

## The effects of aqueous extract of *Melissa officinalis* on some blood parameters and liver of *Oncorhynchus mykiss*

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**Abstract.** The lemon balm (*Melissa officinalis*) has long been considered as traditional medicine which is used as an analgesic and anti-microbial plant. The aim of this study was to investigate the effects of aqueous extract of *M. officinalis* on liver enzyme alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and total protein and to study its pathology effect on *Oncorhynchus mykiss* liver. For this study, fishes with a mean weight of  $85 \pm 5$  g in 3 groups were examined which includes: control (without extract); test group 1 (450 mg per kg of body weight aqueous extract of *M. officinalis*); and test group 2 (1,350 mg per kg of body weight aqueous extract of *M. officinalis*), each with a 2 repetition treatments daily for 30 days, feeding with extracts and concentrates were in extruded form. Growing conditions (water temperature 14°C, the amount of oxygen dissolved in water of 9 mg/mL) was similar for all groups. The results of blood at the end of the 30 day showed a significant reduction of ALT and AST in blood serum in test groups 1 and 2 against control ( $p > 0.05$ ). Comparison of the total protein in blood serum test for all groups indicated significant differences ( $p > 0.05$ ). During comparative pathology examination of the liver, the waste is observed in the test group, compared to the control group.

**Key Words:** blood, biochemical, serum, lemon balm, rainbow trout.

**Introduction.** The medicinal plants are rich of secondary metabolites and they are the sources of many medicinal products of which one or more of their organs contain pharmaceutical active substance. These substances make up less than 1% dry matter, including effective drug properties (Liggins & Burt 2004). Given the number of chemical compounds in the extract, there can be no single mechanism for their antibacterial effects seen in the cell, actually they will have multiple target. These mechanisms do not operate isolated, but some of them are influenced by others (Liggins & Burt 2004). Lemon balm (*Melissa officinalis*) is one of the oldest and most popular medicinal plant that belongs to the Lamiaceae family. Because of the smell of lemon plant family they are called lemon balm. Various properties have been confirmed by numerous studies done on this herb (Wichtl 2004). The most common therapeutic properties of *M. officinalis* can be sedative, antioxidant, antispasmodic, carminative, anti-bacterial, anti-viral and anti-inflammatory (Budzyńska et al 2013). Also, a study conducted by Bunn et al (2001) proved the effect of this plant on the nervous system and its role in decreasing the symptoms of neurological disorders such as stress, anxiety and irritability. The plant protects the nerve cells by eliminating the free radicals (Lopez et al 2009). Moreover, this plant enhances the quality of sleep, as it is combined with valerian (Larsen et al 1992). Methanol and aqueous extracts of this plant function as an inhibitor of monoamine oxidase, underlining that the effect of methanol extract is stronger, compared to the aqueous extract. Hence, it can be construed that this plant has antidepressant effects (Garten et al 2009).

*M. officinalis* is an aromatic and herbaceous perennial plant originating from the East Mediterranean region and it is found in some places of Azerbaijan and Caspian sea (Mimica-Dukic et al 2004). *M. officinalis* leaves contain compounds as: glazes, neral,

geraniol, citral A and B, flavonoids, essential oils as caryophyllene and linalool, primary limonene terpenoids, carboxylic acid, phenol acid, rosmarinic acid, tannin, bitter substance, camphor, gallic acid, wax, resin, various sugars, golden and pectic materials (Dastmalchi et al 2008).

Phenolic compounds consisted of rosmarinic acid, tannins, flavonoids such as epithelial zhenin-7-oxide-glucoside, luteolin-7-oxide-glycoside and three flavonols, including ramosositerin, ramnazin and isococriterin. The results of some studies show that *M. officinalis* also contain rosmarinic acid, more terpenoids, olinolic acid and ovsal acid (Pourmorad et al 2006). Previous studies show zhenin epitopes content in the extract of *M. officinalis* which has anti-tumor properties (Namjoo et al 2013), also was able to reduce the activity of liver enzymes such as AST and ALT (Zeraatpishe et al 2011). Other flavonoids (quercetin and fisetin) have been observed in low concentrations which inhibits cell toxicity, break down DNA and apoptosis induced by hydrogen peroxide (Saleem et al 2010). The studies of some scholars (Namjoo et al 2013; Rostami et al 2012; Saleem et al 2010) demonstrated the toxic effects of high concentrations of ethanol extract of *M. officinalis*, such effects are realized via enzyme variations. The high concentrations of ethanol extract of the *M. officinalis* causes DNA damage, apoptosis and development of damages in liver and kidney tissue of Syrian mice (Namjoo et al 2013). Further researches are needed to be conducted in order to show the side effects that may occur after excessive use of *M. officinalis* extract.

