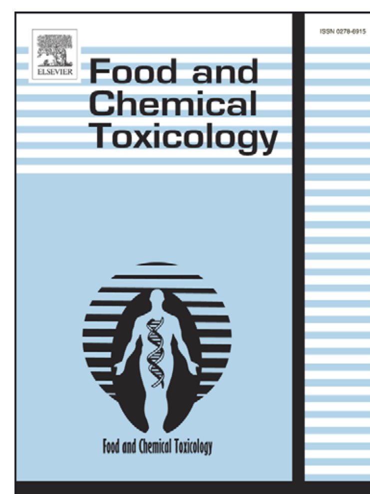
Accepted Manuscript



Lack of cytotoxic and genotoxic effects of *Minthostachys verticillata* essential oil: studies *in vitro* and *in vivo*

Franco Matías Escobar, Laura Noelia Cariddi, María Carola Sabini, Elina Reinoso, Sonia Beatriz Sutil, Cristina Vanesa Torres, Silvia Matilde Zanon, Liliana Inés Sabini

PII: S0278-6915(12)00432-2
DOI: http://dx.doi.org/10.1016/j.fct.2012.06.018
Reference: FCT 6691

To appear in: *Food and Chemical Toxicology*

Received Date: 10 May 2012
Accepted Date: 12 June 2012

Please cite this article as: Escobar, F.M., Cariddi, L.N., Sabini, a.C., Reinoso, E., Sutil, S.B., Torres, C.V., Zanon, S.M., Sabini, L.I., Lack of cytotoxic and genotoxic effects of *Minthostachys verticillata* essential oil: studies *in vitro* and *in vivo*, *Food and Chemical Toxicology* (2012), doi: http://dx.doi.org/10.1016/j.fct.2012.06.018

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Title Page

Lack of cytotoxic and genotoxic effects of *Minthostachys verticillata* essential oil: studies *in vitro* and *in vivo*

Franco Matías Escobar[a,*], Laura Noelia Cariddi[a], María Carola Sabini[a], Elina Reinoso[a], Sonia Beatriz Sutil[a], Cristina Vanesa Torres[a], Silvia Matilde Zanon[a], Liliana Inés Sabini[a].

Affiliation
a *Department of Microbiology and Immunology, National University of Río Cuarto. Ruta 36 Km 601, CP 5800 Río Cuarto, Córdoba, Argentina*

* Corresponding author. Franco Matías Escobar, Tel.: +54 0358 4676433; fax: +54 0358 4676231.
E-mail address: fescobar@exa.unrc.edu.ar.

Abbreviations

FCS: foetal calf serum
GC–FID: Gas chromatography–flame ionization
HD: hydrodistillation
MN: micronucleus
MNPCE: micronucleated polychromatic erythrocytes
Mv-EO: *Minthostachys verticillata* essential oil
NCE: normochromatic erythrocytes
NRU: Neutral Red Uptake
PBMCs: peripheral blood mononuclear cells
PCE: polychromatic erythrocytes

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

Minthostachys verticillata (Griseb.) Epling, also known as peperina, is a species of the Lamiaceae family. Peperina is a South American aromatic and medicinal plant; it grows widely in the central and northwestern region of Argentina, especially in the hills of the Córdoba province between 700 and 1200 m (Ojeda et al., 2001; Schmidt-Lebuhn, 2008). This plant is traditionally used as infusion or added to "mate". According to folk traditional medicine, peperina has been used to treat indigestion, vomiting, diarrhea, abdominal pain and is known for its digestive, carminative, antispasmodic and antirheumatic properties (Bandoni et al., 1972; Bonzani and Ariza Espinar, 1993; Núñez and Cantero, 2000). In addition, it is also used to flavor drinks like liquors, aperitifs or for the preparation of "yerba mate compuesta" (Bonzani and Ariza Espinar, 1993).

