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Safety assessment of essential oil from *Minthostachys verticillata* (Griseb.) Epling (peperina): 90-Days oral subchronic toxicity study in rats

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ABSTRACT

Minthostachys verticillata (Lamiaceae), popularly known as peperina is largely used in popular medicine for its digestive, carminative, antispasmodic and antirheumatic properties. There are no reports of repeated exposure toxicity to guarantee their safety. The present study investigated the chemical composition, analyzed by GC–FID, and the 90-day toxicity and genotoxicity effect of *M. verticillata* essential oil (Mv-EO), using Wistar rats as test animals. The rats were divided into four groups (5 rats/sex/group) and Mv-EO was administered on diet at doses of 0, 1, 4 and 7 g/kg feed. The main components of Mv-EO were pulegone (64.65%) and menthone (23.92%). There was no mortality, adverse effects on general conditions or changes in body weight, food consumption and feed conversion efficiency throughout the study in male and female rats. Subchronic administration of Mv-EO did not alter the weights, morphological and histopathological analyses of liver, kidney and intestine. Genotoxicity was tested by micronucleus and comet assays. Mv-EO up to a concentration of 7 g/kg feed for 90 days did not exert a cyto-genotoxic effect on the bone marrow and cells blood of Wistar rats. These results suggest that Mv-EO appears to be safe and could be devoid of any toxic risk.

1. Introduction

Minthostachys verticillata (Griseb.) Epling, also known as peperina, is a species of the Lamiaceae family. It is a well-known South America aromatic and medicinal plant that has been commonly used to treat indigestion, vomiting, diarrhea and abdominal pain. Peperina is also known for its digestive, carminative, antispasmodic and antirheumatic properties (Bandoni et al., 1972; Bonzani and Ariza Espinar, 1993; Núñez and Cantero, 2000). These plant is widely used as infusion or added to "mate". In addition, it is used for the preparation of "yerba mate compuesta" and added to flavor drinks like liqueurs and aperitifs (Bonzani and Ariza Espinar, 1993).

Peperina has been reported to possess various physiological and pharmacological properties. Most of the beneficial effects of peperina are attributed to the essential oil constituents. Different studies have demonstrated that *M. verticillata* essential oil (Mv-EO) has antivirus, antibacterial, antifungal and immunoenhancing activities (De Feo et al., 1998; Maldonado et al., 2001; Primo et al., 2001; González Pereyra et al., 2005; Cariddi et al., 2007; Bluma et al., 2008; González and Marioli, 2010). Furthermore, it has repellent properties and insecticidal activity (Ruffinengo et al., 2005; Palacios et al., 2009; Rossi et al., 2012). The digestive and respiratory activities of Mv-EO have been attributed to the presence of monoterpenes, mainly comprised of pulegone, menthone, isomenthone, limonene, and to a minor concentration, menthol, α-pinene and β-pinene, carvone, piperitenone, sabinene, myrcene, (E)-b-ocimene, thymol and carvacrol (Fester and Martinuzzi, 1950; Fester et al., 1960; De Feo et al., 1998; Schmidt-Lebuhn, 2008). Previous acute studies have demonstrated that Mv-EO

Abbreviations: FCS, foetal calf serum; GC–FID, gas chromatography–flame ionization; HD, hydrodistillation; MN, micronucleus; MNE, micronucleated erythrocytes; Mv-EO, *Minthostachys verticillata* essential oil; NCE, normochromatic erythrocytes; NOAEL, no-observed-adverse-effect level; PCE, polychromatic erythrocytes.

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was not cytotoxic *in vitro* nor cyto-genotoxic *in vivo* both at low and high concentrations (Escobar et al., 2012). However, little toxicological information is available regarding safety following repeated exposure.

The aim of the present study was to evaluate the 90-days oral subchronic toxicity and genotoxicity of *M. verticillata* essential oil in Wistar rats on diet, through parameters such as body weight, food consumption, feed conversion efficiency, organ toxicity and histopathology of various tissues, micronuclei and comet assay.