To track these liver enzymes changes such as AST, ALT and ALP can be studied. Following the variation in liver and its failures, these enzymes change, therefore the enzymes should be regarded as biochemical index for liver disorders (Namjoo et al 2013). A survey was conducted in Romania in 2011 which proved that the essential oil of *M. officinalis* has antibacterial effect on gram-positive bacteria, but the essential oil had no effect on gram-negative bacteria (Spiridon et al 2011).

A study conducted in 2004 in Serbia (Baričević et al 2004) demonstrated the strong anti-bacterial effect of *M. officinalis* essential oil on *Shigella* bacteria. The *M. officinalis* essential oil has strong anti-fungal effect upon fungus in the food industry like, *Aspergillus flavus* and *Penicillium chrysogenum* (Baričević et al 2004).

Rostami et al (2012) evaluated the antibacterial properties of *M. officinalis* and they found that the plant has a similar antibacterial effect on gram-positive and gram-negative bacteria, when it is used in the form of essential oil. It is worth noting that the antibacterial effect of the extract on gram-positive bacteria is high, compared to gram-negative (Rostami et al 2012). According to another research (Rostami et al 2012), it can be deduced that the use of ethanol extract of the leaves of *M. officinalis* in Hindi pigs prevents the contraction and induction of histamine and barium (200 mg per mL) and it inactivates them.

During testing, the effect of essential oil on mice showed that the essential oil influenced the small intestine, while in guinea pig, it was deduced that the essential oil has an impact on the heart aorta and large intestine. Moreover, this oil weakens the muscles of the bronchi (ED<sub>50</sub> 22 mg per mL) through stimulating the ileum of choroid plexus of the third ventricle with the 8.7 electric induction (50 mg ED<sub>50</sub>) (Namjoo et al 2013). Inhalation of the plant extract to induce a state of relaxation in Syrian mice is poor (Meenatchisundaram et al 2009).

## Material and Method

**Biochemical methods for blood assessment.** In this study 300 *O. mykiss* with mean weight of 85±5 g in 3 test groups including: without extract (control), with 450 mg *M. officinalis* extract/kg body weight (test group 1) and 1,350 mg *M. officinalis*/kg body weight (test group 2), each with one treatment and 2 repetition for 30 days were fed with the extract. After a month, the bleeding from tail vein was accomplished. The fish blood serum was obtained from heparin blood by centrifugation on 4500 rpm (Glynn et al 2012). For measuring the total protein, alanine aminotransferase (ALT), aspartate aminotransferase enzymes (AST), and urea the samples were evaluated by spectroscopy method (Yunoos et al 2014). Blood factors measurement were carried out on the basis of

instructions provided by kit manufacturer (ATLAS). For accomplishment of complete protein test, 20 microliter of *O. mykiss* blood was prepared and examined using Olanow method (Olanow et al 1995). For carrying out the blood urea test, 10 microliter of fish sample blood was prepared and tested using Kukekov method (Kukekov et al 1999). In order to measure the alanine aminotransferase (ALT) and aspartate aminotransferase (AST), 100 microliter of fish sample blood was tested using a method described by ATLAS.

**Liver pathology methods.** To study fish necropsy after general anesthesia, and liver biopsy was performed (Roberts 2001). The tissue fixative solution was prepared. The tissues were prepared for molding after passing the phases, including discharging, elucidation, alcohol extraction and staining with paraffin. Molding was conducted in the form of paraffin, and sections were performed using microtome (Roberts 2001). After staining with hematoxylin and eosin staining technique on the slide glass, using an optical microscope with lens 10 and 40 cases were observed (Roberts 2001). Hematoxylin-eosin stained tissue sections were used for analysis (Roberts 2001).

