

## Hepatotoxic Activity of Essential Oil from Nutmeg (*Myristica Fragens*) Against Tetrachloride-Induced Hepatic Damage In Mice

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**Abstract:** To extracting and purifying the essential oil containing terpenes from dried seeds of nutmeg *Myristica fragrans* available in Iraqi markets. Male albino mice (n= 44), with average weight (25-28 g) of about six weeks old The study also employed an in vivo evaluation of the hepatotoxic effect of essential oil in male albino mice at different concentrations ( 500 and 1000 mg / kg) given orally for 7 days including biochemical functions 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). At day 8 the animal was sacrificed 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 SAST, SALT, SALP and TSB in a dose dependent manner. The nutmeg essential oil carries a marked specific potential toxicity to the liver parenchyma, this is very important to be considered for further experimental and clinical studies.

**Key words:** *Myristica fragrans*, essential oil , Hepatotoxic, hepatic necrosis

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### I. Introduction

Nutmeg (NM) has its origins in the Spice Islands of Indonesia, formerly known as the spice islands, it is also cultivated in the Caribbean, south India, Sri Lanka, Sumatra, and Malaysia, It has been widely popular in Europe and India for its flavoring and medicinal properties [1].

Herbal preparations have become one the fast growing markets in the world and even in Iraq for alternative health products , there is a great potential misuse of these herbs and spices and there is no standardization of active constituents, herbs and spices can be viewed in terms of inherent toxicity like pennyroyal oil is used as an abortifacient but linked with hepatic necrosis similar to acetaminophen in toxic amounts , another example is chaparral which is used as antioxidant but linked with hepatic necrosis , cholestatic hepatitis and hepatic failure . [2].

The essential oil is highly sensitive to light and temperature and yields a colorless, pale yellow or pale green oil with a characteristic odor of nutmeg, the oil is soluble in alcohol and insoluble in water, the essential oil of East Indian nutmeg and West Indian nutmeg differ in their flavor and odor characteristics , the East Indian nutmeg oil is considered superior to the West Indian nutmeg oil, having a better aroma and a higher amount of phenyl propanoid ethers [3] and terpenes [4].

The essential oil extracted from nutmeg is a colorless or pale yellow thin liquid with the characteristic odor and taste of nutmeg Usually, it is obtained by steam distillation from ground nutmeg or from whole nutmegs graded as shrivels, rejects, or broken and wormy [5].

The acute oral LD50 of nutmeg oil in rats has been reported to be 2620 mg/kg [6] and 2600 ± 220 mg/kg [7]. However, in another study, oil of nutmeg have been reported to have a rat oral LD50 value of 160 mg/kg [8] . The Food and Drug Administration has reported oral LD50 values for other species including mice (5620 ± 520 mg/kg) and hamsters ~(6000 ± 230 mg/kg), Cats seem to be highly sensitive to nutmeg and particularly to myristicin. A 5-10 g oral dose of nutmeg will cause death in cats, while 50 mg/kg of myristicin intraperitoneally (i.p) will result in death [9]. Most of the major components of nutmeg have established LD50 values. With the exception of myristicin, which has a reported LD50 of approximately 570 mg/kg, most of the other identified components of nutmeg have LD50 values in excess of 1300 mg/kg [6].

The aim of this study is to evaluating the physicochemical characteristics of the essential oil extract and study the effect in mice compared to that caused by carbon tetrachloride as a hepatotoxic model.

## II. 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 extract was prepared according to Harborne,[10]. 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 then 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°. [11].

The yield percentage of essential oil was determined using the formula described by Rao *et al.*,[12] where the amount of essential oil recovered (g) was determined by weighing the oil after moisture was removed:

$$\text{Percentage yield (\%)} = \frac{\text{Amount of essential oil recovered (g)} * 100}{\text{Amount of plant material distilled (100g)}}$$

The physical features were evaluated according to the methods mentioned Al-Shahhat,[13]. and used for studying hepatoprotective.

Mice weighting between 25-28 gm 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. Hepatoprotective activity of *Myristica fragrans* was evaluated using CCl<sub>4</sub> –induced model Krishnamoorthy and Rema [14]. Group one was kept on normal diet and served as control, the second group received CCl<sub>4</sub> (0.5 ml/kg ) orally to induce liver damage in mice and served as positive control, the third and fourth group received *Myristica fragrans* essential oil extract 500 and 1000 mg/kg respectively once daily, for eight days.

### 2.1. Acute toxicity testing :

The nutmeg essential oil 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 [15].