2. Materials and methods

2.1. Plant material

Leaves and thin stems from *M. verticillata* (800 g) were used to obtain Mv-EO. Peperina was purchased from a local herb store and the voucher specimens were deposited in the herbarium of Universidad Nacional de Río Cuarto. The extraction of Mv-EO was done following the technique of hydrodistillation (HD) proposed by De Feo et al. (1998), using a Clevenger type apparatus. After 2 h distillation, for each 60 g of plant, the Mv-EO was obtained, separated from the aqueous phase, dried over anhydrous Na_2SO_4 and stored in the dark at −20 °C until use. The Mv-EO content was determined on a volume/dry weight basis.

2.2. Gas chromatography–flame ionization (GC–FID)

Quantification of components present in the oil sample was performed monthly following the methodology described by Zygadlo et al. (1996). Briefly, analytical GC was performed on a Shimadzu GC-R1A gas chromatograph fitted with a DB5 capillary column (30 × 0.25 μm). The identification of the compounds was made comparing their retention times against standard pure drugs injected in the same conditions. Operating conditions were as follows: injector temperature 250 °C; FID temperature 250 °C, carrier (gas N_2) flow rate 1 mL/min and split injection mode. Oven temperature was initially 60 °C and then raised to 140 °C at a rate of 2 °C/min, then raised to 250 °C at a rate of 6 °C/min and finally held at that temperature for 20 min.

2.3. Experimental animals and management

Twenty male and twenty female rats of Wistar strain, weighing about 200–240 g each (8-week-old), were divided into four groups consisting of 5 male and 5 female rats in each group, based on their body weight which was measured just before starting study. They were obtained from the Bioterio Central of the Universidad Nacional de Río Cuarto. Animals were maintained in a temperature and humidity controlled room, with a 12-h light/dark cycles. All experimental procedures were conducted in accordance with recent legislation. This study was approved by the Universidad Nacional de Río Cuarto Institutional Review Board.

The diets were formulated with commercial pelleted rat chow (GEPSA FEEDS, Grupo Pilar S.A., Argentina). The centesimal composition of the feed is >24% protein, <7% fiber, 1–1.2% calcium, >6% ether extract, 0.5–0.9% phosphorus, <8% total minerals and <13% moisture. The feed was crushed in a blender and mixed with the Mv-EO at 0, 1, 4 and 7 g/kg of feed mash representing T1, T2, T3 and T4, respectively. Water and feed were provided *ad libitum* throughout the experimental period (90 days). Toxicity signs, body weight and food consumption were monitored daily. The feed conversion efficiency (FCE) was determined by the ratio of feed intake (g)/weight gained (g). The dosages 1, 4, and 7 g/kg were selecting according to preliminary study that resulting in food rejection and daily food consumption decreasement when more than 10 g Mv-EO/kg feed were administrated (data not shown). Mean daily intake of Mv-EO was determined taken into account the amount of Mv-EO present into the feed, the mean body weight of rats and daily amount of feed supplied to rats (mg Mv-EO/kg bw/day).

At the end of the study, the rats were decapitated without being anesthetized, and the blood samples were immediately taken. After dissection, the liver, the kidneys, and a section of intestine were removed, weighed and used for histopathological examinations.

2.3.1. Morphological and histopathological analyses

The macroscopic external features (weight, size and color) of the organs collected during necropsy (liver, kidney and intestine) were registered. The above organs were fixed in 10% neutral buffered formalin (pH 7.4) for paraffin routine processing. These samples were cut at 4 μm thickness and subjected to haematoxylin/eosin (H&E) staining for microscopic histological examination under 400× magnification. Photomicrographs were taken with a Zeiss Axiostar plus microscope using an Electronic Eyepiece camera with MIAS (Micro Image Analysis Software 2008, v 2.2) software and a Canon Power Shot G5 camera (Canon Inc., Japan).

