

Original Article**Genotoxicity and Histopathology Effects of *Melissa officinalis* Aqueous Extract on the Blood and Vital Tissues of *Oncorhynchus mykiss* Fish**Mitra Jafarpour¹, Amir Amniat-Talab^{*2}, Ali Nekuie-Fard³

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ABSTRACT

Background: This study was conducted to investigate both the genotoxicity effects of *M. officinalis* aqueous extract on blood cells and the pathologic changes in the renal, cardiac and splenic tissues of *Oncorhynchus mykiss*.

Methods: 300 fish (*Oncorhynchus mykiss*) were divided randomly into three groups (N=100 each), consisting of group 1 (control), and groups 2 and 3 (experimental), which received 450 mg/kg and 1350 mg/kg of body weight the aqueous extract of *M. officinalis*, respectively. The fish were fed for 30 days, with the experimental groups given three treatments. Micronuclei test and comet assay were used to identify the histopathological damages, simultaneously.

Results: We found significantly more micronuclei (33%) in erythrocytes of group 3 than those in group 2 (5%; $p < 0.05$). Similarly, the results of comet assay were consistent with those obtained for the micronuclei test. The recorded DNA damages to erythrocytes was significantly higher in group 3 (35.75%) compared to that for group 2 (7.15%; $p < 0.05$). The pathologic findings in the spleen, kidneys and heart tissues together with those obtained from the micronuclei test and comet assay confirmed the tissue and DNA damages after exposure to the extract. Abundant and severe cystic and atrophic glomeruli, renal tubular degeneration, hemorrhage and focal lymphocytic inflammation in heart, and increased melanomacrophage centers in the kidneys and spleen were observed at significantly higher frequency in group 3 than those in group 2 ($p < 0.05$).

Conclusion: The findings of this study demonstrated that the extract of *M. officinalis* at doses higher than 450 mg/kg per body weight caused toxic effects with severe tissue and DNA damages in *O. mykiss* fish.

Keywords: Genotoxicity, *Melissa officinalis*, *Oncorhynchus Mykiss*, Pathologic Alterations.

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INTRODUCTION

Nearly, 60-80% of the population worldwide traditionally use herbal medicines as remedies for various ailments [1]. *Melissa officinalis* (a.k.a. lemon balm) is considered an old plant, belonging to *Lamiaceae* family, an endemic herb in the Mediterranean region, south Europe, west Asia and tropical countries. The most common therapeutic application of the *M. officinalis* is for its antioxidant, spasmolytic, carminative, antibacterial, antiviral and anti-inflammatory properties. Specifically, the antioxidant properties are thought to be mainly through the phenolic and flavonoid components that serve to reduce the cellular oxidative stress [2,3]. The aqueous or ethanolic extract of the leaves and stems is also used traditionally as a remedy for headache, epilepsy, hyperthyroidism and fever [4-6].

Some plants may have toxic ingredients and pose direct or indirect harmful effect to cells and their genome [2]. Medicinal plants with toxicity to fish may enter the human body through food chains or as herbal drugs, thus may pose challenges to human health and safety [7]. Recently, it has been determined that many plants that are used traditionally as foods, may have

cytotoxic, mutagenic and genotoxic effects. Generally, genotoxic tests are performed to determine the ability of specific plants to interact with DNA and to alter the hereditary characteristics of the cells through mutations. Also, plants may interact or combine with drugs, resulting in harmful effects to human organs. The properties of some herbal drugs have not yet been fully understood, thus there remain concerns about their toxicity and safety in humans and animals [1]. Upon a careful review of literature, there was no published evidence on the genotoxicity of *M. officinalis* on the erythrocytes and kidneys, renal, cardiac and splenic tissues of *O. mykiss* fish. However; one study has reported that gills are more sensitive to DNA damages than other tissues in fish [8]. The purpose of this study was to investigate the possible histological damages to the cells and organs in *O. mykiss* fish after exposure to the extract of *M. officinalis*.