### 2.2. 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 [16].

### 2.3. 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 [17]. The blood obtained from each mice ranging from 0.7-1 ml.

### 2.4. Histopathological Examination:

Liver tissues were prepared for histopathological examination according to Provan and Krentz [18] 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.

### 2.5. 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) [19].

### III. Results And Discussion

The results shows that the odor of the essential oil from *Myristica fragrans* obtained is strong, turpentine like odor or spicy. Each essential oil is characterized by a special odor which attributed to the presence of some low molecular weight compounds like alcohols, esters, phenols and oxygenated compounds which have the future of being highly volatile at room temperature [13; 20].

The essential oils of plants differ in their colors and the degree of colors, they are either colorless, pale yellow, (like the essential oil of Coriander and anise), light yellow (like the essential oil of peppermint), greenish yellow (like Japanese mint and celery), yellowish brown (like the essential oil of cumin and dill), or blue or greenish blue (like Camomile and Achillea flowers) [21].

The specific gravity of the studied essential oil was 0.890, Sarath-kumara [22] have mentioned that the specific gravity of the East Indian Nutmeg oil ranges from 0.885 to 0.915 and that of west Indian has been 0.860 to 0.88 at 20° C. Leela [14] has also report that the specific gravity of east Indian oil is ranging from 0.880 to 0.913 and that of west Indian is ranging from 0.859 to 0.865.

The refractive index of the nutmeg essential oil is found to be 1.4822, Leela [14], has reported that the refractive index of the East Indian Nutmeg essential oil is ranging from 1.4776 – 1.4861, while that of West Indian Nutmeg essential oil is ranging from 1.4729 - 1.4746. It is consider one of the important features to evaluate the quality and purity of oils and fats, the increasing in the refractive index value is a feature of high quality oils, and it is increased with increasing of the specific gravity of the oil and affected by the change in the concentrations of its components, especially the solid compounds.[23].

Myristicin Purification it was done using silica gel G60 column chromatography technique with a mobile phase n-hexane: benzene: ethyl acetate, methanol to elute fractions according to their affinity to mobile phase. The resultant fractions that give positive ferric chloride test 1% solution (fraction II) are detected by T.L.C silica gel 60 F<sub>254</sub> plate of 0.75 mm thickness using the mobile phase n-hexane-chloroform (3:2), the TLC chromatogram in (Fig 1) for the fraction of the positive result (FII) and the standard have given one brown spot of R<sub>f</sub> 0.71 in n-hexane-chloroform (3:2) as mentioned by Harborn [10].

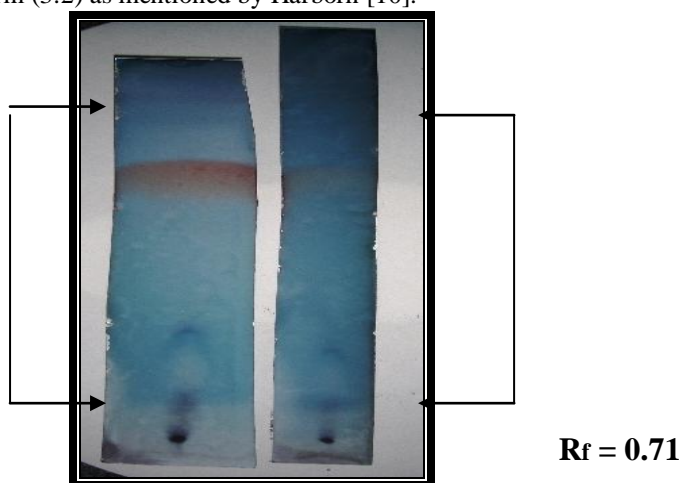


Figure (1): T.L.C. of the standard Myristicin (left) and the purified Myristicin (right) in n-hexane - chloroform mobile phase, the brown spot of R<sub>f</sub> 0.71 indicate the presence of myristicin [10].

Gas Chromatography (GC) for Essential oil and Myristicin

The GC chromatogram for the purified Myristicin as in (Fig 2) shows the presence of one main peak of conc. of 97.6%. (Table 1).

The applied GC for the essential oil and purified myristicin, According to the GC analysis of the essential oil and the purified myristicin, the percentage of myristicin in the essential oil was about 6%. Therefore it can be assumed that the nutmeg is from East India [25]. Particularly may be from Singapore (Table 1)[26]. Although the seeds are imported to Iraq from India but India itself imported it from south-east Asian countries because nutmeg trees are not grown to any large extent in India [27]. Reineccius [25] has reported that the myristicin content of East Indian nutmeg is 3.3 – 13.5% of the essential oil while of West Indian nutmeg is 0.5-0.8% of the essential oil and that of Sri-Lanka origin is 3.8%.

