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Biochemical, liver and renal toxicities of *Melissa officinals* hydroalcoholic extract on balb/C mice

Abdolrasool Namjoo[1], Mohammad MirVakili[2], Hedayatollah Shirzad[3*], Mostafa Faghani[1]

[1]Pathology Department, Islamic Azad University of Shahrekord Branch, Shahrekord, Iran
[2]Veterinary Medicine Department, Islamic Azad University of Shahrekord Branch, Shahrekord, Iran
[3]Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Introduction: *Melissa officinalis* is usually used as antispasmodic, antiaxiety and antibacterial agent. However, its toxicity has not been evaluated, yet. In this study biochemical, liver and renal toxicities of *Melissa officinals* hydroalcoholic extract were evaluated in balb/C mice.

Methods: In an experimental study, 21 balb/C male mice were randomly designated to three equal groups. Group I was treated with normal saline and groups II and III were respectively treated with 0.450 and 1.350 g/kg, hydroalcoholic extract of *Melissa officinals* daily for two weeks, intraperitoneally. Then on 15th day of the experiment, blood samples were obtained from the heart. The blood was centrifuged and then the sera were evaluated for alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea and creatinine, using autoanalyzer and commercial kits. The liver and kidney tissues were also hystopathologically evaluated. The data were analyzed by one-way ANOVA, Tukey's post hoc test, and Kruskal-Wallis at a significance level of $p<0.05$.

Results: *Melissa officinals* dose dependently caused a significant reduction in alkaline phosphatase and alanine aminotransferase levels compared to the control group. Furthermore, *Melissa officinals* extract had no effect on the amount of urea and creatinine compared to the control group. The liver and kidney histopathological changes in the groups that received different doses of the extract showed mild, moderate, and severe tissue injuries.

Conclusion: The biochemical analysis in this study indicates that the extract of *Melissa officinals* causes liver tissue damage in mice; therefore, its consumption in high doses should be avoided.

Implication for health policy/practice/research/medical education:
Melissa officinalis may cause liver tissue damage and therefore, its consumption in high doses should be avoided.

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Introduction

The use of herbal medicines has been increased in the last few decades due to some properties such as variety of chemical biologic activities, effectiveness, higher safety and lower cost in comparison to synthetic drugs (1,2). In Iran the use of herbal medicine in the treatment of deseases is developing but biologic active compounds effects of some plant species on human health has been remained unknown. *Melissa officinalis* (lemon balm) with scientific is a medicinal plant in Lamiaceae family. It is more than 2 thousand years that this plant has been known. This plant grows in some mediterean regions, most Euroupean countries, Central America (3), and widely in Tehran, Golestan, Azarbayjan, Lorestan and Kermanshah (4,5).

In traditional Iranian medicine the lemon balm has been used in the treatment of depression and to reduce nervousness in young daughters (5). Most common therapeutic properties of the lemon balm are sedative, antioxidant, antispasm, antibacterial, antivirus, anti inflammation properties (6). It is also used dor the treatment of stomach disorders, histria, migraine, toothache, headache, hypertension, rheumatism, neuralgia, stiff neck (7), relief of menstrual cramps and relief of fever caused by cold (3). Today this plant is commonly used in cosmetics, perfumary and food industries (3). Experimental studies on laboratory animals have shown that hydroalchohol extract of the lemon balm plant has calming effect on central nerveous sytem. On the other hand this plant reacts to some drugs

*Corresponding author: Hedayatollah Shirzad, Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran.
E-mail: shirzadeh@yahoo.com

like alchohol, tranquilizers, barbiturates, antigaluocoma drugs and selective serotonin reuptake inhibitors (4). More than one hundred chemicals have been identified available in lemon balm. Some herbal chemicals like jeraniol linalool, citronella and citral produce perfume and fragnance in lemon balm (8). Moreover lemon balm plant contains a number of flanovoids and *monoterpenoid aldehydes* (6). Some of phenolic compounds are tannins, rosmarinic acid, fanovids like apigenin-7-oxide-glucoside, luteolin-7-oxide-glucoside and three flavenoids including Rhamnazin, ramnocitrin and isoquercetin (8,9). The results of the last studies have shown that lemon balm extract contains rosmarinic acid, tritrepnoids, olinolic acid and oresal acid that prvent the activities of gamma-amminobutric acid transaminase (GABA-T) (10) and this prevention increase brain accessability to GABA (11). Given lemon balm pharmaceutical properties, studying the toxic effects of this plant is significantly important. The present study is the first experimental study that considers effect of subacute toxicity of lemon balm hydroalcoholic extract on liver and kidney tissues of the female balb/C mice in the terms of anzyme changes and pathology in order to show side effects that can be provided following consumption of this plant.