10-20 mL absolute hematoxylin, 200 g ammonium alcohol or potassium, ammonium alum or potassium with an amount of 20 g, 200 mL distilled water and mercuric oxide (oxidized mercury) with an amount of 0.5 grams were used for preparing hematoxylin color. Moreover, for the preparation of color eosin, 1 gram of eosin, 100 mL of distilled water and a small piece of thymol were utilized. The hematoxylin and alum ammonium were solved in alcohol and distilled water, respectively, in the vicinity of low heat. Then, the resulted solutions were mixed together, poured in a 500 mL Erlenmeyer flask and it was boiled quickly (Roberts 2001).

At the same time mercuric oxide was added and was cooled quickly, and after cooling was placed in cold water. The mixture was dark purple color; this color was used for nuclear staining of tissue cells (Roberts 2001). After that distilled water and a piece of thymol as protective was added. The paint was used to paint the cytoplasm of cells in tissue.

Three dishes containing xylene were provided and the sample slides were put in each of these containers for 3 minutes, this measure was taken in order for removal of paraffin surrounding the samples. At this stage, again containers comprising absolute, 90, 80 and 70 degree alcohols were prepared and the lamps were put in the above-mentioned alcohols (absolute, 90, 80 and 70 degree alcohols respectively) in order for dehydration of the samples (Roberts 2001).

Then samples were placed in distilled water for a few minutes, and after were dried at the lab temperature. At this stage, the vessels containing alcohol acid were prepared and the samples were moved in these containers for 10 seconds in order to remove the additional colors of the hematoxylin. Then each of the samples was placed in distilled water for a few minutes. To stabilize the color of a substance called lithium carbonate, hematoxylin was used. Samples were kept in this substance was 1 minute. Then each of the samples was placed in water for a few minutes. In order to paint staining cytoplasm of cells, eosin stain was used.

After this period, each of the samples was transferred in distilled water for a few minutes, then the vessels containing 70, 80, 90 and absolute degree alcohols were prepared, respectively. Finally, the samples were emerged in these vessels for 1 minute, with regard to the above mentioned order. This was done in order for dehydration of the samples. At this phase, the vessels containing xylene were prepared and each sample was moved in these containers in order of elucidation for 5 to 8 minutes (Roberts 2001).

All test groups were maintained in concrete-made (rectangular) pools with dimensions of 1x10x0.8 m and water source with velocity of 3 cm/s, under 14 centigrade, and 8 ppm dissolved oxygen (oxygen saturation equals to 99%) under controlled condition. The daily feeding was accomplished during three times a day for a month, with chemical composition as presented in Table 1.

Proximate composition of the feed used in test groups

Table 1

<i>Characteristics</i>	<i>Values</i>
Target size (mm)	4.0
Crude protein (%)	39–42
Carbohydrates (%)	22.5
Ash (%)	6-8
Moisture (%)	7.5
Phosphorus (%)	0.8
G.E. (MJ/kg)	21.36
D.E. (MJ/kg)	18.43
Feeding guidelines bodyweight (%/day)	1.88

**Results.** The results of blood serum at the end of the 30<sup>th</sup> day showed a significant reduction of ALT and AST test groups and control group ( $p>0.05$ ). Comparison of the total protein in blood serum test for all groups showed a significant differences ( $p>0.05$ ). According to comparative pathology examination of the liver, the waste is evident in the test group, compared to the control group.