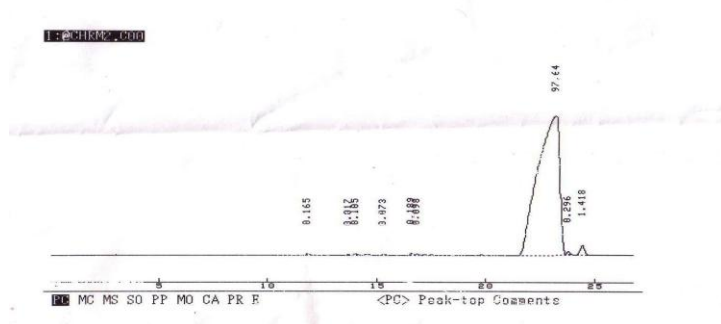


Figure 2 : GC of the purified Myristicin after T.L.C.

Table 1: Retention time for the purified Myristicin (myristicin peak appeared after 23.194 minutes with concentration of (97.6399))

Peak no.	Time	Area	Height	Conc.
1	11.32	2443	231	0.1649
2	13.378	256	30	0.0172
3	14.006	1549	159	0.1046
4	15.317	1080	115	0.0729
5	16.608	2804	274	0.1892
6	16.83	1454	141	0.0981
7	23.194	1446608	1211	97.6599
8	23.783	4380	411	0.2956
9	24.44	21083	1483	1.4176
Total		1481574	24060	100

Table (2) shows the results of body weight (gm) of mice before and after treatment. CCl<sub>4</sub> – treated mice show no significant (p< 0.05) decrease in the mean of body weight compared to control group . Mice treated 500 and 1000 mg /kg essential oil of NM seeds have showed no significant (p< 0.05) differences in the means of body weights at the end of seven days of treatment compared to the control group.

The absence of a significant body weight reduction may be due to the stomachic effect of nutmeg seeds, however the absence of significantly increase in body weight may be due to the short term treatment although relatively highly dosage used [28].

Table 2: Effect of different doses of *myristica fragrans* essential oil extract on the body weight in mice before and after treatment

Treatment	Dose (mg/kg)	Weight( gm) before treatment	Weight(gm) after treatment
Control	---	25.40 ± 1.90 <sup>a</sup>	26.90 ± 1.90 <sup>a</sup>
CCl <sub>4</sub>	---	25.70 ± 2.40 <sup>a</sup>	25.20 ± 3.10 <sup>a</sup>
Essential oil + CCl <sub>4</sub>	500 mg/kg	28.10 ± 1.90 <sup>a</sup>	27.90 ± 1.40 <sup>a</sup>
Essential oil + CCl <sub>4</sub>	1000 mg/kg	27.10 ± 2.20 <sup>a</sup>	28.60 ± 2.00 <sup>a</sup>

Each value represent mean + SD

Values with non-identical superscripted (a, b, c, d, e & f) are considered as significantly different (p<0.05) N= 10 animals each group.

### 3.1. Effect of NM essential oil on the activity of serum aspartate aminotransferase (AST) and alanin aminotranferase (ALT)

Carbon tetrachloride-treated mice (group II) showed a significantly (P>0.05) increase in the serum activity of AST compared with control mice (group I) (Table 3). Mice treated with 500 mg / kg and 1000 mg / kg of essential oil show a significant (p> 0.05) increase in the level of AST as compared to the control group but

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still significantly less than carbon tetrachloride – treated mice . Also there is a significant ( $p > 0.05$ ) increase in the serum activity level of AST between the different doses of NM essential oil (500 mg / kg and 1000 mg / kg) for 7 days in the serum activity level of AST (77.83 and 94.48 U/l respectively) as compared to the control group but still significantly less than CCl<sub>4</sub> treated group.

Mice treated with 500 and 1000 mg / kg of essential oil show a significant ( $p > 0.05$ ) increase in the level of ALT as compared to the control group but still significantly less than carbon tetrachloride – treated mice.

Table 3. show a significant ( $p > 0.05$ ) increase in the serum activity level of ALT between the different doses of NM essential oil (500 mg / kg and 1000 mg / kg for 7 days) in the serum activity (49.85 U/l and 60.95 U/l respectively ) as compared to the control group but still significantly less than CCl<sub>4</sub>-treated group .