Materials and Methods

In this experimental study lemon balm samples were provided from grossary centers were confirmed by botenists of the Medical plants research Center of the Shahrekord University of Medical Siences. Then, a herbarium specimen was prepared and kept in Herbarium unit of Shahrekord University of Medical Sciences under No. 407. The maceration method was used to extract lemon balm plant. Smooth powder was made form dried leaves and branches of the plant by using an electric mill. Then, the powder was mixed with 70 degree alchohol to the extent that alchohol level was 3 centimeters higher than powder level and then this mixture was filtered using glass hopper and filter paper after passing 48 hours. Filtered solution was transferred into balloon and its solvent was evaporated by rotary vaccuim pump (set in 48-50 ċ). Obtained concentrated liquid was dried by a flat glass dish inside avon in 37 ˚C. The obtained powder was collected to be used in providing different doses of lemon balm hydroalchoholc extract (6,12). 40 grams pure lemon balm extract was obtained from 1000 gr dried lemon balm plant. In this interventional study albino female mice with approximate weight of 20-30 g were used to conduct study. They were provided from Tehran Pastor Institute and then maintained in laboratory Animals Resource Center of the Islamic Azad University of Shahrekord in 3±20 ċ, 12 hours light-dark cycle and appropriate humidity. To make them compatible with environmental conditions mice were randomly divided into three groups of seven mice, one week befor starting the test, including a control group and two treatment groups treated with 0.450 and 1.350 mg/kg doses of lemon balm hydroalcoholic extract and were maintained in standard polipropolyne cages. Urban water and standard pelleted food were freely available for mice. All instructions related to work with laboratory animals were performed in accordance with University Regulation and Ethics Committee orders. To determine acute toxity (LD50) with regard to similar studies 18 other female mice were divided into three groups of six mice which received 4, 4.5 and 5g /Kg doses of lemon balm hydroalchoholic extract, intraperitoneally. Mice were cared and supervised for 5 hours at 2.30 h intervals and for 24h at the end of experiment in terms of behaviour, food and water consumption amount and death. Mortality number was determined after 24h (12) and LD50 was obtained 4.5 g/Kg. After specification of acute toxicity dose the studied groups were injected daily with lemon balm hydroalcoholic extract at 10 and 30% doses of acute toxicity, respectively for 14 days at a same time and control group was injected 0.3 ml normal saline, intraperitoneally (6). Mice were not fed for 12 hours after last injection of hydroalcoholic extracts and normal salin then blood samples were collected from the heart after anesthetization by chloroform and opening thoracic cavity. To seprate serum, the sample was centrifuged 10 minutes in 3000 cycles. Measurment of ALP, ALT and AST enzymes and serum levels of urea and creatinine was performed by autoanalyzer BT-3000 model (Biotechnique Company, Italy) using diagnostic kits of the Pars Azmoun Company. To perform pathology studies liver and kidney tissue samples were fixed in 10% buffered formalin (German country). Then according to common histology methods paraffin blocks were provided and some 5 micron cuts were created by microtom Shandon Citadel 315 model (England) and then were stained with haematoxylin and eosin (13). Damages of the kidney and liver tissue in studied groups were randomly selected with magnification 100, in three microscope fields and then were studied. These demages included some abnormalities in structural order and pattern of the liver cells and kidney nephrons. Results were reported as (-), (+), (+2) and (+3) signs, respectively for no changes in the kidney and liver structural order, presence of mild focal amnormalities in the liver lobules and kidney tubules structure and order, 50% (moderate) abnormality in the liver lobules and kidney tubules and sever abnormality in liver and kidney structure. The signs -, +, +2 and +3 were used respectively to show lack of observation of the ducts with hyperplastic epithelial cells, observation of three biliary tracts in each triads, 4 or 5 ducts with connective tissue in each triad and more than 5 biliary ducts in each triad, respectively. Lack of piecemeal necrosis around the portal area and the glomerular tubules, mild piecemeal necrosis, less than 50% involvement (moderate) of the hepatocytes around the portal tract and the curved pipes near and around the cortical area and more than 50% involvement of the hepatocytes around the portal tract and the cortical tubules were considered, respectively as signs -, +, +2 and +3. Lack of inflammatory cells observation in the portal area and between kidney tubules was reported with sign -, mild penetration of inflammatory cells in less than 1/3 of the portals and between renal tubules was reported with sign +, moderate penetration of inflammatory cells in the 1/3 to 2/3 of portals and between renal tubules was reported with sign +2 and sever penetration of compressed inflammatory cells in more than 2/3 of the portal area and between renal tubules was reported with sign +3. The signs -, +, +2 and +3 were assigned respectively to lack of portal fibrosis observation, development of mild portal fibrosis, moderate fibrous bridges and severe cirrhosis. Neutrophils accumulation in dead liver cells regions was defined as hepatocyte focal necrosis. Also apoptotic hepatocytes were specified by acidophilic cytoplasm and hyalinization. The presence of focal