### **Biochemical parameters of blood results**

Biochemical parameters of blood (mean±SD) in experimental groups

Table 2

<i>Parameters</i>	<i>Treatment</i>		
	<i>Control</i>	<i>Test group 1</i>	<i>Test group 2</i>
AST IU/L Aspartate aminotransferase	38.54±8.88 <sup>a</sup>	8.77±1.15 <sup>b</sup>	7.31±0.81 <sup>b</sup>
ALT IU/L Alanine aminotransferase	44.51±6.09 <sup>a</sup>	14.64±3.78 <sup>b</sup>	12.87±1.70 <sup>b</sup>
TP g/dL Total protein	43.93±3.3 <sup>a</sup>	183.4±16.63 <sup>b</sup>	102.4±1.29 <sup>c</sup>
UREA mg/dL	0.27±0.01 <sup>a</sup>	0.11±0.01 <sup>b</sup>	0.19±0.01 <sup>c</sup>

Different letter in the same row indicate significant differences ( $P>0.05$ ). Test 1 = 450 mg aqueous extracts of *M. officinalis*/kg bodyweight, Test 2 = 1,350 mg aqueous extracts of *M. officinalis*/kg bodyweight.

Statistical analysis showed that the enzyme alanine aminotransferase test samples of blood serum of *O. mykiss* in the control group were significantly different against experimental groups 1 and 2 ( $p>0.05$ ), but there was no significant differences between the experimental group 1 and 2 ( $p>0.05$ ). This enzyme is most relevant to the control group (44.51±6.09 units per liter) and the lowest values was evidenced for the treatment group 2 (12.87±1.7 units per liter) (Figure 1).

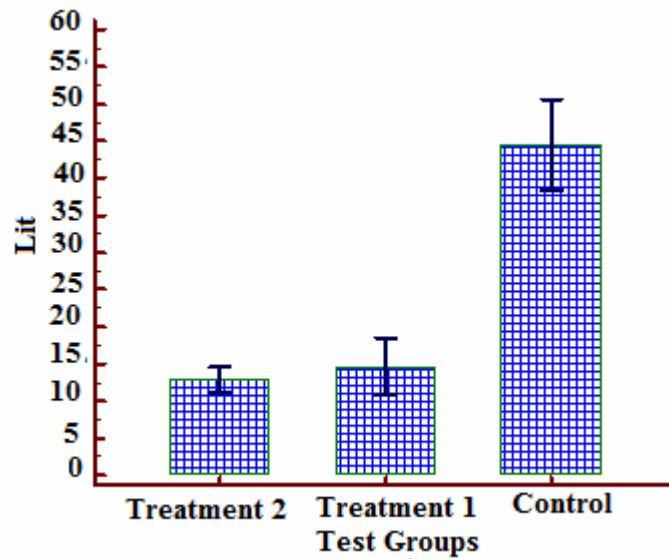


Figure 1. The values of ALT blood studied in examined groups.

Comparing the mean ( $\pm$  standard error) of the enzyme AST in test group and the control group, a significant difference between the control and exposed groups was evidenced ( $p > 0.05$ ). But no significant difference was observed between the groups facing each other ( $p < 0.05$ ), the minimum AST ( $7.31 \pm 0.08$  IU/L) was found in the test group 2 to the and the maximum in the control group ( $38.54 \pm 15.1$  IU /L) (Figure 2).

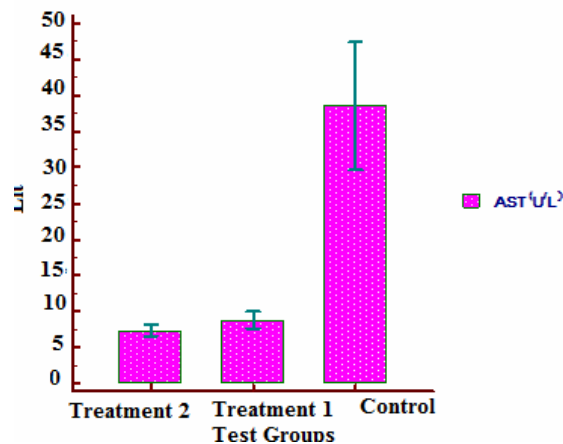


Figure 2. AST enzyme levels in blood samples of *Oncorhynchus mykiss* studied under different treatments.

Comparing the mean ( $\pm$  standard error) of total protein of test and control groups, was evidenced a significant difference which was in the blood index of all experimental groups ( $p > 0.05$ ). The highest value of the total protein was recorded in the experimental group 1 ( $183.4 \pm 16.63$  g/dL) and lowest value in the control group ( $44 \pm 4.23$  g/dL) (Figure 3).