On the other hand, numerous studies have described the bioactivities of the *M. verticillata* essential oil, showing in *in vitro* studies that the plant has antiviral activity against herpes and pseudorabies viruses, and antibacterial activity against several bacterial strains (De Feo et al., 1998; Maldonado et al., 2001; Primo et al., 2001; González and Marioli 2010). Also, its antifungal effect against *Aspergillus* section *Flavi* sporulation and inhibition of aflatoxin B1 production by 85-90% was reported (Bluma et al., 2008). Further, its immunomodulatory potential on effector cells of humoral and cellular immune system derived from patients with asthma, bronchitis, atopic eczema and from patients allergic to cow milk and environmental anemophile fungi has been reported (González Pereyra et al., 2005; Cariddi et al., 2007). Recently, Cariddi et al. (2011) reported that *M. verticillata* essential oil and its monoterpenes, principally limonene, induced a potent immunomodulator effect in *in vitro* and *in* vivo assays.

The essential oil constituents of the *Minthosthachys* are primarily responsible for digestive and respiratory activities. Most species of this genus contain the dominant essential oil components, such as menthone, pulegone, carvacrol, carvone. These compounds are likewise chiefly employed for medical purposes, such as digestive, carminative, antispasmodic, and antitussive activities (Schmidt-Lebuhn, 2008). Essential oil constituents of *M. verticillata* include pulegone, menthone, isomenthone, limonene, menthol, α-pinene and β-pinene, carvone, piperitenone, sabinene, myrcene, (E)-β-ocimene, thymol and carvacrol (Fester and Martinuzzi, 1950; Fester et al., 1960; De Feo et al., 1998). However, pulegone and menthone usually are the two most abundant monoterpenes (Banchio et al., 2005; Schmidt-Lebuhn, 2008).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

In view of its popular use and the lack of knowledge about cyto-genotoxic properties of *M. verticillata* essential oil, the aim of the present study was to investigate the main chemical composition, as well as its cytotoxic, apoptotic and genotoxic effects by using Vero cells, human PBMCs and mice bone marrow cells.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

2. Material and methods

2.1. Plant material

Leaves and thin stems from *Minthostachys verticillata* (Griseb.) Epling were collected in Santa Rosa (Córdoba province, Argentina) in April 2004. The plant was identified and voucher specimens deposited in the RCV (Río Cuarto Vasculares) herbarium as file #1955, Universidad Nacional de Río Cuarto. The morphological characterization of the plant was executed macro and microscopically to confirm the identity of these specimens. For the extraction of Mv-EO the technique of hydrodistillation (HD) using a Clevenger type apparatus was applied (De Feo et al., 1998; De Logu et al., 2000). After 3 h of distillation, Mv-EO was obtained from 60 g of plant, 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.

In order to perform the *in vitro* assays, the oil was emulsified in dimethylsulfoxide and diluted in RPMI-1640 or Eagle's minimum essential medium (MEM). In the micronucleus assay the Mv-EO was emulsified in Tween-80 and diluted in saline (0.5% v/v).

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

Quantification of components present in the oil sample was made by measuring the area under each peak of the chromatogram (Zygadlo et al., 1996). Briefly, analytical GC was performed on a Shimadzu GC-R1A gas chromatograph fitted with a DB5 capillary column (30 x 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.

ACCEPTED MANUSCRIPT

2.3. Cell culture and cytotoxic assay

Vero cells (African green monkey kidney cells) were obtained from ABAC (Asociación Banco Argentino de Células). Cells were propagated in MEM supplemented with 8% foetal calf serum (FCS), gentamycin 50 µg/mL and 2 mM glutamine. Vero cells viability was measured by Neutral Red Uptake assay (NRU) in a modified form as described by Borenfreund and Puerner (1985). Cells were seeded in 96-well culture plates at 10^4 cells/well and, after monolayer formation, were treated with increasing concentrations of Mv-EO (10-1000 µg/mL) during 48 h. Untreated cells were used as controls. The medium was replaced with 150 µL of a 50 mg/mL solution of neutral red in MEM. After incubation at 37ºC for 3 h, medium containing dye was removed and wells were washed twice with warmed PBS (150 µL/well). The dye within viable cells was released by extraction with a mixture of acetic acid, ethanol and water (1:50:49). After the cultures were shaking for 10 min, absorbance values were read at 540 nm. Relative cell viability was expressed as percentage NRU of untreated control groups.