2.3.2. Micronuclei assay

The assay was carried out following standard protocols as recommended by Schmid (1975). At the end of study the animals were sacrificed and the femurs were immediately excised from the body. Using a syringe, the bone marrow was then flushed into a glass tube containing 4 mL foetal calf serum (FCS). The collected cells were centrifuged at 1000 rpm for 5 min and the supernatant was carefully removed from the pellet. The cells were re-suspended in the remaining fluid, slides were prepared and air-dried. Then the slides were stained with May-Grunwald–Giemsa. To establish the genotoxic capacity of Mv-EO, 1000 polychromatic (PCE) and corresponding normochromatic erythrocytes (NCE) were scored for the presence of micronuclei (MN) from each animal. To detect possible cytotoxic effects, the effect on the proportion of 1000 PCE with respect to the number of normochromatic erythrocytes (NCE) per rat (PCE/NCE index) was observed. The slides were scored blindly using a light microscope at a 1000× magnification. The average number of micronucleated erythrocytes (MNE) in individual rats was used as the experimental unit, with variability based on differences among animals within the same group.

2.3.3. Comet assay

The comet assay was performed as was described by Tice et al. (2000) with some modifications. Briefly, the blood samples were diluted in 1 mL FCS/RPMI mixture (1:1) and the cells were precipitated with centrifugation (5 min, 1000 rpm). The pelleted cells were re-suspended in 100 μL of 0.75% low melting point agarose at 37 °C. Immediately, 75 μL was spread onto 2 microscope slides per concentration pre-coated with 0.75% normal melting point agarose. The slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were gently removed and 75 μL of 0.75% low melting point agarose at 37 °C was added. Again, the slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were removed and the slides were immersed in cold, freshly prepared lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with 1% Triton X-100 and 10% DMSO (Merck). The slides, which were protected from light, were allowed to stand at 4 °C for 1 h. They were placed in a gel box, and left in high pH (>13) electrophoresis buffer (300 mM NaOH, 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA) at 4 °C for 20 min before electrophoresis to allow the DNA to unwind. Electrophoresis was carried out in ice bath (4 °C) for 20 min at 250 mA and 30 V (0.722 V/cm). The slides were submerged in neutralization buffer (0.4 M Tris–HCl, pH 7.5)

for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were dried and stored overnight or longer before staining. For staining the slides were briefly rinsed in distilled water, covered with 25 μL 1× ethidium bromide staining solution prepared from a 200 μg/mL 10× stock solution, and coverslipped. The material was evaluated immediately at 400× magnification using fluorescence microscope (Axiophot, Carl Zeiss, Germany) attached to the image-analysis system (Powershot G6, 7.1 megapixels, Canon INC., Japan with software AxioVision Release 4.6.3, Carl Zeiss, Germany), with 515–560 nm excitation filter and a 590 nm barrier filter. From each treatment, images from 100 "nucleoids" were captured with a camera attached to the fluorescent microscope and linked to the CometScore® 1.5 software. Highly damaged cells were not included in the scoring (clouds were not analyzed). Tail moment (TM) was used to estimate DNA damage (arbitrary units).

2.4. Statistical analysis

All data were expressed as mean and standard deviation (SD). Body weight, organ weight, hematology and micronucleus assays were tested by conducting a one-way analysis of variance (ANOVA). Results obtained by comet assays were submitted to a Kruskal–Wallis test. Dunn's Multiple Comparison Test was used to determine statistical significance ($p < 0.05$) among the control and treatment groups using the GraphPad Prism software.

3. Results

3.1. Identification of M. verticillata essential oil compounds

The yield of the Mv-EO obtained by hydrodistillation of leaves and thin stems was 5.1% (w/v). The chemical composition of the Mv-EO used in the experiments is shown in Table 1. The main components were pulegone (64.65%) and menthone (23.92%). Other minor ingredients were eugenol (2%), iso-pulegone (1.62%), limonene (1.4%), spathulenol (0.79%), piperitone (0.6%), 2-methil-2 (3-methil-2-oxibuthil) cyclohexanone (0.6%), iso-menthone (0.42%), β-germacrene (0.39%), β-pinene (0.37%), α-pinene (0.3%), ocimene (0.18%), terpineol (0.16%), sabinene (0.15%), β-myrcene (0.15%), trans-calamenene (0.13%), α-tujone (0.12%), 1,8 cineole (0.09%) and others (2.47%).

3.2. General observations and behavior

The animals were healthy and no signs of toxicity or death were observed throughout the study following administration of Mv-EO on diet at doses of 1, 4 and 7 g/kg feed for 90 consecutive days. The dosage of the Mv-EO at 1, 4 and 7 g/kg feed was equivalent to a mean daily intake of 70, 260 and 460 mg/kg bw/day, respectively.