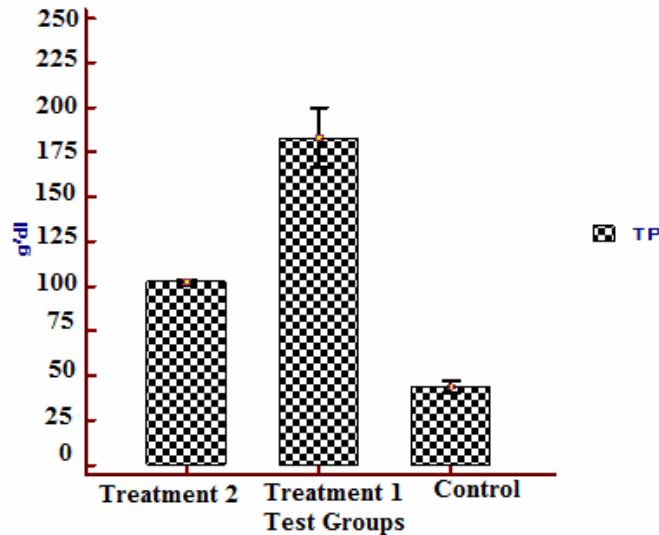


Figure 3. *Oncorhynchus mykiss* blood total protein levels studied under different treatments.

Comparing the mean value ( $\pm$  SD) of blood urea between experimental and control groups, significant differences was showed between all the groups ( $p > 0.05$ ). The minimum amount of blood urea was recorded in the test group 1 ( $0.2 \pm 0.019$  mg/dL), and the highest value (0.2 mg/dL) was observed in the control group (Figure 4).

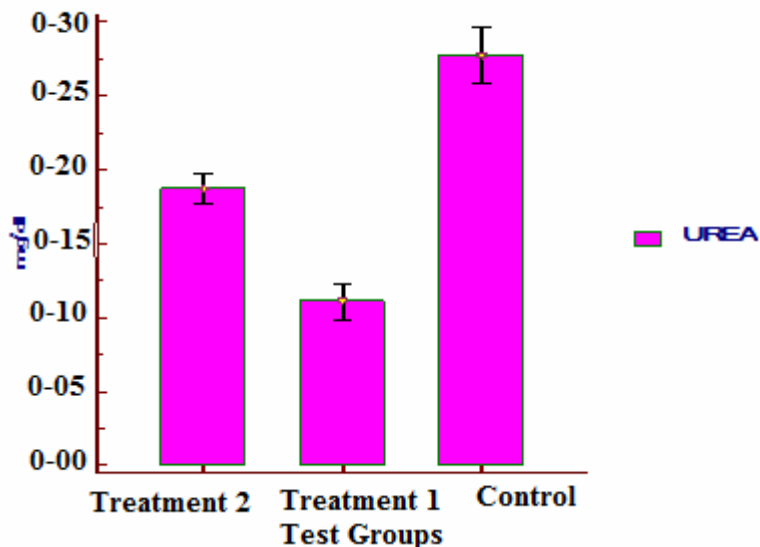


Figure 4. Amounts of blood urea in *Oncorhynchus mykiss* under different treatments.

**Results of liver pathology.** With regard to the pathology findings linked to the test group liver tissue exposed to the various concentrations of the *M. officinalis* aqueous extract, significant damages in the liver tissue of the test group 2 (Figures 5 and 8) were observed, compared to the liver tissue of control group. So that, as the *M. officinalis* concentration was increased, the tissue damage was developed.