MATERIALS AND METHODS**Sample Collection**

This study was performed in the pools of Berkeh Tala-ee farm in Urmia, Iran. Rainbow trout fish (N=300) with average weight of 85 ± 5 g was used in three test

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groups (N=100, each) as follows: **group 1:** no extract given; **group 2:** 450 mg/kg body weight (BW) of the extract; and **group 3:** 1350 mg/kg BW of the extract. The experimental fish in each group were treated with one initial dose and two subsequent treatments. The fish were fed for 30 days with diets containing aqueous extract of *M. officinalis* (450 or 1350 mg/kg BW) daily at water temperature 13°C with a water circulating system [9]. All fish were kept in pools (1.5×10×0.8 m) with the water circulating at 3 liter per second.

Preparation of *M. Officinalis* Extract

Upon collection of *M. officinalis* from the academic botanist at Urmia University, Urmia, Iran, the leaves and stems were dried for 3 days by air circulation at 40°C. One hundred grams of the dried and powdered material was thoroughly mixed in one liter of distilled water for 10 minutes. The mixture was then filtered twice (paper filter) and the solution was lyophilized. Two doses of the aqueous extract (450 and 1350 mg/kg) were considered appropriate for the experiments [6,10,11].

Micronucleus Test and Comet Assay

For the micronucleus test, blood samples were withdrawn by puncturing the vein in the fish tail or taking peripheral blood smears after 30 days into the study, using heparinized syringes. The blood smears were fixed in absolute methanol for 10 minutes and stained with 5% Gimsa. The red blood cells were visualized under light microscopy (×40 & ×100) and the micronuclei were counted and examined for structural damages. Also, the stained smears were evaluated under light microscopy (Nikon ECLIPSE-50i, Japan) to identify the abnormal erythrocytes and the micronuclei, using the established micronucleus test and comet assay [12]. These are sensitive methods to determine the genotoxicity of chemicals used widely in the industry and agriculture [7,13].

Briefly, 10 µl of each blood sample was diluted in one liter of 5% bovine serum albumin (BSA) and mixed with 120 µl of 0.5% agarose at 37°C. This was coated on the slide and refrigerated for one hour. The slides were immersed in the lysis buffer containing NaOH, sodium lauryl sarcosinae, Triton X100, DMSO, NaCl, EDTA and Tris. Following the lysis process, slides were incubated for 20 minutes in NaOH and EDTA buffer (pH>13) to denature the DNA. The slides were then subjected to electrophoresis (25 V, 300 mA) for 20 minutes. The slides were stained by ethidium bromide (0.02 mg/ml) [12] and 100 nuclei were analyzed. Red blood cell count was performed from the blood smears and photomicrographs were taken under fluorescence microscopy (Olympus BX51, Japan). Also, one hundred erythrocytes were counted in each blood smear for all 100 fish in each group and the percentage of damaged versus intact DNA was determined [14]. The photomicrographs were processed and filtered by Image J 1.46 software (Bethesda, USA) for accurate determination of the erythrocytes with damaged DNA.

Pathologic Procedures for Tissues from Kidneys, Heart and Spleen

For pathological evaluation of the kidneys, heart and spleen, 30 fish in each group were selected randomly. After necropsy and tissue sampling, the collected tissues were placed in 10% buffered formalin as the fixative solution. The fixed tissues were processed by a processor (Leica TP1020; Nussloch, Germany) for dehydration, clearing and infiltration followed by preparing paraffin blocks and cutting sections by a rotary microtome (Leica RM2125 RTS; Nussloch, Germany) in 5µm thickness. Finally, the sections were stained by hematoxyline and eosin. The histopathologic study of the stained tissue sections was performed under light microscopy (Olympus CX 23, Japan). Ordinal grading of the histopathologic lesions was identified as absent (0), mild (+), moderate (++) and severe (+++) based on an established method [15].

