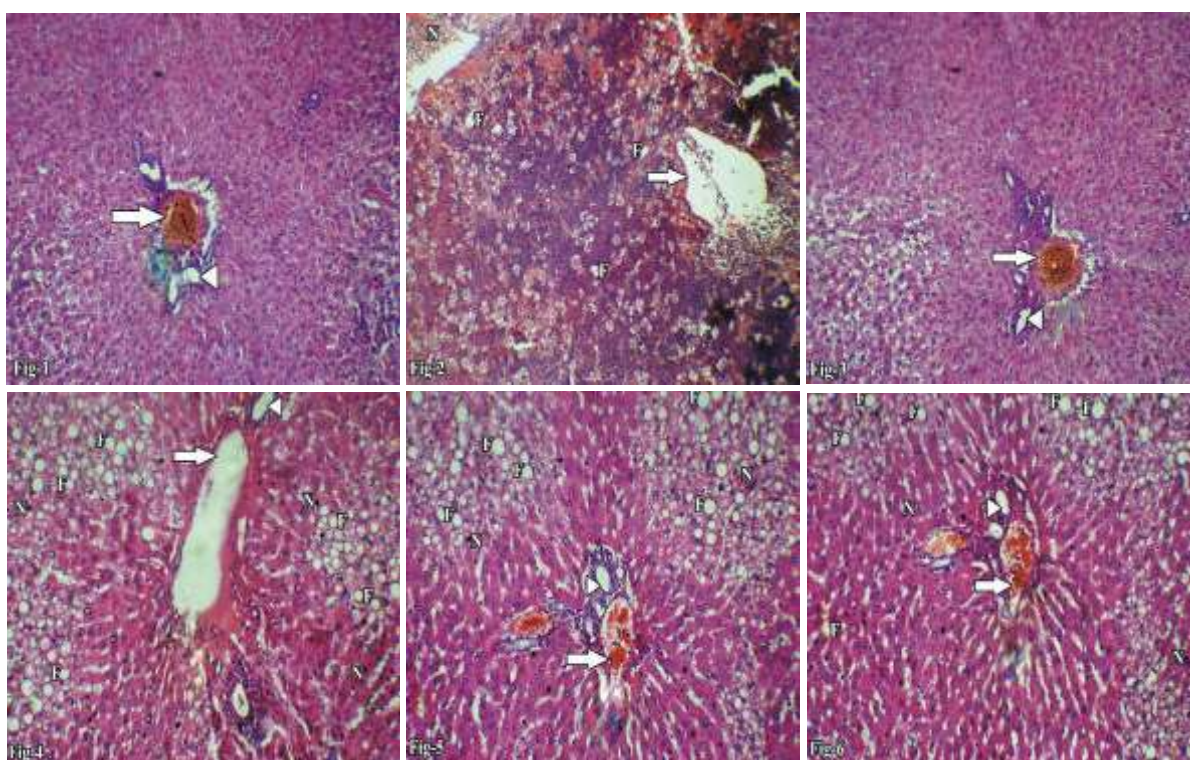


Plate - 1

- Fig. 1: Section of the liver tissue of control animal showing normal histology, portal triad showing portal vein (V), portal artery (arrow), hepatic duct (arrow head). (H and E, 100X).
- Fig. 2: Section of the liver tissue of animal treated with CCl_4 showing necrosis (N) and fatty vacuole (F) and central vein (V). (H and E, 100X).
- Fig. 3: Section of the liver tissue of Silymarin treated animals showing normal hepatocytes, portal vein (V), portal artery (arrow), bile duct (arrow head). (H and E, 100X).
- Fig. 4: Section of the liver tissue of Ethanol seed aril extract treated animals showing normal arrangement of hepatocytes around the portal vein (V), necrosis and moderate accumulation of fatty vacuoles (F). (H and E, 100X).
- Fig. 5: Section of the liver tissue of Chloroform fraction treated animals showing normal arrangement of hepatocytes around the portal vein (V), portal artery (arrow), bile duct (arrow head), necrosis and few fatty vacuoles (F). (H and E, 100X).
- Fig. 6: Section of the liver tissue of Benzene fraction treated animals showing normal arrangement of hepatocytes around the portal vein (V), portal artery (arrow), bile duct (arrow head), absence of necrosis and few fatty vacuoles (F). (H and E, 100X).

