

Original Article**Determination of Safety Margin for Hepatotoxic Effect of *Mentha Longifolia* Essential Oil in Rat**Mohammad Hossein Hesami¹, Seyed Ebrahim Sajjadi², Ali Hosseini-Sharifabad*¹

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ABSTRACT

Background: *Mentha longifolia* is one of the aromatic medicinal plant belongs to Lamiaceae family. There are some active ingredients in the essential oil of *M. longifolia*, which potentially could impair the hepatic function. The aim of this study was to find the maximum dose of essential oil of *M. longifolia* (EOML) that does not show any hepatic deterioration.

Methods: Adult Wistar rats fed different doses of EOML as 50, 100, 200, 300, 400 or 600 mg/kg, for two wk. After the completion of administration, the serum activity of ALT, AST, and ALP as the well-known liver toxicity enzymes and the serum total bilirubin were measured, by spectrophotometer. The study was done at 2016 in Isfahan Pharmacy School, Isfahan, Iran.

Results: Totally, 400 mg/kg of EOML significantly raised all of the evaluating factors compare to the control group. We found complete mortality in animals that received 600 mg/kg of EOML.

Conclusion: The essential oil of *M. longifolia* is not entirely safe especially for the liver. Administration at the dose of 400 mg/kg leads to the hepatotoxic effect. The death occurred in the higher doses. The possible mechanisms for the EOML liver toxicity are triggering of oxidative stress or apoptosis by its ingredient like pulegone compound.

Keywords: Essential Oil, Hepatotoxic Effect, *Mentha Longifolia*, Safety.

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INTRODUCTION

“The wild mint (*Mentha longifolia* L. (Lamiaceae)) grows extensively in different part of Iran and other regions such as Mediterranean, Europe, Australia, and North Africa” [1].

M. longifolia is used in the pharmaceutical, food and cosmetic industries. Different parts of the plant including its leaves, flowers, stem, bark, and seeds have been also used widely in traditional medicine as antimicrobial, carminative, diuretic, antitussive, mucolytic, antispasmodic agent. Besides, it is used for the treatment of various diseases such as headaches, digestive disorders, amenorrhea, gout, colds, increased micturition, and for skin diseases [2, 3]. The essential oil of *M. longifolia* (EOML) as an aromatic compound contain terpenoids like pulegone, isopiperiten-one, 1,8-cineole, carvone and limonene [4]. The aromatic ingredients, lead to pleasant scent and taste of *M. longifolia*, encourages the manufacturer to add its different parts especially essential oil to drinking or eating products. It is frequently incorporates to the dairy product to make them more desirable, in Iran.

Liver is the major organ responsible for the metabolism of drugs and toxic chemicals, thus it is the primary exposed organ for nearly all orally toxic chemicals. Therefore, the toxicity of liver as the most important organ of detoxification could lead to a wide range of disorders in the biologic processes [5]. The hepatotoxicity agents that resulting in membrane leakage and increase the hepatic biochemical markers were including AST, ALT, ALP and bilirubin in the serum [6, 7].

Pulegone agents and its derivatives cause hepatic disruption leading to its toxicity [6]. Based on the existence of pulegone and its derivative agents in the EOML, it can potentially cause the liver deteriorations.

The aim of the current study was to find the maximum dose of EOML that did not show any hepatic disorders and accordingly to determine its partial safety for the liver.

MATERIAL AND METHODS**Chemicals**

The essential oil of aerial parts of *M. longifolia* was purchased from Barij Essence

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Company (Iran, Barij Essence Pharmaceutical Co.) The AST, ALT, ALP and total bilirubin assay kits were prepared from Bionik Company.