3.3. Body weight, food consumption and feed conversion efficiency

The performance of Wistar rats fed with Mv-EO at different concentrations is presented in Table 2. The differences in body weight, food consumption and FCE between females and males were observed as expected. Administration of Mv-EO on diet for 90 days did not produce any significant difference in food consumption of rats when compared to control animals. The average food intake for the control group was 15.54 g/day/animal and 20.51 g/day/animal in female and male rats, respectively. There was a slight but not significant increase ($p \geqslant 0.05$) in the average food intake when

Table 1
Minthosthachys verticillata essential oil components obtained by hydrodistillation.

Compounds	RI	% v/v
α-Pinene	948	0.33
Sabinene	987	0.15
β-Pinene	993	0.37
Myrcene	1001	0.15
Limonene	1043	1.4
1,8 Cineole	1047	0.09
Ocimene	1058	0.18
α-Tujone	1111	0.12
Menthone	1170	23.92
Iso-Menthone	1180	0.42
Borneol	1187	tr
Iso-Pulegone	1190	1.62
Terpineol	1211	0.16
Pulegone	1260	64.65
Piperitone	1269	0.6
2-methyl-2 (3-methyl-2 oxobutyl) cyclohexanone	1299	0.6
Ascaridol	1331	0.06
Eugenol	1353	2
β-Elemene	1393	tr
β-Germacrene	1508	0.39
β-Bisaboleno	1512	0.1
Spathulenol	1592	0.79
Trans-calamenene	1646	0.13

RI: retention index.
tr, trace amounts (<0.05).

Table 2
Performance of Wistar rats fed with different concentrations of *M. verticillata* essential oil included in diet.

Parameters	T1 0 g/kg Mv-EO	T2 1 g/kg Mv-EO	T3 4 g/kg Mv-EO	T4 7 g/kg Mv-EO
Males				
Initial body weight (g)	210.50 ± 7.9	208.41 ± 9.2	214.61 ± 10.5	209.82 ± 10.1
Final body weight (g)	342.62 ± 9.2	378.81 ± 10.5*	360.63 ± 9.5	337.66 ± 12.1
Body weight gain (g)	132.12 ± 8.3	170.40 ± 8.9*	146.02 ± 11.5	127.0 ± 10.3
Total feed intake (g)	1867 ± 45.8	2002 ± 57.9	1806 ± 49.2	1770 ± 51.8
Daily feed intake (g)	20.52 ± 6.1	22.01 ± 5.8	19.85 ± 6.4	19.45 ± 5.1
Feed conversion ratio	14.13 ± 4.4	11.75 ± 6.0*	12.37 ± 7.5	13.85 ± 4.7
Females				
Initial body weight (g)	214.60 ± 8.3	215.40 ± 10.1	211.40 ± 7.5	204.33 ± 11.1
Final body weight (g)	300.20 ± 7.2	321.80 ± 12.2*	305.20 ± 8.1	288.67 ± 10.2
Body weight gain (g)	85.60 ± 6.3	106.40 ± 9.8*	93.80 ± 10.5	84.33 ± 12.3
Total feed intake (g)	1414 ± 40.2	1508 ± 55.1	1389 ± 62.2	1332 ± 38.8
Daily feed intake (g)	15.54 ± 5.4	16.57 ± 4.4	14.86 ± 6.1	14.63 ± 6.5
Feed conversion ratio	16.52 ± 7.3	14.17 ± 5.1*	14.80 ± 9.1	15.79 ± 3.5

Values are mean ± SD for 5 rats/sex/group.
* Significantly different from control, $p < 0.05$. The differences between control and treated groups were evaluated by Dunnett's Multiple Comparison Test.