Effect of seed aril ethanol extract and its benzene and chloroform fractions of *Myristica malabarica* on carbon tetrachloride induced liver damage in rats with reference to biochemical changes in serum is shown in Table 4. At the end of 14 days treatment, blood samples of carbon tetrachloride treated animals showed significant increase ($p < 0.001$) in the levels of total bilirubin (2.683 ± 0.11), alanine transaminase (2148.3 ± 0.134), aspartate transaminase (1490.7 ± 0.115) and alkaline phosphatase (433.15 ± 1.144) but the total protein level decreased (5.813 ± 0.003) reflecting the liver injury caused by carbon tetrachloride, whereas blood samples from the animals of group IV, V and VI treated with hydro-alcoholic, chloroform and benzene fraction showed significant decrease in the levels of serum markers (Table 2) indicating the recovery of hepatic cells. Among the three groups treated extracts studied, the benzene fraction showed significant reduction in total bilirubin (0.543 ± 0.004), alanine transaminase (215.13 ± 0.143), aspartate transaminase (206.31 ± 0.1), alkaline phosphatase (185.13 ± 1.025) and increase in total protein (9.104 ± 0.020) indicating the potency of benzene fraction against carbon tetrachloride induced hepatic damage.

Histological profile of control animal showed normal hepatocytes (Plate 1, Fig. 1), the section of liver of the group II animals exhibited severe intense centrilobular necrosis (N), vacuolization and macrovesicular fatty changes (F) (Plate 1, Fig. 2). The liver tissue sections of Silymarin treated animals showed normal hepatic architecture (Plate 1, Fig. 3). The liver tissue sections of the animals treated with ethanol extract and chloroform extract exhibited moderate accumulation of fatty lobules (Plate-1, Figs. 4 and 5, respectively) indicating the partial recovery where as the liver section of animals treated with benzene fraction showed significant liver protection against carbon tetrachloride induced liver damage as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration (Plate-1, Fig-6).

DISCUSSION

Carbon tetrachloride induced hepatic injury is the common model used for hepatoprotective drug screening [16]. Several studies have demonstrated that CCl_4 modulate toxic effects through its haloalkane metabolites. These reactive metabolites are produced during biotransformation of CCl_4 and these metabolites may cause the oxidative damage of lipids, lipoproteins and other cellular components, such as enzymes, DNA and proteins. The oxidative damage due to excessive

production of haloalkane radicals, can damage tissues and cells by alteration of lipid peroxidation, protein or nucleic acid structure and function. Thus, abnormal levels of the liver enzymes in plasma are usually indicative of the hepatic cellular injury in experimental animals.

The extent of hepatic damage is assessed by the elevated level of biochemical parameters which are attributed to the generation of trichloromethyl free radicals during metabolism by hepatic microsomes which in turn cause peroxidation of lipids of cellular membrane [17]. Hepatocellular necrosis leads to very high level of aspartate transaminase and alanine transaminase released from liver to blood. Among the two, alanine transaminase is a better index for liver injury, as its activity represents 90 % of total enzyme present in the body [18]. ALP activity on the other hand is related to the functioning of hepatocytes and increase in its activity is due to increased synthesis in presence of increased biliary pressure [19].

Recent findings indicate that the hepatitis could be handled effectively if the drug possesses antioxidant and anti-inflammatory property [20], as the liver protection and proliferation of hepatocytes accelerate in the presence of antioxidants. In the present study it was evident that, the benzene fraction and the crude ethanol seed aril extract has potent antioxidant activity. Hence the hepatoprotective potency of the plant could be attributed to its antioxidant property.

Based on the above results of the pharmacological screening, it can be concluded that Benzene seed aril extracts of *Myristica malabarica* possesses significant hepatoprotective activity, which provides scientific evidence to the ethnomedicinal use of this plant species used by the tribal group of Western Ghats in treating hepatitis.

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