GC-MS Analysis:

Gas chromatography combined with mass spectrometry was used for identification of the oil components. The analysis was performed on a Hewlett-Packard 5972A mass selective detector coupled with a Hewlett-Packard 6890 gas chromatograph, equipped with a HP-5MS capillary column (30 m x 0.25 mm; film thickness 0.25 μ m). The oven temperature was programmed from 60-280 °C at 4 °C/min. Helium was used as carrier gas at a flow rate of 2 mL/min. Injector and detector temperatures were 280 °C. The MS operating parameters were ionization voltage, 70 eV; ion source temperature, 200 °C. Identification of components of the oil was based on GC retention indices relative to n-alkanes and computer matching with the Wiley 275.L library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature [8, 9].

Animals

Wistar rats weighing (200 \pm 20 g) obtained from the animal house of Faculty of Pharmacy, Isfahan University of Medical Sciences. The animals were kept under standard condition (25 \pm 5°C, 12:12 light/dark cycle). All experimental procedures were conducted during the light phase of the cycle. The free access of food and water were allowed.

All procedures were reviewed and approved by the Animal Care Committee in Isfahan University of Medical Sciences. The study was done at 2016 in Isfahan Pharmacy School, Isfahan, Iran.

Experimental Design

Rats were randomly divided into 7 groups (n=6) and the groups were treated according to the following schedule:

Group1: They received daily orally saline as the vehicle for 2 wk.

Groups 2, 3, 4, 5, 6, 7: They were daily-administered 50,100, 200, 300, 400, 600 mg/kg of EOML for two wk by gavage according to their respective group.

After 2 wk, the animals were anesthetized and cardiac blood samples were collected for the biochemical hepatic marker determination.

Besides, hepatic tissue sample was taken and stored in 10% formalin buffer solution for next histopathological examination.

Biochemical Determination

The collected blood samples were centrifuged at 3000 rpm for 15 min for separation of serum. The serum activity level of AST (Aspartate aminotransferase), ALT (Alanine aminotransferase), ALP (Alkaline phosphatase) and total bilirubin were measured by UV-Vis spectrophotometric method using Bionik assay kits.

Histopathological Examination

The fixed liver tissues were embedded in paraffin. The tissue sections underwent eosine and hematoxyline staining process. The degree of hepatic damages was scored as described by following method [10]:

Score 0: No visible cell damage;

Score 1: Focal hepatocyte damage on less than 25% of the tissue;

Score 2: Focal hepatocyte damage on 25%-50% of the tissue;

Score 3: Extensive, but focal, hepatocyte lesions;

Score 4: Global hepatocyte necrosis;

The sections were investigated for the hepatocyte necrosis, inflammation, cell swelling, fatty degeneration, infiltration of Kupfer cells and lymphocytes. These pathological evidences were used to classify the scores of damage [10, 11].

Statistical Analysis

The calculations and statistical analysis were carried out using the SPSS ver. 12.0 software (Chicago, IL, USA). The results of biochemical analysis were represented as mean \pm S.E.M. Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Statistical probability of $P < 0.05$ was considered to be significant.

RESULTS

Essential Oil Composition

The constituents of the oil and their percentages are presented in the Table 1. Twenty-eight components were identified in the essential oil of *M. longifolia*. Pulegone (54.1%), menthofuran (12.5%), 1,8-cineole (8.0%) were the main components of the oil. However, more than half percentage of the oil consists of pulegone.

Table 1. Composition of the essential oil of aerial parts of *Mentha longifolia* L.

