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HEPATOTOXIC EFFECT OF METHANOLIC EXTRACT FROM NUTMEG (*MYRISTICA FRAGRANS*) COMPARED TO CARBON TETRACHLORIDE- INDUCED HEPATIC DAMAGE IN MICE

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ABSTRACT
This study was conducted with the aim of extracting methanolic extract containing flavonoids, alkaloids, tannins, terpenes, resins and phenols from dried seeds of nutmeg (*Myristica fragrans)* available in Iraqi markets. The study also employed an *in vivo* evaluation of the hepatotoxic effect of methanolic extract in male albino mice at different concentrations (500 and 1000 mg / kg) given orally for 7 days including biochemical functions SGPT, SGOT and SALP, bilirubin and histopathlogical changes. At day 8 the animal was sacrificed by anesthetic ether and the liver is weighed and kept in 10% formalin for preparation of histopathological sections. The serum was isolated from the blood for the biochemical tests. Statistical results showed the absence of any significant changes on body weight and liver weight of nutmeg treated mice. However nutmeg treated mice showed statistically significant alteration in the biochemical indicators of liver function including significant elevation in SGPT, SGOT, SALP and TSB in a dose dependent manner. Examination of the liver tissue confirmed potential histopathological effects for nutmeg as evident fatty changes and necrosis in nutmeg treated mice in a dose dependent manner.

KEY WORDS: Carbon tetrachloride, Hepatotoxic, Methanolic extract, *Myristica fragrans*,

INTRODUCTION

Nutmeg belongs to the family Myristicaceae, with about 18 genera and 300 species, the genus *Myristica* is distributed from India and South-east Asia to North Australia and the Pacific Islands. The whole or ground nutmeg, the dried seed of the evergreen nutmeg tree is readily and widely available common household spice. Besides nutmeg and mace, a number of other products, namely oleoresin, nutmeg butter and essential oils, these value-added products find different uses in the food, medicine and perfume industries (Krishnamoorthy and Rema, 2000). Extensive analyses have been carried out on the methanolic extract of nutmeg and these have provided the major classes of compounds constituting the oil as: monoterpene hydrocarbons, 61–88%; oxygenated monoterpenes, aromatic ethers, sesquiterpenes, aromatic monoterpenes, alkenes, organic acids and miscellaneous compounds (Leela, 2008).

The ethanolic extract of nutmeg kernels showed hypolipidaemic effect in albino rabbits, the administration of 500 mg/kg of the extract daily for a period of 60 days in the hyperlipidaemic rabbits resulted in significantly lower levels of lipoprotein lipids (total cholesterol: 574 ± 61 versus 210 ± 27 mg/dl; low-density lipoprotein (LDL) cholesterol: 493 ± 57 versus 131 ± 25 mg/dl; and triglycerides: 108 ± 14 versus 67 in control versus experimental) (Ram *et al*., 1996). Aqueous extract of nutmeg protected rats against hyperglycaemia, hyperlipidaemia and cardiac tissue damage following myocardial infarction (Abdul Kareem *et al*., 2009). This is could be due to effective quenching of free radicals by active phytochemicals in nutmeg such as flavenoids and cardiac glycosides (Olaleye *et al*., 2006) in conjunction with its antioxidant properties (Suchandra *et al*., 2007). The aim of this study to show the effect of the methanolic extract on male mice compared to that caused by carbon tetrachloride as a hepatotoxic model.

MATERIALS AND METHODS

Dried seeds of *Myristica fragrans* were collected from local market in Baghdad during September / 2009 and identified by the botanist professor Ali Almosawi, Department of Biology, College of Sciences / Baghdad University. The covers of the seeds were removed by hand and the dried kernel was crushed to small pieces by mortar and pestle then grinded by a coffee grinder to a fine powder and stored in a closely tight container until used. (It is preferred to be used directly after grinding). The extract was prepared according to the method used (Ozaki *et al*., 1989) with some modification. 50 gram of the crude powder of seeds was refluxed with 350 ml of 70% methanol (1:7) in soxhlet apparatus for 8 hours. The extract was than filtered through a filter paper and evaporated to dryness under vacuum at 40° C, and the dried extract was weighed and stored at 4° C and used for studying hapatotoxic.

Mice weighting between 25-28 g about six weeks old obtained from the Institute of Embryo Researches and Infertility Treatment/ Al-Nahrain University were used as animal models. The mice divided into four groups, each group consisting of 10 animals. Hepatotoxic effect of *Myristica fragrans* was evaluated using CCI_4 –induced model (Torres-Duran *et al*.,1998). Group one was kept on normal diet and served as control, the second group received CCI_4(0.5 ml/kg)orally to induce liver damage in mice and served as positive control, the third and fourth group received *Myristica fragrans* methanolic extract 500 and 1000 mg/kg respectively once daily, for eight days.