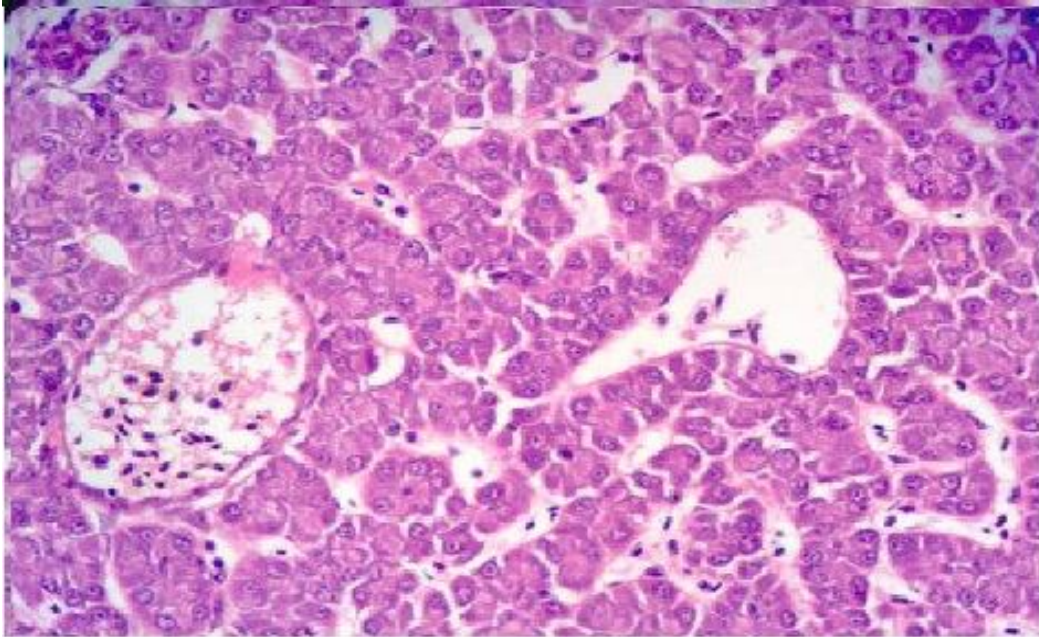


Figure 5. Liver of the control group. The structure of parenchyma and hepatocytes is normal and there is no sign of hyperemia, bleeding and melanomacrophage centers (H & E X400).

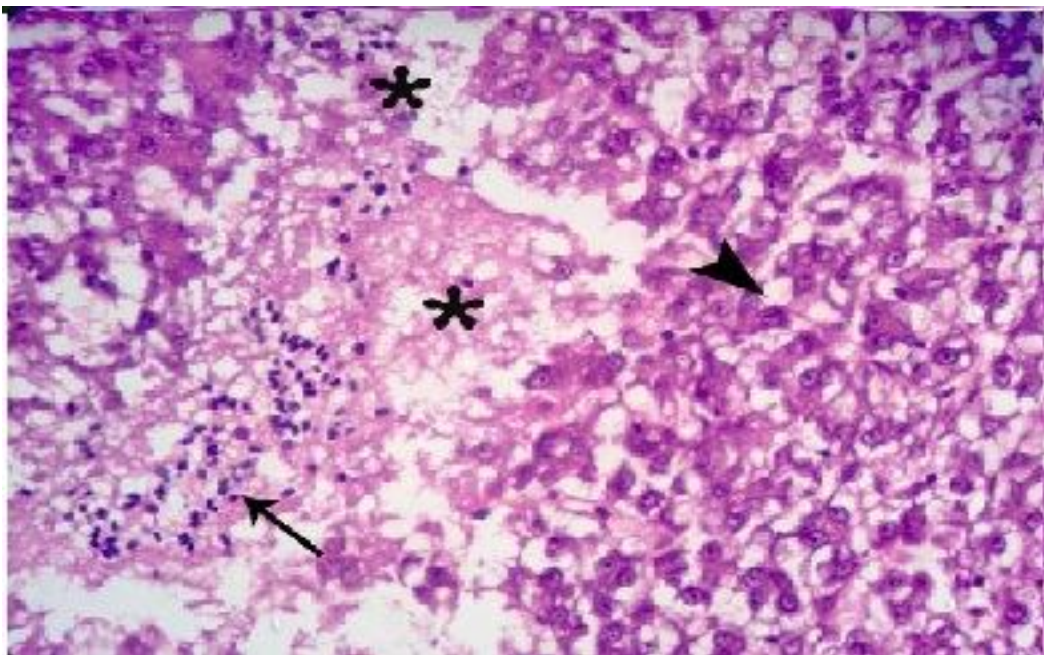


Figure 6. Hepatocytes necrotic liver test 2 and their remaining cellular debris (debris) (stars), the accumulation of red blood cells and fewer lymphocyte cells (arrow) in the area of necrosis and transparent vacuole (arrowhead) in hepatocytes are determined (H&E X400).