| No. | RT | Compound | % | RI |
|-----------|--------------|--------------------------------------|-------------|-------------|
| 1 | 3.66 | A-thujene | t | 929 |
| 2 | 3.81 | A-pinene | 3.5 | 937 |
| 3 | 4.07 | Camphene | 0.3 | 952 |
| 4 | 4.55 | Sabinene | 0.8 | 976 |
| 5 | 4.61 | β -Pinene | 2.1 | 978 |
| 6 | 4.88 | Myrcene | 1.1 | 991 |
| 7 | 5 | 2-octanol | t | 996 |
| 8 | 5.87 | 1,8-cineole | 8 | 1034 |
| 9 | 5.98 | <i>Cis</i> - β -ocimene | 0.2 | 1039 |
| 10 | 6.29 | <i>Trans</i> - β -ocimene | 0.2 | 1052 |
| 11 | 6.5 | γ -Terpinene | t | 1060 |
| 12 | 6.78 | <i>Cis</i> -sabinene hydrate | 0.3 | 1070 |
| 13 | 7.29 | Terpinolene | 0.1 | 1087 |
| 14 | 7.53 | Isoamyl isovalerate | 0.3 | 1099 |
| 15 | 9.21 | Menthone | 3.3 | 1154 |
| 16 | 9.58 | Menthofuran | 12.5 | 1165 |
| 17 | 9.92 | <i>Cis</i> -isopulegone | 3.5 | 1175 |
| 18 | 10.71 | Γ -terpineol | 1.4 | 1201 |
| 19 | 12.32 | Pulegone | 54.1 | 1239 |
| 20 | 12.64 | Piperitone | 0.3 | 1254 |
| 21 | 12.94 | Geraniol | 0.2 | 1270 |
| 22 | 13.47 | 8-hydroxy-delta-4(5)-p-menthen-3-one | 1.1 | 1289 |
| 23 | 15.05 | Piperitone | 1.8 | 1341 |
| 24 | 15.86 | Piperitoneoxide | 2.6 | 1367 |
| 25 | 16.81 | <i>Cis</i> -jasmone | 0.4 | 1395 |
| 26 | 17.37 | β -Caryophyllene | 0.6 | 1416 |
| 27 | 18.55 | <i>Trans</i> - β -farnesene | 0.1 | 1455 |
| 28 | 19.22 | Germacrene-d | 0.3 | 1481 |

RI: Retention indices on HP-5MS capillary column

t: trace ($\leq 0.05\%$)

%; Calculated from TIC data

Effect of Different Doses of EOML on the Serum AST and ALP Level in Rat

Orally administration of 400 mg/kg EOML for 2 wk significantly increased the AST ($P < 0.01$)

and ALP ($P < 0.05$) serum activity in rats compare to the control groups. None of the other treated doses of EOML showed change in the AST and ALP level compared to control (Figure 1A, B).

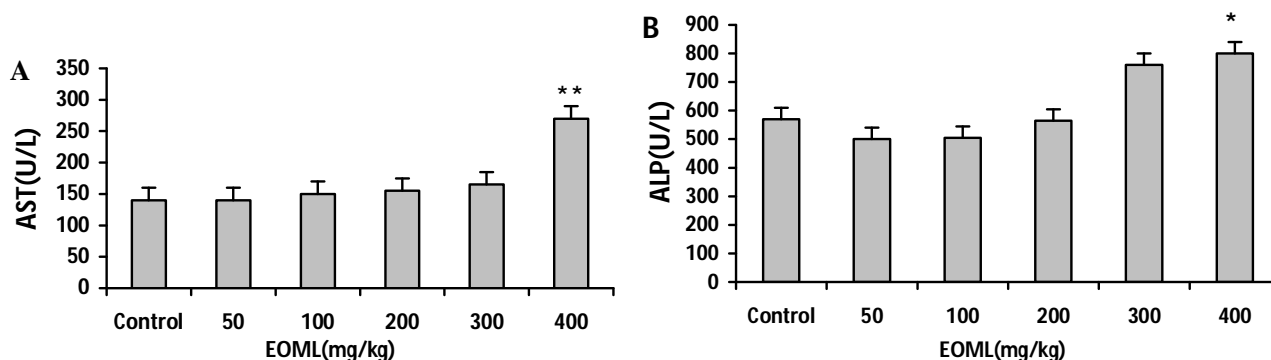


Figure 1. Effect of different doses of *Mentha longifolia* essential oil on the serum AST and ALP activity in rat.

Serum AST (A) and ALP (B) level in experimental animal treated with different doses of EOML. Data are presented as Mean \pm SEM. (** $P < 0.01$, * $P < 0.05$ significantly different compare to control group) (EOML: essential oil of *Mentha longifolia*, AST: aspartate transaminase, ALP: alkaline phosphatase, U/L: unit/liter).