necrosis, diffuse necrosis, apoptosis, Kupffer cells hyperplasia, sinusoidal dillation and hyperemia was specified with the sign + and the sign – was used to show their absence (14). Based on the results obtained from the Kolmogorov-Smirnov test to estimate equality of continuous, one-dimensional probability distribution, all data were distributed normally (P>0.05). Data were presented as mean±standard deviation and the one-way ANOVA statistical test was used to test significant difference between control and treatment groups. The Tukey's Post Hoc test was used if significant difference was obtained. Also to test histopathological data, the data obtained as qualitative between groups were analysed by Kruskal-Wallis test using SPSS statistical software.

Resuls

The results of biochemical parameters tests showed that serum activity of enzyme AST, as well as creatinine and urea following intraperitoneal injection of 450 and 1350 mg/kg doses of lemon balm had not changed significantly compared to control group (P>0.05), but the enzyme ALT in groups of mice treated with 1.350 g/kg dose of lemon balm extract showed significant reduction compared to control group and group of 450 mg /kg dose of lemon balm (P<0.05). Also activity of ALP enzyme in groups treated with hydroalcoholic lemon balm extract at doses of 10 and 30% acute toxicity showed a significant decrease (P<0.01) compared to the control group (Table 1).

According to histopathologic results of the liver tissue the lemon balm has increased relatively mild the pleomorphism hepatocyts in group recieving 30% lemon balm compared to other groups (P<0.05). Also in group receiving 30% lemon balm, moderate necrosis of the liver parenchymal, sinusoids dilation and moderate increase of hepatocytes with eosinophilic cytoplasm and hyperchoromatic chromatin (apoptosis) was more than the other groups (P<0.05) (Table 2 and Figure 1).

Microscopic studies conducted on liver tissue in groups receiving lemon balm at doses of 10 and 30% acute toxicity showed secretion of mononuclear inflammatory cells in the portal space (Figure 1), irregular hepatocytes plates and

Table 1. Effect of lemon balm hydroalcoholic extract on biochemical parameters of the mice serum

Biochemical parameters Groups	Urea (Mg/dl)	Creatinine (Mg/dl)	Alkaline phosphatase (U/l)	Alanine aminotransferase (U/l)	Aspartate aminotransferase (U/l)
Control	49±11.91	0.08 0.42 ±	360.2±89.15	44.8±5.07	180.8±53.28
Group 10%	42.6±11.12	0.050.36 ±	**185.2±38.48	34.4±5.36	131±29.93
Group 30%	40.33±9.33	0.040.38±	**148.17±54.03	*27.33±7.84	137.67±22.1

The number of mice in each group was 7, the data are as Mean ± SD,
*P<0.05 compared to control group, **P <0.01 compared to control group.

Table 2. Intensity of histopathological changes in liver tissue of the albino mice in different doses of lemon balm

Groups Liver changes	Lemon balm 10%	P value	Control	Lemon balm 30%
Hepatocytes degenerations	2+	0.001	+	2+
Sinusoidal congestion	-	0.389	-	+
Sinusoidal dilatation	-	0.032	-	2+
Portal congestion	+	0.147	-	+
Portal dilatation	2+	0.114	+	3+
Liver vein blood	+	0.082	-	2+
Bile ducts proliferation	-	1.00	-	-
Mononuclear cell secretion around the central vein	+	0.411	-	+
Mononuclear cell secretion around the portal area	2+	0.035	+	2+
Poleomorphism hepatocytes	-	0.047	-	+
Distinct nucleoli in hepatocytes	2+	1.00	2+	2+
Hepatocytes with eosinophilic cytoplasm	+	0.042	+	2+
Necrosis of the liver parenchymal cells	2+	0.002	-	2+
Irregular plates	+	0.026	-	+
Increase of fibrous tissue around the portal area	-	0.876	-	+