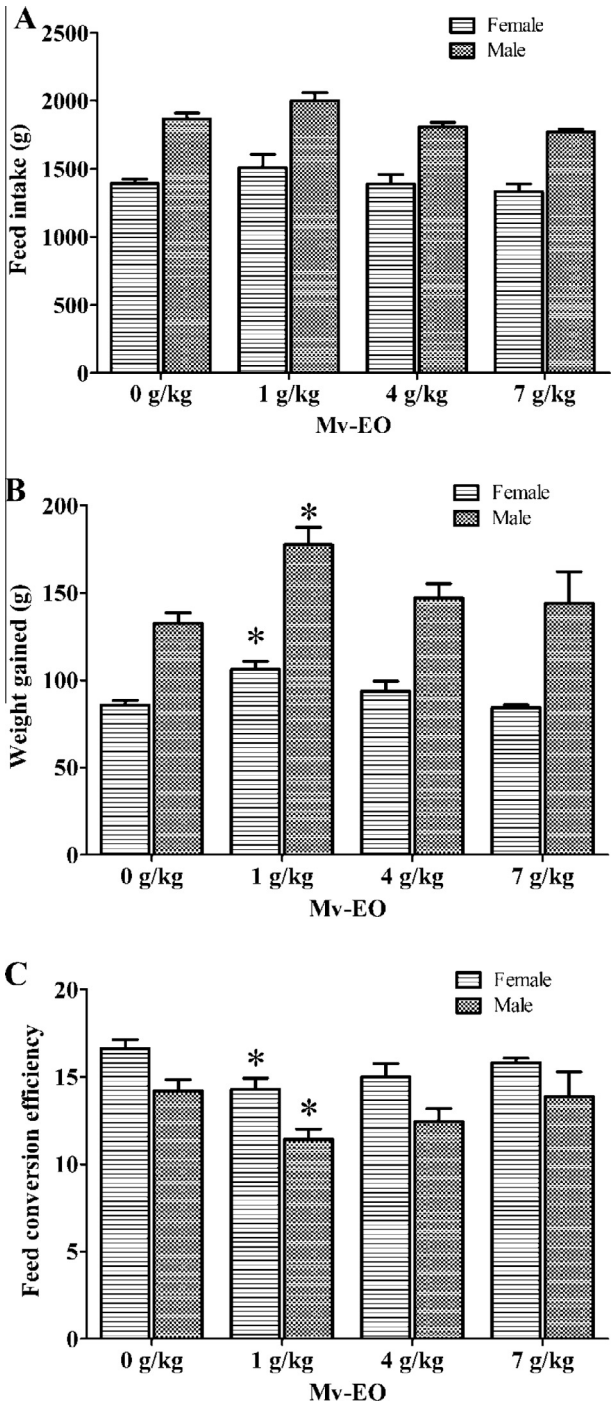

Fig. 1. Effects of subchronic administration of *M. verticillata* essential oil on food intake, gain in body weight and feed conversion efficiency (FCE) in Wistar rats. (A) Change in food intake (B) Change in body weight over 90 days. (C) FCE was determined from the gram of feed consumed per gram of weight gain. All data are mean ± SEM for 5 rats/group/sex. $^{*}p < 0.05$ (Dunnett's multiple comparison test).