Effect of Different Doses of EOML on the Serum ALT and Total Bilirubin Level in Rat

Although 50 or 100 mg/kg EOML did not show any considerable change in the serum ALT level, two wk oral administration of 200 or 300 or 400 mg/kg of EOML remarkably increased the

ALT activity compare to the control ($P<0.05$, $P<0.05$, $P<0.01$ respectively) (Figure 2A). Besides, 300 or 400 mg/kg EOML significantly increased the serum total bilirubin level in comparison to the control group ($P<0.05$ and $P<0.001$ respectively) (Figure 2B).

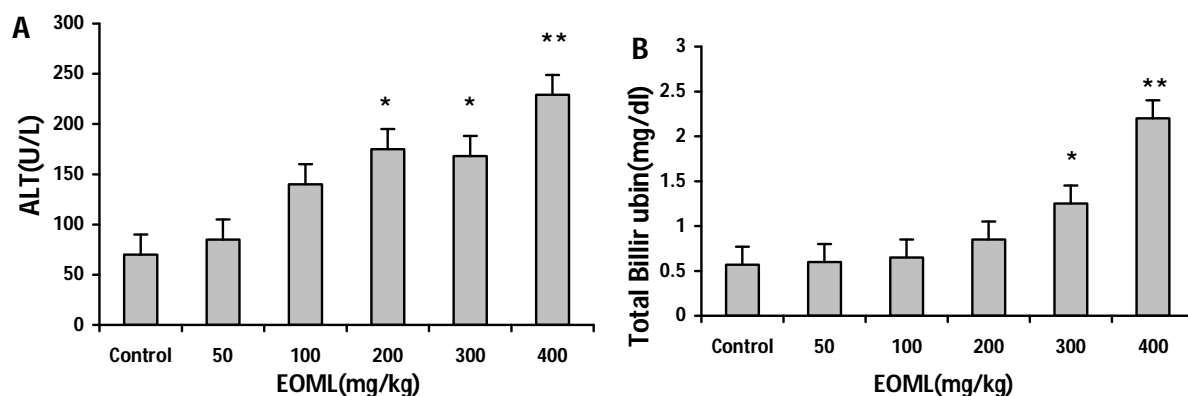


Figure 2. Effect of different doses of *Menthalongifolia* essential oil on the serum ALT activity and total bilirubin in rat.

Serum ALT (A) and total bilirubin (B) level in experimental animal treated with different doses of EOML. Data are presented as Mean±SEM. (***) $P<0.001$, (**) $P<0.01$, (*) $P<0.05$ significantly different compare to control group) (EOML: essential oil of *Menthalongifolia*, ALT: alanine transaminase, U/L: unit/liter).

Histopathological Findings

The liver sections of the normal control group (Figure 3A) had normal parenchymal structure, and there was no significant histological changes (score=0).

Liver tissues which received 50mg/kg of EOML showed no fatty changes, no necrosis and inflammations and slightly cell swelling (Score=2) (Table 2 and Figure 3B).

Liver tissues which treated by other increasing doses of EOML displayed histologic findings according to the following schedule:

EOML 100mg/kg: score=4 (Table 2 and Figure 3C),

EOML 200 mg/kg: score=6 (Table 2 and Figure 3D),

EOML300mg/kg: score=5 (Table 2 and Figure 3E),

EOML400mg/kg: score=4 (Table 2 and Figure 3F).