- Lack of histological changes observations (normal structure), + mild damage, 2+ moderate damage, 3+ sever damage

liver paraenchymal necrosis than control group (P<0.05). Histopathologic results of the kidney tissue in group receiving lemon balm at dose of 30% of acute toxity showed relatively mild atrophy of the glomerulus, and increase in urinary space than other groups (P<0.05) (Figure 2 and Table 3).

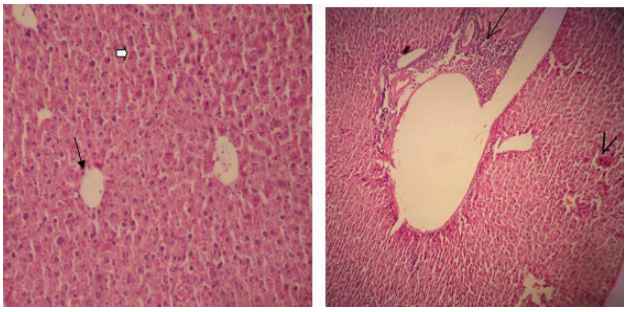

Figure 1. Histopathological picture of the liver in lemon balm group. Right side shows secretion of the lymphocytic cells in the portal space (arrow) and dilation of portal vein, apoptic cells (arrow peak), (H & E × 250); left side shows liver lobules structure in the control group, central vein (dark arrow) and natural hepatocytes (light arrow), (H & E × 400).

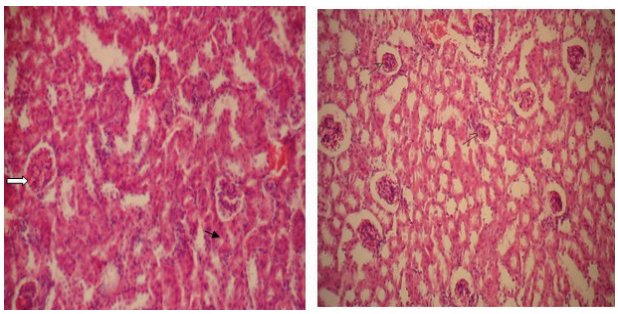

Figure 2. Histopathological image of the kidney tissue in the group receiving 30 % acute toxicity lemon balm.
Right side: Atrophy of the kidney tubules and glomeruli (arrow), (H & E × 250); leftside: tubules structure (dark arrow) and kidney glomeruli (light arrows) in control groups (H & E × 400).

Discussion

Liver enzymes such as ALT, AST, GGT and ALP are enzymes that significantly reflect liver hepatocytes necrosis and cholestasis and can be used in detection of sever liver diseases such as liver inflammation (15,16). Enzyme ALT in dog, cat, rabbit and rat is a liver hepatocyte cytosol specific enzyme that is increased in blood plasma in result of change in liver activity (17). In addition, AST has been recommended to identify liver abnormalities (17). ALP is a membrane-bound glycoprotein enzyme found in different tissues such as liver, bone and in kidney, intestine and placenta in less amounts. Also, it is a valuable biochemical index used in detection of hepatobiliary disorders, osteoporosis, and fatty liver disease (18,19). In this research, the acivity of ALT enzyme in group recieving 1350mg/kg lemon balm showed significant decrease compared to control group and injection of 450 and 1350 mg/kg doses of lemon balm hydroalcoholic extract decreased significantly enzyme ALP compared to control group. Researches of Wei et al showed that apigenin had anti-tumor properties, especially on breast tumors (13,14), and prevent Ornithine decarboxylase activity (20). Le Bail et al also presented the effective role of apigen in preventing activity of aromatase (human estrogen synthetase) and 17-Beta hydroxysteroid dehydrogenase available in the human placental microsomes (21). On the other hand change in the activity of liver aminotransferases encourages the renovation of glucose from amino acids and changes activity of plasma of AST and ALT. Activity of these two enzymes can be changed with a variety of chemical and biological substances and physiological factors or disorder in Krebs cycle. Decreased activity of the Krebs cycle causes decrease in some compounds and finally decreases in AST and ALT (22). It seems that decrease in activity of ALT and ALP enzymes in treatment groups is due to presence of apigenin compound that prevents activity of the ATP precursors and aminoteransferses. This hypothesis needs more studies. Histopathological changes of liver tisuue in groups receiving 450 and 1350 mg/kg doses of