Acute toxicity testing: The nutmeg methanolic extract was administered in a dose of 500, 1000, mg/ kg to groups of mice (n= 5) and the number of animals dying within 24 hr was observed in each group (Sonavane *et al*., 2001).

Preparation and analysis of samples for evaluation of hepatic injury:
The serum enzymes tests that have proved useful for evaluation of experimentally- induced hepatic injury according to (Said, 2005).

Preparations of Post-mortum Serum Samples: After sacrificing the animals by anesthetic ether, blood was collected from the animals by intracardiac puncture using insulin syringe. The clot was dispersed with glass rod and then centrifuged at 3000 rpm for 15 minute; the serum was used for the estimation of serum glutamic pyruvic transaminase (SGPT),serum glutamic oxaloacetic transaminase (SGOT) and serum alkaline phosphatase (SALP) as parameters of liver function tests and serum total bilirubin (TSB) as excretory function test (Provan and Krentz , 2002). The blood obtained from each mice ranging from 0.7-1 ml.

Histopathological Examination:
Liver tissues were prepared for histopathological examination according to (Junqueira *et al*., 1995) using paraffin sections technique. Liver samples were fixed in 10% formaldehyde solution, and then dehydrated using increasing strengths of ethanol (70%, 80%, 90% and 100%) for two hours each. Cleaning of tissues using xylene were done, then impregnated with paraffin wax, heated for two hours in the oven at 60 C° and blocked by pouring in embedded models. Blocks were cut by microtome (Reichert Jung) into 5 micron thick sections, floated in water bath and left in oven for dewaxing, hydrated using decreasing strengths of ethanol (90% , 80% and 70%) for 10 minutes each , stained with haematoxyline and eosin , and examined under light microscope.

Statistical Analysis: The significance of differences between the mean values was calculated using unpaired Students't-test. Multiple group comparisons were made using analysis of variance (ANOVA) (Dunean *et al.*, 1983).

RESULTS AND DISCUSSION
The phytochemical detection of methanolic extract showed the presence of flavonoids, alkaloids, tannins, terpenes, phenols, glycosides, resins and the absence of saponins and coumarines. Table (1) shows the results of mean body weights (g) for mice before and after treatment. CCl_4 – treated mice (group II) showed no significant ($p>0.05$) decrease in the mean of body weight compared to the control group (group I).

Table 1. Effect of different doses of *Myristica fragrans* methanolic extract on enzyme liver activity in mice.
Each value represent mean + SD
Values with non-identical superscripted (a, b, c, d, eand f) are considered as significantly different

Treatment	Liver (wt/100g body wt)	Dose (mg/kg)	SGOT U/L	SGPT U/L	SALP U/L	TSB mg/dI
Control	5.95+ 0.12	-	61.9+0.8e	33.5+1.1f	64.03+1.5f	0.34+0.02f
CCl_4	9.13±0.20^{a}	-	195.0±4.3^{a}	110.10+2.8^{a}	157.00+5.6^{a}	1.30+0.06^{a}
Methanolic extract + CCl_4	6.27±0.20^{b}	500mg/kg	68.05±0.7^{d}	39.25+0.6^{e}	71.18+1.3^{e}	0.42+0.01^{e}
Methanolic extract+ CCl_4	6.58±0.10^{b}	1000mg/kg	73.48±1.8^{c}	43.42+1.4^{d}	79.26+0.8^{d}	0.49+0.007^{d}

($p<0.05$)
N= 10 animals in each group.

Mice treated with 500 and 1000 mg / kg of methanolic extracts (group III and IV respectively) showed no significant ($p> 0.05$) decrease in the mean of body weights as compared to control group (group I). The absence of significant decrease in the mean body weights of CCl4 treated mice may be attributed to the short period of treatment. The non significant decrease in the mean body weights for mice treated with different doses of methanolic extract may be due to the presence of tannins in the extract.(James *et al*., 2010). Table (1) shows no significant ($p>0.05$) change in the mean

of liver weights of the treated groups compared to the control group, except the CCl4 treated group (group II) which showed a significant ($p<0.05$) increase in the liver weights compared to the control group (group I), this may be due to the inflammatory response caused by CCl_4 as a result of the severe damage in the hepatic tissue.

James *et al.*, (2010) showed that the toxicity of CCl4 was associated with a significant body weight loss and abnormal tissue weight increase in rats. Tajuddin et al., (2005) observed that the size of the liver was enlarged significantly in CCl4-intoxicated rats at a dose of 1.25 ml/kg.