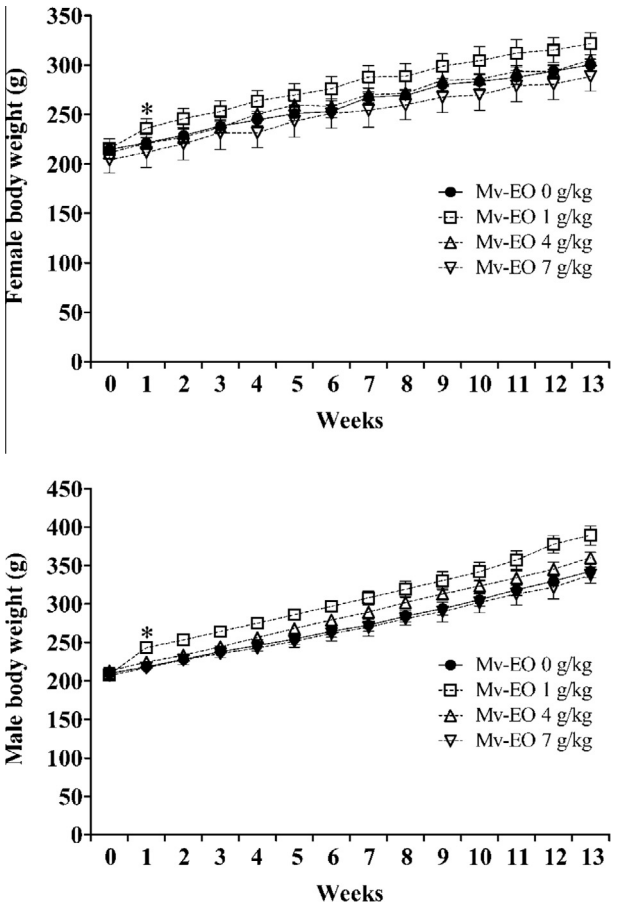

Fig. 2. Effect of subchronic administration of *M. verticillata* essential oil on body weight changes of female and male Wistar rats during the 90 days toxicological assessment. All data are mean ± SEM for 5 rats/group/sex. $^{*}p < 0.05$.

Mv-EO was supplemented at 1 g/kg feed. However, the addition of Mv-EO at 1 g/kg improved the body weight gain and the feed conversion efficiency in both sexes; therefore, it was required less feed to increase 1 g of body weight (Fig. 1).

The body weight changes of male and female rats fed with Mv-EO at 1 g/kg feed showed a significant increase after the first week compared with control group (Fig. 2).

3.4. Morphological and histopathological analysis

The relative weights of organs were measured. There were no significant differences in organ weights among all the experimental groups (data not shown). Gross pathological examination of the organs such as liver, kidney and intestine of animals treated with Mv-EO did not show any difference when compared with untreated groups in both sexes. Histopathological examination revealed that all the features were within the normal parameters and no detectable abnormalities were attributed to the Mv-EO administration (Fig. 3).

3.5. Micronuclei assay

The frequency of MNE and the rate of PCE/NCE in Mv-EO treated and control groups are presented in Table 3. No significant induction of micronuclei was observed by Mv-EO after 90 days at all the dose levels studied compared with the control. Also, no significant difference among the test groups was observed ($p \geqslant 0.05$). As shown in Table 3, the ratio of PCE/NCE in bone marrow preparations showed no significant differences among all the groups tested. There were no sex-dependent changes in any treatment.

3.6. Comet assay

Fig. 4 shows that after 90 days administration of Mv-EO at different concentrations, there was no significant Tail moment in the cells blood of the treated Wistar rats of both sexes when compared