Statistical Analysis

In this study, the data were analyzed by SPSS, version 19 software. Chi-square test was used followed by Bonferroni's post-hoc comparisons test to analyze the data. A $P \leq 0.05$ was considered significant throughout the statistical analyses. Ethical considerations in this study were based on the approved national protocol for animal care and research.

RESULTS

Micronuclei Test

The results of microscopic evaluation for presence of micronuclei in erythrocytes are presented in Table 1. The highest frequency of micronuclei presence in the erythrocytes was 33% in group 3 compared with 17% in group 2 (17%) and 0% in control group. Figure 1 represents the erythrocytes of and the micronuclei.

Genotoxicity Tests

The evaluation of the fish blood smears by fluorescence microscopy revealed that DNA damages in the RBCs of group 3 were more severe and frequent than those for group 2. No DNA damages were observed in the RBC's of the control group. The results of comet assays are presented in Table 2 and Figure 2.

Histopathologic Findings

The highest and lowest percentages of erythrocyte micronuclei were recorded for groups 2 and 3 at %5 and %33, respectively, but micronuclei were not observed in the control group. The histopathologic lesions detected in the three groups are presented in Table 3. The severity of the lesions in the kidneys, heart and spleen were mild in group 2 and severe in group 3. The observed pathologic lesions found in the evaluated tissue sections were as follows. **Kidneys:** cystic and atrophic glomeruli, abundant melanomacrophage centers and tubular degenerations were observed (Figure 3).

Melanomacrophage centers are aggregates of highly pigmented phagocytes, found primarily in the head, kidney and spleen, and occasionally the liver of many vertebrate species. **Heart:** nuclear pyknosis,

hemorrhage and lymphocytic infiltration were observed (Figure 4). **Spleen:** The most notable changes observed were increased number of melanomacrophage centers (Figure 5)

Table 1. Comparison of the presence of micronuclei in *O. mykiss* fish.

Groups	Total number of slides evaluated	Micronuclei (+)	Micronuclei (-)	P-value
(1) Control Group	100	0	100	0.041
(2) <i>M. officinalis</i> (450 mg/kg, BW)	100	5	95	
(3) <i>M. officinalis</i> (1350 mg/kg, BW)	100	33	67	

BW = Body weight

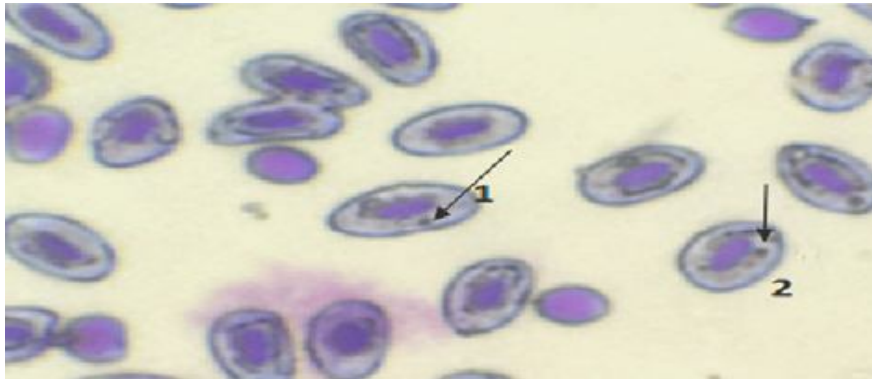


Figure 1. Erythrocytes of *O. mykiss* containing micronuclei (arrows) in group 2 (Gimsa, $\times 400$).

Table 2. Results of comet assay in two experimental groups compared to the control group.