Table 2. Degree of histological hepatic injury in different treated groups with essential oil of *Mentha longifolia*.

| Groups | Score of injuries ^a | | | | |
|-----------------|--------------------------------|----------|---------------|--------------|--------------|
| | Fatty degeneration | Necrosis | Cell swelling | Inflammation | Total scores |
| Control | 0 | 0 | 0 | 0 | 0 |
| EOML(50 mg/kg) | 0 | 0 | 2 | 0 | 2 |
| EOML(100 mg/kg) | 0 | 2 | 0 | 2 | 4 |
| EOML(200 mg/kg) | 0 | 3 | 0 | 3 | 6 |
| EOML(300 mg/kg) | 0 | 3 | 0 | 2 | 5 |
| EOML(400 mg/kg) | 0 | 3 | 0 | 1 | 4 |

^aLivers were scored for hepatic injury with score 0= no visible cell damage; score 1=focal hepatocyte damage on less than 25% of the tissue; score 2= focal hepatocyte damage on 25-50% of the tissue; score 3=extensive, but focal, hepatocyte lesions; score 4=global hepatocyte necrosis.

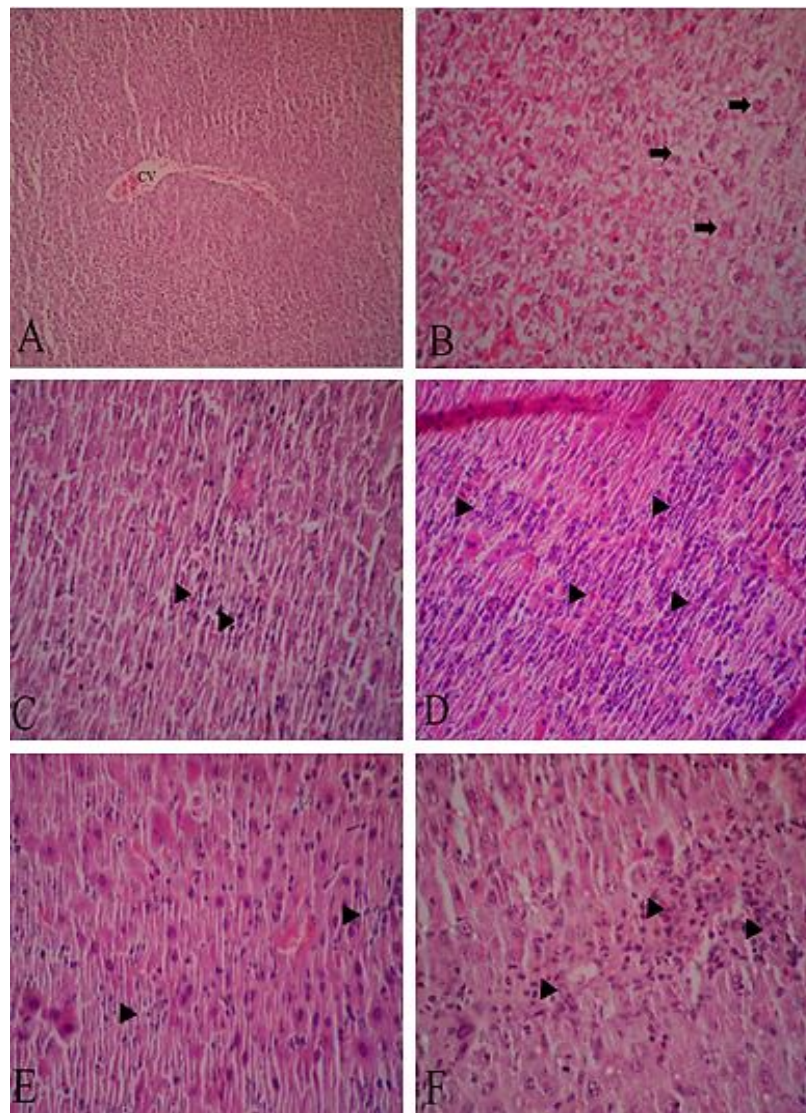


Figure 3. Effect of essential oil of *M. longifolia* on the liver of rat.