The Vero cells viability was also determined by trypan blue dye exclusion assay. Cells were cultured as described previously. After incubation with increasing concentrations of Mv-EO (10-1000 µg/mL) during 48 h, Vero cells were detached from their dishes with trypsine, incubated during 5 min with a solution of trypan blue and viable (unstained) and non-viable (stained blue) cells were counted with a Neubauer chamber. Results were expressed as percentage of cells which exclude the vital trypan blue/total cells.

In addition, Maximum Non Cytotoxic Concentration (MNCC) was determined microscopically by daily observation of morphological changes of cells at 24, 48 and 72 hours of incubation.

2.4. Isolation of human PBMCs

Peripheral blood was drawn from healthy volunteers (18 to 25 years old). PBMCs were isolated from blood samples using Hystopaque®-1077 centrifugation (Sigma Aldrich, St. Louis, USA). From

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

an optimal suspension of $1x10^6$ cells/mL, cell viability was determined by trypan blue dye exclusion assay (Mongini and Waldner, 1996). The study was approved by the Comité Institucional de Ética de la Investigación en Salud (CIEIS). In accordance with ethical standards, the healthy volunteers were properly informed of the study and signed an agreement authorizing the test.

2.4.1. Human PBMCs viability

The cells (2×10^5) in a final volume of 200 µL, were cultured in a sterile 96-well microplates containing RPMI-1640 medium, added with 25 mM Hepes , 2 mM L-glutamine, 5% FCS, 50 mM 2-mercaptoethanol, 100   g/mL streptomycin and 100   g/mL penicillin. Cells were exposed to different concentrations of Mv-EO (10-1000 µg/mL). Cell cultures with RPMI-1640 alone were used as control. The system was incubated at 37° C with 5% CO_2 and humidity for 18-24 h. After that time, cell viability was evaluated by trypan blue dye exclusion using Neubauer chamber for counting of viable cells, as described by Militão et al. (2006). Each experiment was done in triplicate.

2.4.2. Analysis of apoptosis by Hoechst staining

Cell morphology was evaluated by fluorescence microscopy following Hoechst 33258 DNA staining (Sigma Aldrich, St. Louis, USA), as described (Montaner et al., 2000) with modifications. Briefly, PBMCs were cultured as described previously and exposed to Mv-EO (10-100 µg/mL) and to hydrogen peroxide (1mmol/L), as positive control, for 18-24 h. After that time cells were centrifuged and fixed with cold methanol (at -20ºC). Then, cells were stained with Hoechst 33258 to a final concentration of 20 µg/mL, and incubated for 5 min at room temperature in the dark. After incubation, cells were examined with a light 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). Apoptotic cells were identified by

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

characteristic features of apoptosis (e.g. nuclear condensation, formation of membrane blebs and apoptotic bodies).

2.4.3. DNA fragmentation analysis

The isolation of fragmented DNA from cells cultivated in 24-well plates was carried out according to the procedure of Amirghofran et al. (2007) with modifications. In brief, cells ($2x10^5$ cells per well) were exposed to different concentrations of Mv-EO (10-1000 µg/mL) and then collected by centrifugation (2600 rpm, 15 min).Cells cultured with media alone were used as control. The pellet was resuspended in 0.5 mL of DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris-Hcl, pH 8.5). The lysate was immediately incubated with 0.1 mg/mL proteinase k (Sigma Aldrich, St. Louis, USA) and 0.5 mg/mL RNase A (Boehringer Mannheim, Germany) for three hours at 37°C, added with 200 µL of ClNa 3 M and centrifuged at 3000 rpm for 15 min. After addition of isopropanol, the DNA was precipitated with 70% ethanol. The samples were then loaded into 2% agarose gel and electrophoresed. The DNA band pattern was visualized under UV light using ethidium bromide staining.