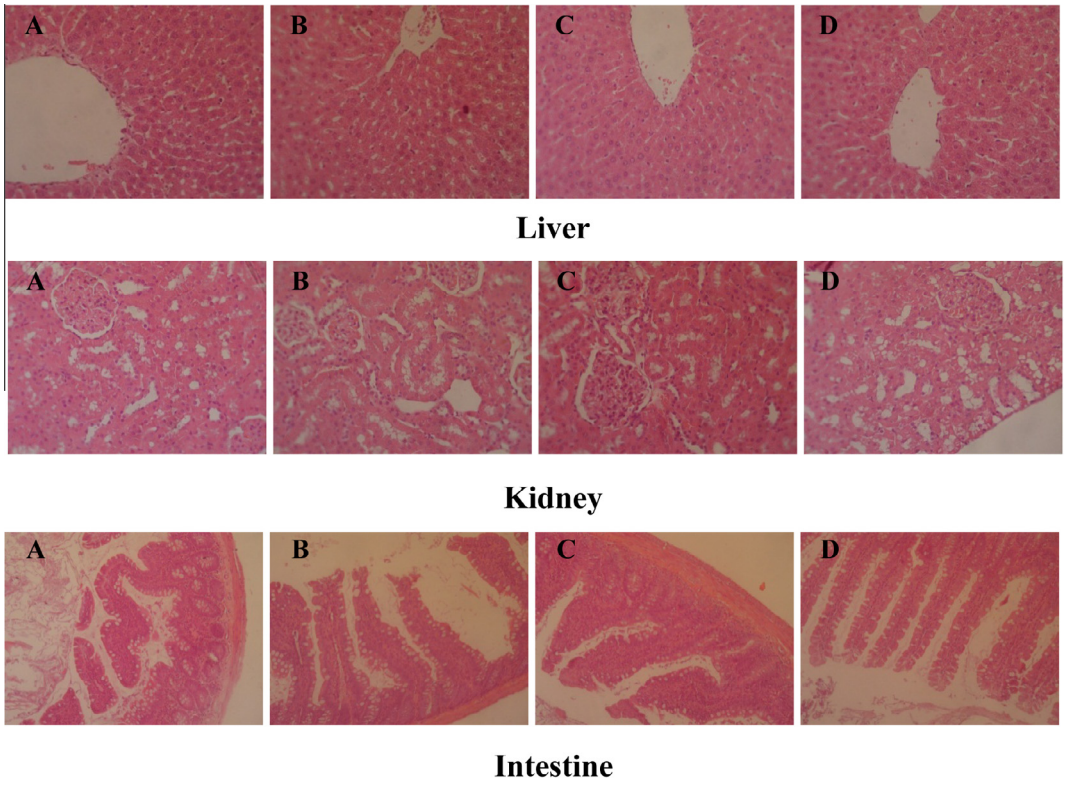

Fig. 3. Histological examination of internal organs of Mv-EO-treated and non-treated (control). Wistar rats fed on Mv-EO at doses of 0, 1, 4 and 7 g/kg feed (A, B, C and D, respectively). Representative sections of liver, kidney (×400) and intestine (×200). All the structures were found to be conserved.

Table 3
Mean of micronucleated polychromatic erythrocytes observed in the bone marrow cells of Wistar rat treated with *Minthostachys verticillata* essential oil, and respective control.

Treatments	Number of MNE per animal										‰ MNE (mean ± S.D.)	PCE/NCE (mean ± S.D.)
	M_1	M_2	M_3	M_4	M_5	F_6	F_7	F_8	F_9	F_{10}		
0 g/kg Mv-EO	3	6	5	6	4	5	3	7	6	7	5.2 ± 1.4	1.5 ± 0.3
1 g/kg Mv-EO	2	5	3	5	2	3	5	2	4	2	3.6 ± 1.3	1.4 ± 0.1
4 g/kg Mv-EO	3	5	8	4	2	4	4	8	2	3	4.1 ± 2.2	1.5 ± 0.2
7 g/kg Mv-EO	4	4	6	6	6	4	5	5	5	7	5.2 ± 1.0	1.6 ± 0.1

For each animal 1000 polychromatic erythrocytes were analyzed. S.D. = standard deviation; PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes; MNE = micronucleated erythrocytes. No statistically significant differences between control and any of the treatment groups. M = male; F = female.

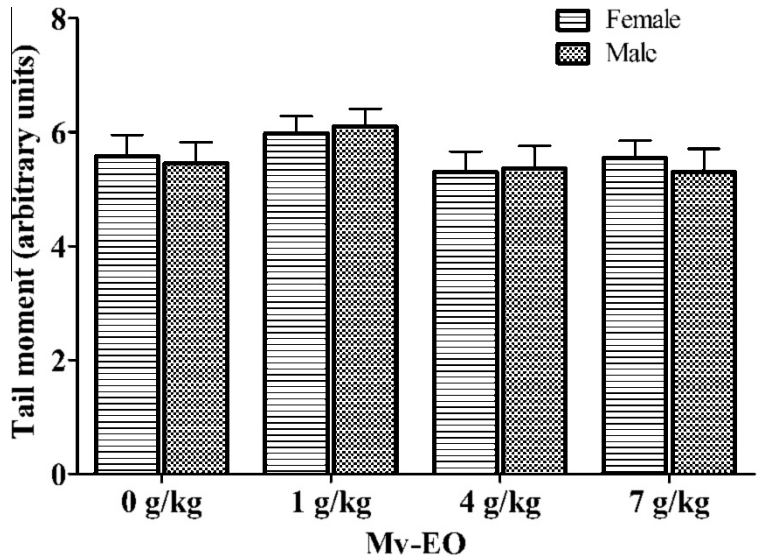

Fig. 4. DNA damage in cells blood of Wistar rats treated with *M. verticillata* essential oil. Results are expressed as tail moment. All data are mean ± SEM for 5 rats/sex/group.

with untreated groups indicating Mv-EO did not produce any DNA damage.