Character	Blood smear	RBC Count in smear	Group 1 Control	Group 2 450 mg/kg	Group 3 1350 mg/kg	P-value
Undamaged DNA (Head)	100	100	100	92.85	64.25	0.061
Damaged DNA (Tail)	100	100	0.0	7.15	35.75	0.047*

*= $P < 0.05$

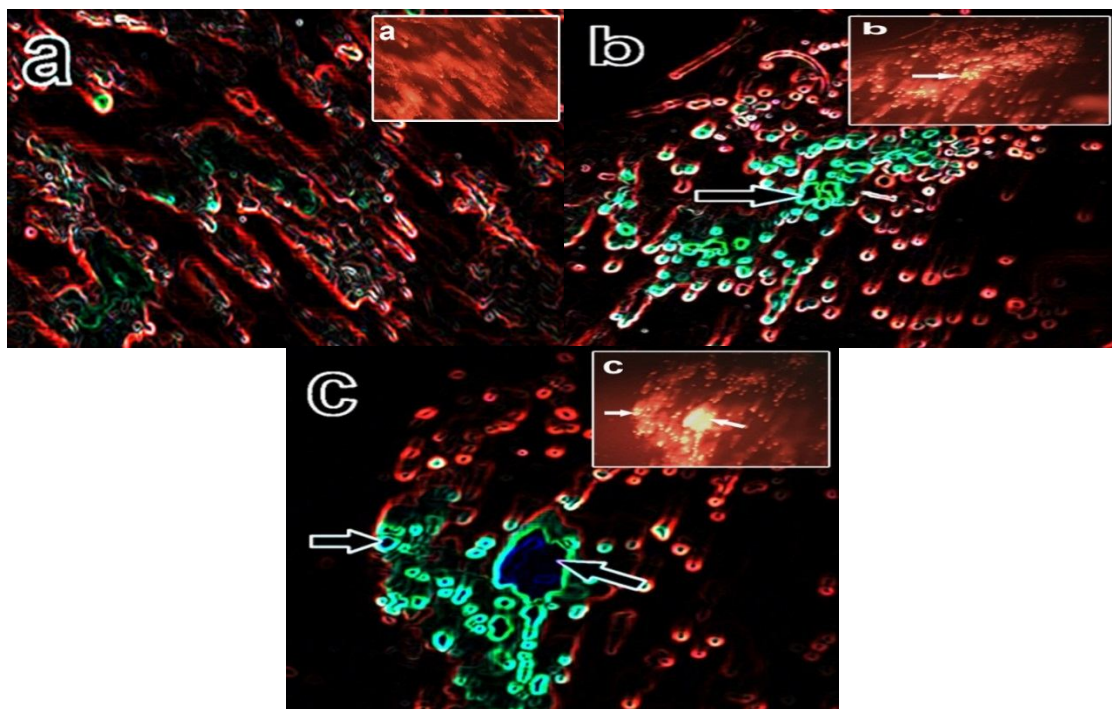


Figure 2. Photomicrographs of comet assay under fluorescence microscopy. (a): No comet was observed in the erythrocytes of the control group. (b): DNA damage (tail) as comet. Arrows indicate aggregation of tail DNA in erythrocytes of group 2 shown in white color in the inset figure, and green color in the main figure (450 mg/kg). (c): Tail DNA with higher density (arrows) in group 3 (1350 mg/kg) than in group 2. Mag. $\times 100$.

Table 3. Pathologic changes caused by *M. officinalis* aqueous extract in tissues of *O. mykiss*.

Tissue	lesion	Group 1 Controls	Group 2 450 mg/kg	Group 3 1350 mg/kg	P-value <0.05
Kidney	Cystic and atrophic glomeruli		13	69	0.044
	Melanomacrophage centers	100	22	84	0.039
	Tubular degeneration		13	68	0.048
Heart	Pyknosis		15	22	0.05
	Hemorrhage	100	8	46	0.028
Spleen	Focal lymphocytic inflammation		5	64	0.036
	Melanomacrophage centers	100	24	88	0.040