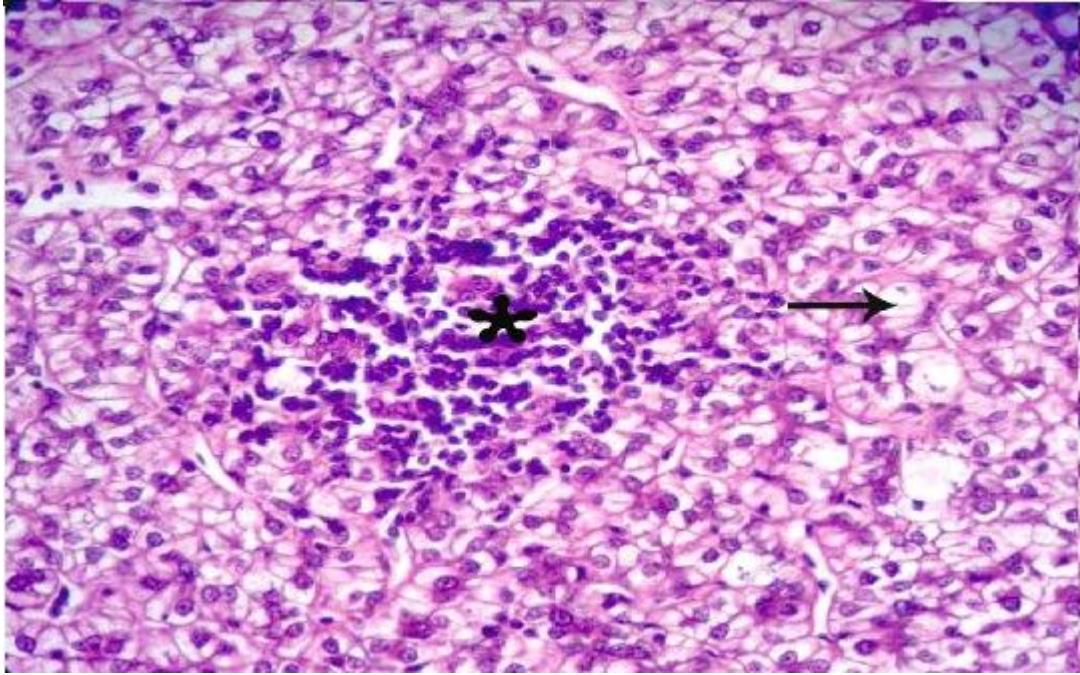


Figure 7. Aggregation focal liver test 2 lymphocytes (star) and transparent vacuole (arrow) are found in liver cells (H&E; X400).