Acute toxicity test

No mortality were observed in all the treated and control groups of mice up to a dose of 3000 mg / kg orally after 24 hours of treatment (three times more than the maximum treated doses) of the methanolic extract. Tajuddin *et al.*, (2005) reported no mortality in rats received 50% ethanolic extract at doses 500, 1000, 2000, and 4000 mg/ kg body weight. This suggests that the short term uses of nutmeg methanolic extract is relatively safe.

Effect of nutmeg seeds methanolic extract on the activity of serum aspartate aminotransferase (SGPT) and alanin aminotranferase (SGOT):

Carbon tetrachloride-treated mice (group II) showed a significantly ($P<0.05$) increase in the serum activity of SGPT compared to control mice (group I) (Table 1).

Mice treated with 500 and 1000 mg/kg of methanolic extract showed a significant ($p<0.05$) increase in the level of SGPT (group III and group IV respectively) as compared to the control group but significantly ($p<0.05$) less than carbon tetrachloride – treated mice.

Table (1) shows a significant ($p<0.05$) increase in the serum activity level of SGPT in mice treated with 1000 mg/kg of nutmeg methanolic extract for 7 days (group IV) as compared to the level in mice treated with 500 mg/kg (group III) but significantly ($p<0.05$) less than the CCl4 treated group.

Mice treated with 500 and 1000 mg / kg of methanolic extract showed a significant ($p<0.05$) increase in the level of SGPT (group III and IV respectively) as compared to the control group but significantly ($p<0.05$) less than carbon tetrachloride – treated mice.

Table (1) showed a significant ($p<0.05$) increase in the serum activity level of SGPT in mice treated with 1000 mg/kg methanolic extract (group IV) for 7 days as compared to the mice treated with 500 mg/kg of the extract (group III) but significantly ($p<0.05$) less than the CCl_4-treated group.

The data presented in the present work clearly demonstrate the state of oxidative stress induced in hepatic tissues by CCl_4, as a result of the increased lipid peroxidation and subsequent degradation of biomembranes, the permeability of the plasma membranes was severely affected, and may lead to leakage of SGOT and SGPT and an increasing in their activities in the serum. This idea was observed in the CCl_4-treated mice compared to controls (Table-1), and the high values of serum activities of both cytosolic enzymes (SGOT and SGPT) may be attributed to the alteration in the structure and function of the hepatocellular membrane as a result of binding of toxic metabolites of CCl_4 to the lipid and protein components of the membrane (Iganzio *et al*., 2002).

SGPT, SGOT are excellent markers of parenchymal liver damage caused by toxic substances (Al-Hamz *et al.*, 2004) the significant increase in these enzymes refers to a specific potential toxicity to the liver parenchyma.

The effect of nutmeg seeds methanolic extract on serum alkaline phosphatase (SALP) activity and total serum bilirubin (TSB):

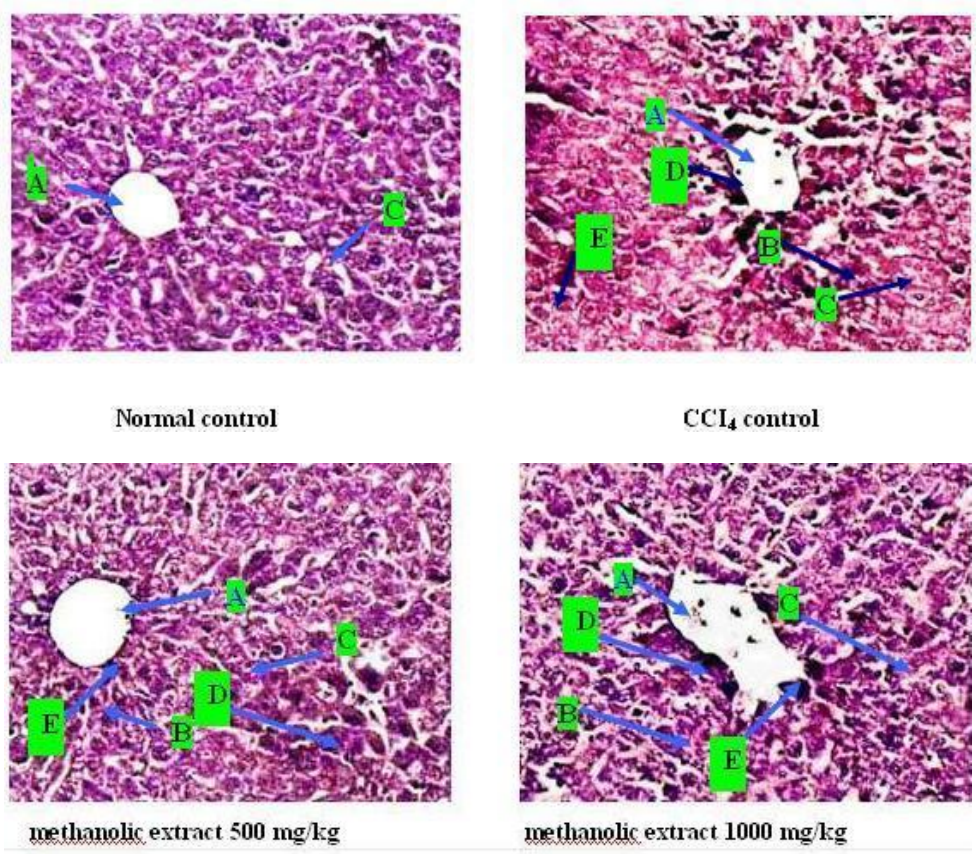
Carbon tetrachloride –treated mice (group II) showed a significant ($p<0.05$) increase in the serum activity level of SALP as compared to control mice (group I). (Table 1)