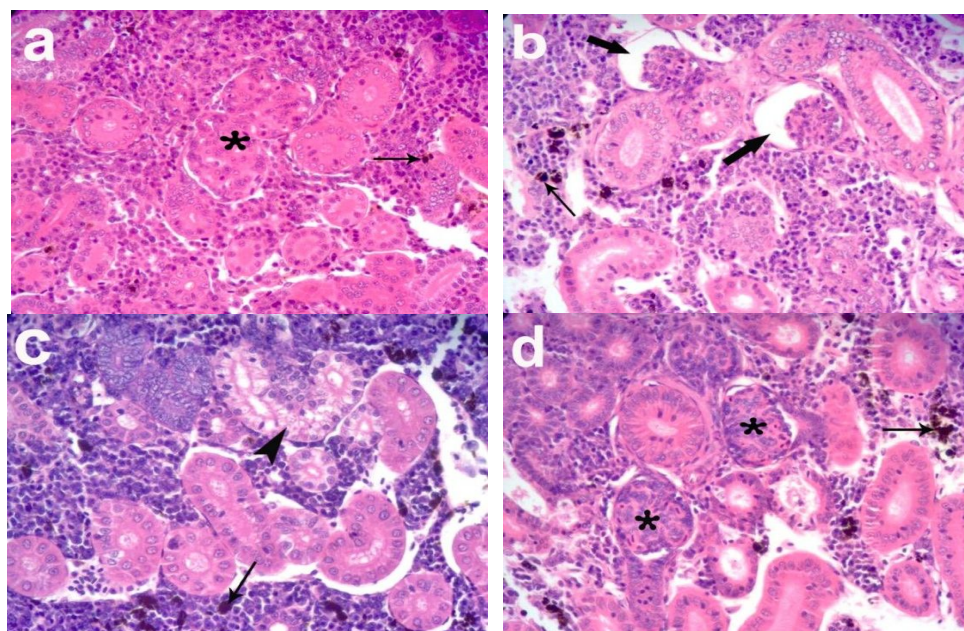


Figure 3. (a): kidney of the control group with normal glomerulus (*asterisk*) and a few number of melanomacrophage centers (*arrow*). (b): Cystic and atrophied glomeruli (*thick arrow*) with melanomacrophage centers (*arrow*) more than control group in renal parenchyma of group 3 (1350 mg/kg extract). (c): Tubular hydropic degeneration (*arrowhead*) and presence of many melanomacrophage centers (*arrow*) in group 3 (1350 mg/kg extract) compared to other groups. (d): Normal glomeruli (*asterisks*) and tubules with increase of melanomacrophage centers (*arrow*) in group 2 (450 mg/kg extract) compared to control group. H&E stained; mag. $\times 400$.

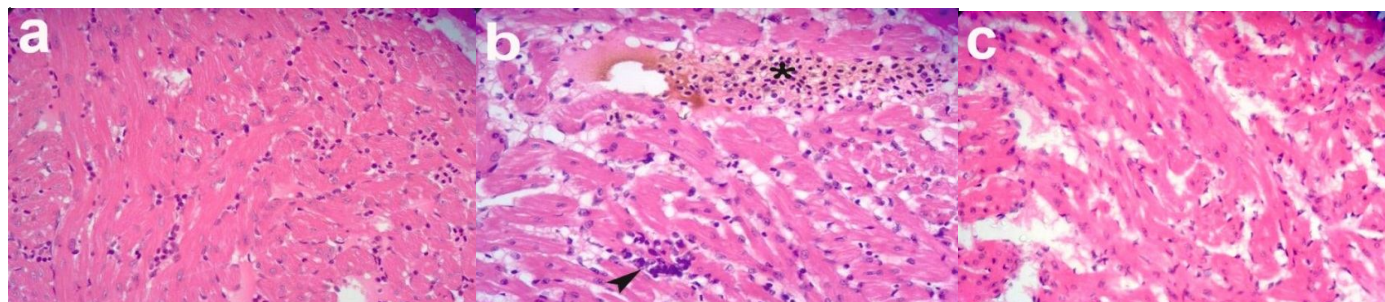


Figure 4. (a): Heart tissue of the control group without pyknosis or hemorrhage in cardiac myocytes. (b): Presence of local hemorrhage (*asterisk*) and focal infiltration of lymphocytic inflammatory cells (*arrowhead*) between cardiac myocytes in group 3 (1350 mg/kg, extract). (c): Normal heart tissue similar to control group, without hemorrhage or inflammation in group 2 (450 mg/kg, extract). H&E stained; mag. $\times 400$.