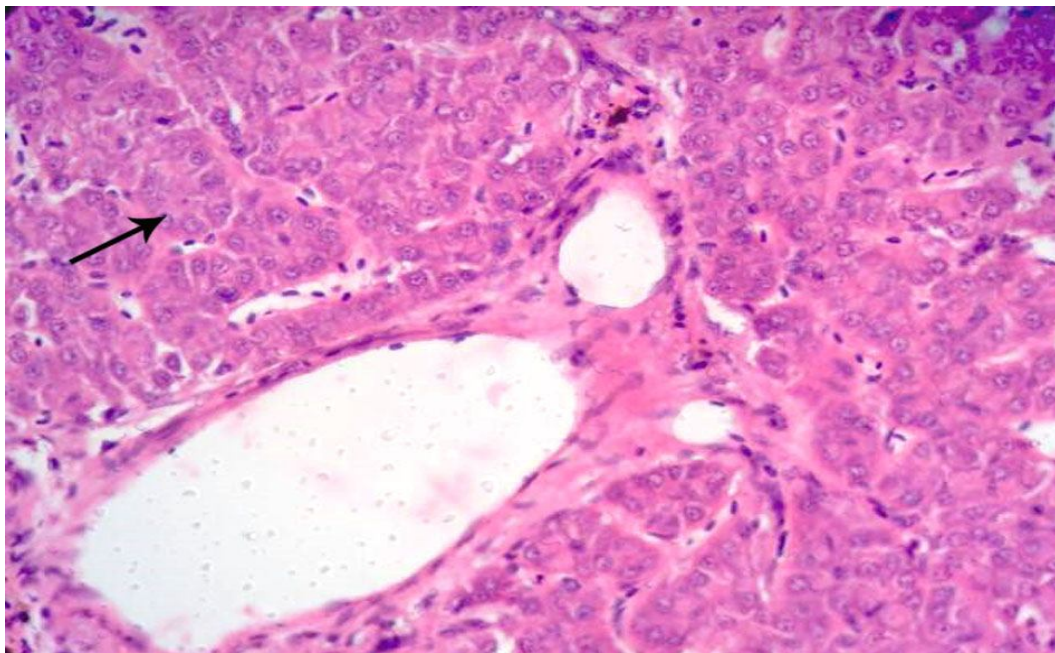


Figure 8. Liver tissue of test group 1. The structure of the liver parenchyma and hepatocytes (arrow) are normal and similar to that of the control group (H&E; X400).

**Discussion.** Farahi et al (2012) studied the impact of dietary supplement of *M. officinalis* and *Aloe vera* on blood, fat oxidation and carcass features in *O. mykiss*. Body condition and growth of fish was not affected by the herbal supplement in fish feeds, but there were significantly decrease in mortality compared with the control group. Moreover, no significant differences in relation to the RBC (red blood cells) Hb (hemoglobin) was observed in the tested groups.

The results of the present study are in contrast with the results of the research conducted by Farahi et al (2012), since there is a significant difference between the blood parametwrs of the treatments fed with *M. officinalis* extract and that of the control group.



The impact of medicinal plant extracts and essential oils and a variety of growth factors were investigated by Syahidah et al (2015).

Since, the blood parameters and immune system were improved, the growth rate of the test groups was increased. The mortality rate of the test groups was lower than that of the control group, so that, these results and findings confirm the results obtained by other scholars, including Salah et al (2008) and Farahi et al (2012).

Moreover, the results of the researches conducted by the mentioned scholars are in line with that of the current study in terms of growth factors. There are no significant differences between the test groups, in terms of growth indices.

Namjoo et al (2013) conducted a study which was mainly focused on investigation of subacute toxicity effects of ethanol extract of *M. officinalis* on the liver and kidneys of Syrian mice.

According to the results obtained from the measurement of the biochemical parameters of the blood samples, it can be construed that there was no significant variation ( $p > 0.05$ ) between serum activity of AST, creatine and urea following the intraperitoneal injection with 450 mg and 1,350 mg doses for each kg of the *M. officinalis* body weight, compared to the control group. But, the ALT enzyme in mice under *M. officinalis* extract treatment with dose of 1,350 mg, was reduced ( $p < 0.05$ ) for each kg of the body weight, compared to the control group and *M. officinalis* group with dose of 450 mg. Moreover, the activity of ALP enzyme in the groups under treatment of ethanol extract of the *M. officinalis* with doses of 10% and 30% acute toxicity showed a very significant reduction, compared to the control group ( $p > 0.01$ ).

**Conclusions.** In this study, low-dose and high dose of aqueous extracts of *M. officinalis* is proved to be toxic to liver cells and blood of *O. mykiss*. The adverse effects are high in group 2 (1,350 mg/kg of body weight), compared to group 1 (450 mg/kg of body weight). The *M. officinalis* concentration higher than 450 mg led to liver tissue damage of the *O. mykiss*, while the higher concentration has a decreasing effect on the blood biochemical parameters. Therefore, it is recommended studying the effect of *M. officinalis* extract on the various tissues, like assessment of the cell DNA damage. Generally speaking, the results of the present study incite to use *M. officinalis* extract with concentration lower than 450 mg/kg bodyweight to *O. mykiss*, which does not lead to destructive effects on the liver tissue, even it has led to reduction of the liver enzymes in the blood, like AST and ALT enzymes.

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