4. Discussion

The consumption of peperina has increased in recent years without taking into account the toxicity aspect. Thus, it is critically important to evaluate the toxicity of *M. verticillata* to protect the consumer's health and to make appropriate use. Previously, Escobar et al. (2012) demonstrated that Mv-EO in acute dose (up to 500 mg/kg bw) administrated by intraperitoneal route, did not produce any sign of toxicity or death in mice. In this study, a 90 days subchronic toxicity and genotoxicity assay in Wistar rats for the evaluation of the Mv-EO safety at different doses was conducted. No significant adverse changes in food intake, food efficiency, body weight and organ weights were associated in male and female rats or among treatment groups. Moreover, there was an increase in weight gain and feed conversion efficiency in rats fed with Mv-EO at 1 g/kg feed. It is important to highlight that the observed effect on weight gain and feed efficiency did not follow a dose–response pattern because of the lower Mv-EO concentration, equivalent to a mean daily intake of approximately 70 mg/kg bw/day, was the most efficient. The essential oils may influence the palatability of feed, the processes in the digestive tract or the immune response. Several researchers have demonstrated the essential oils can be used as food additives because they improved the productive parameters of farmyard animals (Windisch et al., 2008; Yan et al., 2010; Li et al., 2012).

Recent reports informed that ginger oil and *Curcuma longa* L. essential oil are safe in rats up to an oral dose of 500 mg/kg bw for 13 weeks (Jeena et al., 2011; Liju et al., 2013). Similarly, in the present work, the dosage of the Mv-EO at 7 g/kg feed did not induce any adverse toxicological effects and was equivalent to a mean daily intake of approximately 460 mg/kg bw/day. This concentration markedly exceeded the no-observed-effect levels (NOELs) of flavoring substances present in the Mv-EO (15–300 mg/kg bw/day) described by Munro and Danielewska-Nikiel (2006). These authors showed that the compounds with less NOEL were borneol and pulegone (15 and 20 mg/kg bw/day respectively), and the compounds with higher NOEL were limonene, α-pinene and β-pinene (300 mg/kg bw/day).

In this study, the main components of *M. verticillata* essential oil were pulegone and menthone, representing 64.65% and 23.92% of the total oil, respectively. Previously, pulegone has been shown to be hepatotoxic in mice (>75 mg/kg/day) and in rats (>10 mg/kg/day) (NTP, 2002a,b) and a depression in animal growth could be expected. However, in the present study the Mv-EO with pulegone as the main component did not exert any negative influence on the animal growth. This could be explained by the fact that essential oils have other constituents interacting as a whole with synergistic/antagonistic phenomena. Pavlidou et al. (2004) have shown that menthone, the other major component present in the Mv-EO, inhibited the toxic effect caused by pulegone suggesting antagonist interactions in other animal model.

Morphological and histopathological analysis of organs is a powerful tool to study the manifestations of diseases. Here, no alteration in liver, kidney and intestine weights were observed, as well as histological examination revealed that there were not significant changes. These results indicate that Mv-EO administration up to a concentration of 7 g/kg feed for 90 days did not induce any adverse toxicological effects in these organs.

The micronucleus and comet assays are the most recommended tests to detect genotoxic substances. The present results showed that the Mv-EO up to a concentration of 7 g/kg feed for 90 days did not exert a cyto-genotoxic effect on the bone marrow and cells blood of Wistar rat.

5. Conclusions

Present study reports the non-toxicity of Mv-EO given on diet up to a concentration of 7 g/kg feed for 90 days. Mv-EO did not produce any toxicity as seen from body weight changes, food consumption, feed conversion efficiency as well as organ toxicity and histological examinations of main organs that could eventually be affected by long-term administration of peperina. Mv-EO did not produce any genotoxicity to rats as seen from the micronuclei in the bone marrow cells as well as from the single cell gel electrophoresis (comet assay) in the blood cells. These results clearly indicate that administration of Mv-EO did not promote toxic effects in rats up to on diet administration of 7 g/kg feed for 90 days.

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