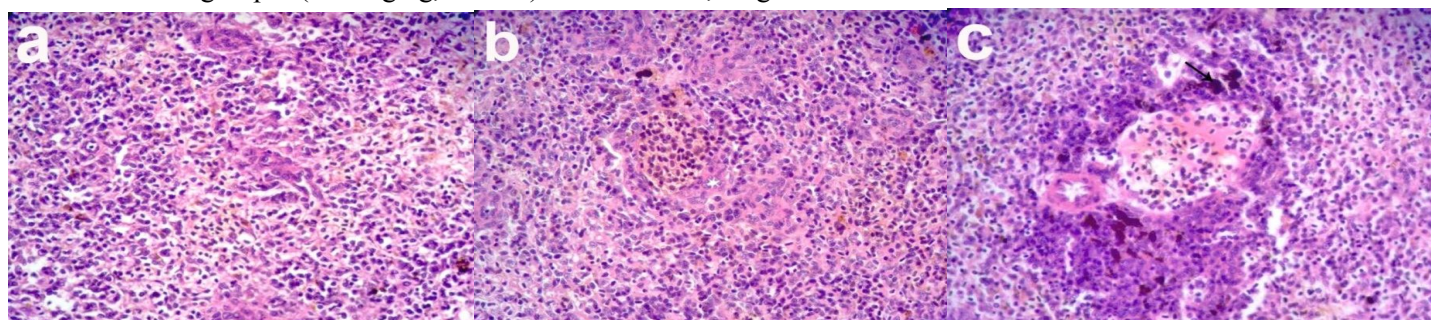


Figure 5. (a): Normal structure of spleen in control group. (b): Spleen of group 2 (450 mg/kg extract) with normal structure of white and red pulp similar to control group. (c): Prominent increase of melanomacrophage centers (*arrow*) in splenic parenchyma of group 3 (1350 mg/kg extract) compared to group 2 (450 mg/kg extract). H & E stained; $\times 400$.

DISCUSSION

Initially, herbal products that are derived from medicinal plants were thought to have no adverse and toxic effects. Nevertheless, the results of Singh *et al.* [7] indicated that some of the products of medicinal plants have genotoxic and mutagenic properties; therefore, they should be used cautiously. For instance, if the extract of *J. gossypifolia* is used in high doses, it will have genotoxic effects [7]. These authors have suggested that the toxic effects of *J. gossypifolia* can be detected in the crude extract [7]. Also, the oral consumption of the extract from *M. officinalis* at high doses has caused pathologic changes in the liver and kidneys of mice [11]. Arguments in support of the toxicity and pathologic effects of the extract involve reduction or inhibition of the antioxidant activity of enzymes that scavenge free radicals [11]. In fact, some of the ingredients of medicinal plants, such as quercetin and naringenin, are able to cause cellular damages by inhibiting the activity of cytochrome enzymes (CYP1A1 and CYP3A4) [11].

Genotoxic & Tissue Damage

In our study, we discovered that the oral administration of *M. officinalis* extract at high dosage caused significant genotoxic effect on the erythrocytes of the fish treated with 1350 mg/kg (group 3) compared with 450 mg/kg of the same extract. Our results (Tables 1, 2) demonstrated that the extract at dosages higher than 450 mg/kg caused more genotoxic damage in fish than did the lower doses. These findings are consistent with those reported by Namjoo *et al.* in mice [11]. Consistent with our findings, another study has also reported that the extract of *Althaea officinalis*, if added to the diet of common carp fish in dosages higher than 5000 mg/kg, had toxic effects on the biochemical indices of blood in the fish. However, the study reported no adverse effects for doses of the extract below 5000 mg/kg in fish exposed to *Aeromonas hydrophila* infection [16,17]. With respect to the dose-relationship, we had similar findings for the pathologic effects of *M. officinalis* extract on the fish liver, comparing the response observed in group 2 versus group 3 (450 mg/kg Vs 1350 mg/kg, extract). The fish in group 3 exhibited more serious damages, such as hemorrhage, fatty changes, necrosis and lymphocytic infiltration in the liver than those in group 2 [18].