2.5. Animals and treatment

Male and female Balb/c mice aged 8–12-weeks old, (weighing 20-25 g), 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 cycle and were allowed standard granulated food and water *ad libitum*. 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 mice were divided into 5 experimental and 3 control groups of 6 animals each (3 males and 6 females). The Mv-EO was diluted and administered in a single dose of 0.2 mL by intra-peritoneal injection at concentrations of 25, 50, 100, 250 and 500 mg/kg b.w. The negative control group received saline solution and the positive control group

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

received cyclophosphamide at 30 mg/kg b.w. The vehicle control group was injected with saline and Tween-80 (0.5% v/v).

2.6. Micronuclei assay

The assay was carried out following standard protocols as recommended by Schmidt (1975). The animals were sacrificed by cervical dislocation at 24 or 48 h post-injection. Immediately after, the femurs were excised from the body. Using a syringe, the bone marrow was then flushed into a glass tube containing 4 mL FCS. The collected cells were centrifuged at 1000 rpm for 5 min and the supernatant was carefully removed from the pellet. The cells were then 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, we determined the number of micronucleus (MN) in 1000 polychromatic erythrocytes (PCE) per mouse. To detect possible cytotoxic effects, we observed the effect on the proportion of 1000 PCE with respect to the number of normochromatic erythrocytes (NCE) per mouse (PCE/NCE index). The slides were scored blindly using a light microscope at a 1000X magnification. The average number of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability based on differences among animals within the same group.

2.7. Statistical analysis

Values were expressed as the mean ± standard deviation (SD) of the means. The data obtained for cytotoxicity assays were evaluated using the program GraphPad Prism version 5.00.288 (San Diego, USA, 2007) and compared with the parametric t-test for twin samples. The data obtained for micronucleus assays were submitted to a one-way analysis of variance (ANOVA) and the Tukey's multiple comparison test using the GraphPad Prism software. The level of significance was established at $p < 0.05$.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

3. Results

3.1. Identification of M. verticillata essential oil compounds

The recovery of the essential oil was processed by conventional HD yielding 5.1% (w/v) of the oil. The chemical composition of *M. verticillata* essential oil obtained by HD was studied by GC–FID analysis. Table **1** show the major compounds of *M. verticillata* essential oil compositions. These six compounds represent 83.03% of the total amount of chromatographic peaks. The Mv-EO was particularly rich in pulegone (60.5%), menthone (18.2%) and limonene (3.76%).

3.2. Cytotoxicity assays

The viability tests were performed in order to assess the toxicity of peperina essential oil. The Vero cells viability was determined by trypan blue dye exclusion and by measuring the amount of neutral red taken up into the cells. The human PBMCs viability was determined by trypan blue dye exclusion. Figure 1 show the dose-response curves of cells exposed to Mv-EO (10-1000 µg/mL) for 48 h. No cytotoxicity is observed in Vero cells system at all concentrations of Mv-EO assayed. The treatment of human PBMCs with Mv-EO at high concentrations showed a slight decrease in the viability. PBMCs treated with the highest concentration (1000 µg/mL) exhibited 72% viability. Many authors consider that a drug is not toxic, weakly toxic or toxic on cells, when cell viability percentage is >70, between 50-70% or <50%, respectively. (Abdillahi et al., 2012).

3.3. Apoptotic assays

By Hoechst 33258 DNA staining, was observed in the fluorescence microscope several nuclei of the PBMCs treated with hydrogen peroxide contained small bright blue dots representing chromatin

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

condensation and/or nuclear fragmentation. In addition, membrane blebs and apoptotic bodies were observed. The PBMCs treated with essential oil at all concentrations assayed did not show the characteristic features of apoptosis (Figure 2). The cells treated with essential oil (10-1000 µg/mL) did not show the typical DNA laddering in agarose gel (data not shown). These results strongly suggest that MV-EO does not induce *in vitro* cytotoxic nor apoptotic effect.