**Table 3:** Effect of different doses of *myristica fragrans* essential oil on the activity of serum AST and ALT in mice

Treatment	Dose (mg/kg)	AST ( SGOT) U/l	ALT ( SGPT) U/l
Control	---	61.9 ± 0.8 <sup>e</sup>	33.5 ± 1.1 <sup>f</sup>
CCl <sub>4</sub>	---	195.0 ± 4.3 <sup>a</sup>	110.10 ± 2.8 <sup>a</sup>
Essential oil + CCl <sub>4</sub>	500 mg/kg	77.83 ± 1.8 <sup>c</sup>	49.85 ± 0.89 <sup>c</sup>
Essential oil + CCl <sub>4</sub>	1000 mg/kg	101.48 ± 3.4 <sup>b</sup>	60.95 ± 1.9 <sup>b</sup>

Each value represent mean + SD

Values with non-identical superscripted (a, b, c, d, e& f) are considered as significantly different ( $p < 0.05$ ) N= number of animals.

In (Table 3) there is a significant ( $p > 0.05$ ) increase in the serum activity level of ALT between group IV and V (methanolic extract 1000 mg/kg and essential oil 500 mg / kg respectively ) (43.42 U/l and 49.85 U/l respectively).

The data presented in the present work clearly demonstrate the state of oxidative stress induced in hepatic tissues by CCl<sub>4</sub>, 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 AST and ALT and an increasing in their activities in the serum [29].

Mice treated with 500 and 1000 mg/kg of Nutmeg essential oil showed an increase in the level of AST and ALT, as compared to control group, the level of these biochemical indicators was increased significantly with the increasing dose of the extract and was more with oil treated mice even between the 500 mg/kg of oil and 1000 mg /kg .

These findings agreed with Al-Hamzi *et al* [29] who reported that , treatment of mice with 20,40 and 80 mg/kg of myristica fragrans extract intraperitoneal (i.p) showed statistically significant alteration in the biochemical indicators of liver function including , and reduction of total protein and serum albumin and significant elevation of SGOT and SGPT .

**3.2. The effect of NM seeds essential oil on serum alkaline phosphatase (ALP) activity and total serum bilirubin (TSB).**

Carbon tetrachloride –treated mice show a significantly ( $p > 0.05$ ) increase in the serum activity level of ALP as compared to control mice .( Table 4)

Mice treated with 500 and 1000 mg/kg of essential oil show a significant ( $p > 0.05$ ) increase in the serum activity level of ALP (group V and VI respectively) as compared to control group, but significantly less than CCl<sub>4</sub>-treated group.

Table (4) shows a significant increase in the serum activity level of ALP between the different doses of essential oil (500 and 1000 mg / kg for 7 days) (81.50 and 95.60 U/l respectively).

Total serum bilirubin level (TSB) has shown a significant increase in CCl<sub>4</sub> treated mice. As compared to control mice (Table 4).

Mice treated with 500 mg / kg and 1000 mg / kg of essential oil show a significant ( $p > 0.05$ ) increase in the level of TSB (0.52 and 0.69 mg / dl respectively ) as compared to the control group but still significantly less than carbon tetrachloride – treated mice .

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Table (4) shows a significant (  $p > 0.05$ ) increase in the serum level of TSB between the different doses of NM essential oil (500 and 1000 mg / kg for 7 days) in the serum level (0.52 and 0.69 mg /dl respectively ) as compared to the control group but still significantly less than CCl<sub>4</sub>-treated group .

Table 4: Effect of different doses of *myristica fragrans* essential oil on the activity of serum ALK and TSB in mice

Treatment	Dose (mg/kg)	ALK U/l	TSB mg/dl
Control	--	64.03 ± 1.5 <sup>f</sup>	0.34 ± 0.02 <sup>f</sup>
CCl <sub>4</sub>	--	157.00 ± 5.6 <sup>a</sup>	1.30 ± 0.06 <sup>a</sup>
essential oil + CCl <sub>4</sub>	500 mg/kg	85.50 ± 1.1 <sup>c</sup>	0.52 ± 0.01 <sup>c</sup>
essential oil + CCl <sub>4</sub>	1000 mg/kg	95.60 ± 1.5 <sup>b</sup>	0.69 ± 0.001 <sup>b</sup>

Each value represent mean + SD

Values with non-identical superscripted (a, b, c, d, e& f ) are considered as significantly different (  $p < 0.05$ )

N= 10 animals in each group.

ALP has widespread tissue distribution, although serum level are thought to be primarily from liver and bone, the increased hepatic ALP is usually associated with biliary system damage, elevated serum ALP can be caused by increased synthesis or release of ALP or by accumulation of bile acids because of biliary obstruction, bile acids can also damage cellular membranes, cause releasing of intracellular ALP. [30].