Inflammatory Effect

In this study, microscopic results from the tissue sections showed that in group 3 melanomacrophage centers were more abundant than those observed for group 2. Notably, these changes were rarely present in the control group. Increase in melanomacrophage centers may be due to the deposition of exogenous or endogenous materials secondary to the toxic and/or inflammatory effects of *M. officinalis* extract at high doses [19,20].

Renal, Hepatic & Intestinal Lesions

Renal pathologic changes, such as cystic and atrophic glomeruli, increased melanomacrophage centers and tubular degeneration were seen in group 3 more severely than those in group 2. The epithelia of renal tubules play an important role in the excretion of ions, pollutants and

antibiotics [19]. This study found that high and toxic doses of *M. officinalis* extract were able to cause degenerative and necrotic lesions in renal tubules of fish. Results of a research on the evaluation of toxic and pharmacokinetic properties of the aqueous extract of Rosmary (*Rosmarinus officinalis*) in *Cyprinus carpio* fish identified 1,8-Cineole as the most important toxic ingredient of *Rosmarinus officinalis*. This ingredient has caused dose-dependent pathological changes in the liver, kidneys and intestinal tissues [21]. The study found that the fish showed more severe pathologic changes in the liver, kidneys and intestine if they were fed more than 40 ml extract per 100 g of their feed, compared to those that were fed 20 ml of the same extract. The pathological findings reported by that study [21] included nuclear pyknosis and cellular degeneration in the liver (≥ 20 ml extract) compared to abundant cytoplasmic clear vacuoles and renal tubular necrosis (≥ 40 ml extract). Based on the results of the above study, oral feeding of the fish (*Cyprinus carpio*) with high doses of *Rosmarinus officinalis* aqueous extract leads to hepatic and renal lesions [20]. The renal pathological findings observed in our study associated with high doses of *M. officinalis* were consistent with those reported by Zoral *et al.* [21] except for the tubular necrosis that was not observed in our study and the tubular degenerative lesions were less severe.

Comet Assay

Comet assay is preferred over other methods for the detection of DNA damages in heterogeneous cell population and its sensitivity even at low DNA concentrations [22]. The simultaneous use of micronuclei test and comet assay has been successful previously in the evaluation of the genotoxic effects of herbal drugs in fresh water fish [7]. Further, the fish blood with excellent cellular homogeneity (97% being erythrocytes) is considered ideal for comet assay experiments [7]. The use of comet assay has also proven in other studies, examining such topics as the acute and chronic genotoxicity of oxytetracycline in rainbow trout [22-24]. Consistent with a previous report, our results suggest that the pathologic and genotoxic effects of *M. officinalis* extract are linked to the dose and duration of exposure (Tables 1-3) [25]. However, we have not found a clear consensus on the toxic dose of *M. officinalis* extract in various aquatic animals. The mechanism of action for the toxicity of *M. officinalis* extract is not clearly established.

CONCLUSION

The results revealed that the aqueous extract of *M. officinalis*, despite having medicinal properties, caused genotoxic and histopathologic damages on such vital organs as liver, kidneys, heart and spleen if consumed by *O. mykiss* fish at doses greater than 450 mg/kg. The extent of pathologic damage was non-linear and dependent on the dose and duration of exposure to the *M. officinalis* extract. To prevent or minimize the toxic effect, we recommend that the extract should be administered orally at doses below 450 mg/kg with long enough intervals to be safe to the fish. Further studies are required to identify other pathologic effects of *M. officinalis* on bodily organs and tissues of animals and humans.

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CONFLICT OF INTEREST

There was no conflict of interest declared by the authors.

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