Table 3. Intensity of histopathological changes in the kidney tissue of the albino mice in the different doses of lemon balm

Groups Changes in kidney tissue	P value	**Control**	Lemn balm 30%	Lemn balm 10%
Degeneration of tubular epithelial cells	0.095	+	2+	+
Tubular epithelial cell necrosis	0.073	-	+	+
Glomerular atrophy	0.029	-	+	-
Tubules atrophy	1.00	-	-	-
Protein secretion in the tubules	1.00	-	-	-
Mononuclear cell secretion around the interstitial tissue	0.45	-	-	-
Increase in fibrous tissue	0.45	-	-	-
Glomeruli congestion	0.92	+	+	+
Vascular congestion in interstitial tissue	0.17	-	+	-
Renal venous congestion	0.32	+	+	2+

- Lack of histological changes observation (normal structure), + mild damage, moderate damage, +2, +3 sever damage.

lemon balm showed that presence of eosinophilic hepatocytes and nucleus with chromatin hyperchrome (apoptosis) may be related to the effects of quercetin and apigenin in producing apoptosis. Apigenin increases reactive oxygen species and causes production and release of cytochrome C from mitochondria into the cytosol and subsequently induction of procaspase-3 process, and finally apoptosis through rapid induction of caspase 3 activity and decrease in transmembrane potential (23). Although, antioxidant activity of the plants chemical compounds is well known (24) but under special conditions like high doses or presence of metal ions can reveal the perooxidants activity.

Furthermore, perooxidant or antioxidant activity of plant chemical compounds has near relationship with consumed concentration (25) that corresponds with liver pathologic lesions at doses of 1350 mg/kg. Studies on the cellular models have shown that some antioxidant polyphenols like quercetine, catechins (*Epigallocatechin-3-gallate* and *epicatechin*) and gallic acid have perooxidative activity (26). Also studies of Robaszkiewicz et al reported that high doses of quercetine (50 µmol) produce super oxide radicals in cultured cells and isolated mitochondria. In other studies, the antioxidant activity of quercetine has been observed only at low doses (1 to 20 µmol) whereas concentrations above 50 µmol can cause decrease in cell viability, thiol levels, the total antioxidant capacity and activity of superoxide dismutase, catalase and glutathione S-transferase (27). Studies of Watjen et al also showed that flavonoids (quercetine and fisetin) at low concentrations (10 to 20 µmol) protect H4IIE Class cells against cell toxicity, DNA breaks and apoptosis induced by hydrogen peroxide. Also, high concentrations of flavonoids (50 to 250 µmol) caused cell toxicity, demage to DNA and planned death (25). It has been proved that high concentrations of flavonoides increase production of reactive oxygen species by autooxidation (28). These cases show that consumption of high doses of lemon balm hydroalcohoic extract in the short period causes toxicity effects on liver cells. It seems that reduction in activity of antioxidant enzymes with internal origin in liver and kidney tissues in the groups treated with high doses of lemon balm extract may be responsible for tissue damages (27). Furthermore, reactive oxygen species, hydrogen peroxide, superoxide anion and hydroxyl radical are important intermediates of cell damage that can react to macromolecules such as lipids, proteins, nucleic acids, carbohydrates and unsaturated fatty acids in cell membrane and cause chain cell damage and death after the initial reaction (3). Studies have shown that a number of antioxidants such as quercetein and narinjenin can prevent activity of cytochrome enzymes (CYP1A1 and CYP3A4) and cause cell damage (29). For this reason, various liver injuries was observed in 1.350 mg/kg dose of lemon balm group because liver has main and important role in the different regulatory and metabolic pathways of drugs metabolism (activation and detoxification), excretion of exogenous and endogenous substances. Also changes of enzymes creatinine and urea are caused by lack of *Melissa officinalis* extract effect on kindy structures.

Conclusion

According to enzyme and pathology results it can be concluded that consumption of lemon balam extract dose dependently causes toxicity in liver tissue.

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This paper has been derived from the thesis of the second author.

Authors' contributions

All authors participated in the study, have read the manuscript, and provided their final approval.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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