Mice treated with 500 and 1000 mg/kg of nutmeg methanolic extract showed a significant ($p<0.05$) increase in the serum activity level of SALP (group III and IV respectively) as compared to control group, but significantly ($p<0.05$) less than CCl_4-treated group.

Table (1) shows a significant ($p<0.05$) increase in the serum activity level of SALP in mice treated with 1000 mg/kg methanolic extract (group IV) for 7 days as compared to mice treated with 500 mg/kg of the extract (group III) but significantly ($p<0.05$) less than CCl_4-treated group.

Table (1) Total serum bilirubin level (TSB) has shown a significant ($p<0.05$) increase in CCl_4 treated mice (group II) as compared to control mice (group I). Mice treated with 500 and 1000 mg / kg of methanolic extract showed a significant ($p<0.05$) increase in the level of TSB (group III and IV respectively) as compared to the control group but still significantly ($p<0.05$) less than carbon tetrachloride – treated mice.

The serum activity of alkaline phosphatase (SALP) that is present in the lining membrane of the hepatocytes has been increased in the CCl_4-treated mice as compared to the control animals, and the results of the present study (Table 1) are consistent with other investigators (Vadivu *et al*., 2008). SALP has widespread tissue distribution, although serum level are thought to be primarily from liver and bone, the increased hepatic SALP is usually associated with biliary system damage, elevated serum SALP can be caused by increased synthesis or release of SSALP or by accumulation of bile acids because of biliary obstruction, bile acids can also damage cellular membranes, cause releasing of intracellular SALP. Total bilirubin is also used to evaluate liver function, high TSB indicate a deficiency in bilirubin metabolism of the hepatocytes (Harris, 2008).



Normal control CCI_4 control ME 500 mg/kg ME 1000 mg/kg

Figure 1: Histopathological studies of the mice liver treated with methanolic extracted (ME). Magnification: (10x20), staining: Haematoxylline and Eosin
A: central vein B: glycoprotein granules C: hepatic cells (hepatocytes) D: inflammatory cells E: ballooning degeneration

Total serum bilirubin (TSB) was increased significantly in CCl_4-treated mice (Table 1) and it is due to hepatic cellular damage which leads to disability of liver cells to metabolize and excrete bilirubin (Sethurman *et al*., 2003). Regarding cholestasis, a form of liver injury results from either decrease in the volume of bile formed or an impaired secretion of specific solutes into bile that is characterized biochemically by a sharp elevation in serum activities of enzymes localized to bile ducts, particularly alkaline phosphetase, in addition to serum levels of bile salts and bilirubin (Raja *et al*., 2004). Sections of mice liver treated with 2% tween 80 (control group) showed normal application of hepatocytes cells with slight accumulation of glycoprotein arranged around the central vein (no significant pathological changes) (Figure 1).

The histological examination of liver sections from each animal treated with CCl_4, showed a wide area of sever ballooning degeneration, necrosis of hepatocyte (bridging necrosis) , bile duct proliferation, sever cholestasis especially around central vein with inflammatory cells and steatosis (Figure- 1) as compared with control group . Figure 1. shows that sections of the liver treated with 500 and 1000 mg / kg of Nutmeg methanolic extract for 7 days showed mild degenerative changes with accumulation of glycoprotein granules inside the hepatic cells and dilatation of sinusoids, these findings were evident in all methanolic extract treated mice but the severity increased with the increasing dose. Vadivu *et al.*, (2008) were reported that the treatment of mice with 20, 40 and 80 mg /kg of *myristica fragrans* extract intraperitonial (i.p) showed that liver tissue confirmed a potential histopathological effect for nutmeg as evidenced by hydropic and fatty degeneration in treated mice, these hepatotoxic effects of Nutmeg have been corrected structurally to some extent but not functionally by vitamin C administration, this concludes that the extract has been toxic to the liver.

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