Total serum bilirubin (TSB), have been increased significantly in CCl<sub>4</sub>-treated mice (Table 4) due to hepatic cellular damage which leads to disability of liver cells to metabolize and excrete bilirubin [31].

**3.3. Histological examinations of the liver sections:**

Section of mice liver treated with 2% tween 80 (control group) show normal application of hepatocytes cells with slight accumulation of glycoprotein arrange around the central vein (no significant pathological changes) (Figure 3).

The histological examination of liver sections from each animal treated with CCl<sub>4</sub>, has 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 3) as compared with control group .

Section of mice liver treated with 500 mg/kg of *myristica fragrans* essential oil for 7 days has showed focal area of degeneration and necrosis with infiltration of mononuclear cells and bile duct proliferation and also section of mice liver treated with 1000 mg/kg has showed more degenerative changes and necrosis of hepatocytes with focal mononuclear cells infiltration and bile duct proliferation (Figure 3).

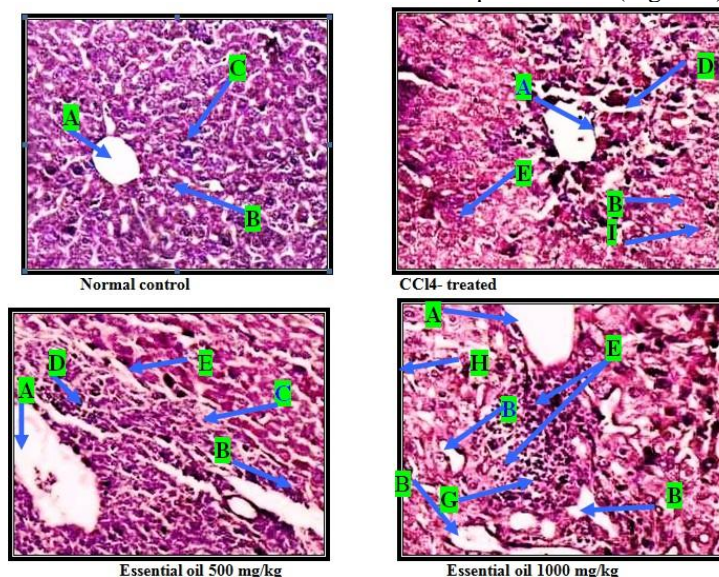


Figure 3: Histopathological studies of the mice liver treated with essential oil. Magnification: (10x20), staining: Haematoxylline & Eosin.

A: central vein      B bile duct proliferation      C: hepatic cells ( hepatocytes)  
B: necrosis      I: fatty change      D: inflammatory cells      E: ballooning degeneration  
G: inflammatory cells      H: fatty changes      E: necrosis

Concerning histological examination seen in the livers of animals treated with CCl<sub>4</sub>, the current work showed that, there was hepatic degeneration with necrosis in portal zone, fatty changes with wide dispersion of inflammatory cells (Figure 3).

Fatty degeneration (steatosis), observed in liver of mice intoxicated by CCl<sub>4</sub> may be attributed to the inhibitory effect of CCl<sub>4</sub> on lipoproteins secretion especially very low density lipoprotein (VLDL) from hepatocytes into circulation [32].

While sections of liver treated with 500 and 1000 mg / kg of Nutmeg essential oil show more destructive changes i.e. more degenerative changes and necrosis of hepatocytes with infiltration of mononuclear cells and bile duct proliferation. Although nutmeg is used as aphrodisiac in traditional medicine , the oral administration of its essential oil lead to antifertile state and histopathological changes in male rats testes due to the interference of the oil in the testicular androgen level , which could recover after termination of the treatment [33].

The actual mechanism by which nutmeg induced cellular degeneration observed in this experiment needs further investigation. The necrosis observed is probably due to the high concentration of nutmeg on the liver. Pathological or accidental cell death is regarded as necrotic and could result from extrinsic insults to the cell as osmotic thermal, toxic and traumatic effect ,Physiological cell death is regarded as apoptotic and organized programmed cell death (PCD) that is mediated by active and intrinsic mechanisms [34].

## V. CONCLUSION

It can be concluded that nutmeg essential oil carries a marked specific potential toxicity to the liver parenchyma and to less extent the methanolic extract, this is very important to be considered for further experimental and clinical studies. The medicinal use of nutmeg and its use as a spice suggest that it contains some constituents which are responsible for the reported biological activities. Some of these active principles may at the same time possess adverse effects when dosage is abused.

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