Liver morphology was characterized by light microscopy (*400) using routine hematoxylin and eosin (H&E) staining. Histopathological changes were evaluated and were compared with healthy rats liver as follows: (A) liver of normal untreated control showing intact basic liver structures like central vein (CV) and hepatocytes, (B) liver in 50 mg/kg treated EOML showing: cell swelling (arrow), (C) liver of 100 mg/kg treated EOML group: showing hepatocytes necrosis (arrow head) and infiltration of inflammatory cells, (D-F) liver in 200, 300, 400 mg/kg treated EOML animals: show that hepatocyte necrosis (arrow head) and infiltration of inflammatory cells (H&E *400).

DISCUSSION

Liver diseases are one of the serious health problems. Unfortunately, so many deaths occur annually from the liver cirrhosis [12]. Liver injuries are commonly caused by viruses, environmental toxicants, drugs and overuse of alcohol that finally leads to different types of hepatic disorders [13, 14]. In contrast to the people who usually believe the medicinal plants does not possess the adverse effects; their

undesirable effects in the body especially for liver were recognized in several studies [15]. The aim of the present study was to evaluate the possible adverse effects of essential oil of *M. longifolia* aerial parts, as a severally used additive plant to the beverages in Iran, on the hepatic tissues. On the other hand, we were going to define the maximum dose of EOML, which did not show any hepatic deterioration as a safety remark. We selected the EOML because it freely and in the

large amount adds to the beverages, baked goods, candies, ice creams, cosmetics and oral hygiene products as a flavor agent in Iran [16, 17].

The administration of EOML in the lower doses up to 400 mg/kg did not cause considerable increase in the serum level of the AST, ALT, ALP and total bilirubin as the well-known markers for hepatic dysfunction. Interestingly, we find a remarkable raise in the described factors at the dose of 400 mg/kg compared to the control group elucidated the hepatic impairment in this dose. We also found completely mortality when EOML was administered 600 mg/kg. This proves the toxicity of EOML and rejects its safety in the higher doses. While the AST and ALT are normally present in large quantity in the liver, the hepatotoxic agents lead to their release from the hepatocytes cytosol to the blood stream indicating cellular degeneration or destruction. On the other hand, increase in the serum level of ALP, suggested the increase permeability of plasma membrane or cellular necrosis of liver [18]. Furthermore, the histopathological observations displayed 400 mg/kg of EOML lead to the hepatic cell injuries indicated by focal necrosis, inflammation, cell swelling and fatty degeneration. The outcome of the biochemical and histological examination illustrated that two weeks exposure to 400 mg/kg of EOML caused a clear hepatotoxicity in rats [17]. The GC-MS analysis data illustrated that the main ingredients constructing EOML are pulegone (54.1%), menthofuran (12.5%) and 1,8 cineole (8.0%).

“The amount of pulegone in the various oils depending on several factors such as origin of the plant, yearly weather conditions, harvest date, plant age, fertilization, location and planting time” [19]. EOML as an aromatic compound contains terpenoids ingredient including pulegone, isopiperiten one, menthofuran, 1,8-cineole, carvone, limonene which is consistent to the current results [4]. Pulegone and its derivatives cause the hepatic dysfunction and its injuries [4]. Liver microsomal CYP450 converts the pulegone to the oxidative metabolites like menthofuran associated with severe hepatic toxicity from centrilobular degeneration and massive necrosis [20]. These metabolites bind to cellular proteins and deplete hepatic glutathione [21-23]. Menthofuran is again metabolized through the CYP450 system, forming reactive species, including epoxy-menthofuran which continues the

oxidative stress from the pulegone [24]. In addition, pulegone itself remarkably depletes glutathione in both liver tissue and plasma [23]. 1, 8-cineole caused structural changes on the hepatic cells including mitochondrion swelling, vacuolar degeneration, rough endoplasmic reticulum fracture and degranulation [25].

CONCLUSION

The essential oil of *M. longifolia* is not entirely safe especially for the liver in high doses. Administration at the dose of 400mg/kg leads to the hepatotoxic effect. The death was occurring in the higher doses. The possible mechanisms for the EOML liver toxicity are triggering of oxidative stress or apoptosis by its ingredient like pulegone and menthofuran compound.

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