3.4. Micronuclei assay

The frequency of MNPCE and the rate of PCE/NCE in Mv-EO treated and control groups are presented in Table **2**. The average number of MNPCE for the negative control group was 0.233 ± 0.05 and 0.183 ± 0.14 at 24 and 48 h respectively. The vehicle did not influence the frequency of MN since their behavior was similar to that exhibited by the negative control group. The positive control induced statistically significant MNPCE ($p < 0.001$) at both time intervals compared to controls groups. In relation to the presence of MNPCE in bone marrow of mice treated with Mv-EO, there was a slight but not significant increase in the frequency of micronuclei in any of the Mv-EO treated group at the time intervals tested compared with the control. Also, no significant difference between the test groups was observed. As shown in Table 2, the ratio of PCE/NCE in bone marrow preparations showed no significant differences between all the groups tested. In all cases, the behavior in relation to the proportion of EPC was practically uniform and the values recorded are part of those reported as historical and considered normal by this method (Krishna and Hayashi, 2000). There were no sex-dependent changes in any treatment. No treatment related clinical signs of toxicity, mortality or changes in body weights, body weight gain or food consumption were observed throughout the study.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

4. Discussion

In order to provide safety information on *M. verticillata* essential oil, we carried out studies to determine the main chemical composition by GC–FID, and to evaluate the toxic effects of Mv-EO on normal cells both *in vitro* and *in vivo* assays.

The therapeutic importance of peperina has been highlighted both in folk medicine and in research. The peperina essential oil constituents are primarily responsible for the digestive properties of this plant (Schmidt-Lebuhn, 2008). Different biological activities have been described to Mv-EO, such as antiviral, antibacterial and antifungal (De Feo et al., 1998; Maldonado et al., 2001; Primo et al., 2001; Bluma et al., 2008; González and Marioli, 2010). It has also been shown that it has anti-allergic and immunomodulating properties *in vitro* and *in vivo* (González Pereyra et al., 2005; Cariddi et al., 2007; 2011).

Although oil composition appears to be subject to high intraspecific variability, and essential oil composition depends on developmental state and environmental growth conditions (season, stress, climate, soil) (Bakkali et al., 2005), in most of the studies mentioned above, oil composition was identified, showing pulegone and menthone as the main components. Our results further contribute to the previous studies, as the main components of *M. verticillata* essential oil were found to be pulegone and menthone, representing 60.5% and 18.2% of the total oil, respectively.

Given the lack of data currently available, the study of the potential cytotoxicity and genotoxicity of *M. verticillata* essential oil is justified. The neutral red and the MTT reduction assays are used to evaluate the ability of toxic substances as induce early damage as the alteration of the lysosomal or mitochondrial function, respectively. Staining of cells with exclusive dyes as trypan blue, detect whether a toxic substance causes a serious damage in the cell as is the loss of plasma membrane integrity (Fentem, 1994). The results obtained in this study revealed that Mv-EO does not induce early or severe damage on Vero cells. A similar observation was made by Sutil et al. (2006), who found *in vitro* cytotoxic properties on Vero and HEp-2 cell cultures at high concentrations. In a previous study realized by our research group was demonstrated by MTT reduction assay that the Mv-EO did not induce early damage on human PBMCs (Cariddi et al., 2011). In this study we have showed that the Mv-EO neither induces severe damage on human PBMCs.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Data from the apoptosis assays in human PBMCs demonstrated that peperina essential oil was not apoptotic. The negative results were obtained by Hoechst staining assay and the absence of the DNA damage. To our knowledge, no previous study on the apoptosis of the essential oil from *M. verticillata* and its major constituents such as pulegone and menthone was performed. However, numerous investigations have demonstrated the anti-tumour proprieties of limonene. This monoterpene was able to induce apoptosis in various cancer cells lines by activating proapoptotic signaling cascades (Nakaizumi et al., 1997; Lu et al., 2004; Rabi and Bishayee, 2009; Kutan et al., 2011).

Our results in the micronucleus assay, the *in vivo* test most recommended to detect genotoxic chemicals, showed that the concentrations of Mv-EO, rich in pulegone, menthone and limonene, did not exert a cyto-genotoxic effect on the bone marrow of Balb/c mice. This was evidenced by the ratio PCE/NCE and by the frequency of MNPCE. Sun (2007) considered that limonene have fairly low toxicity. However, previous *in vivo* studies indicated that pulegone was the most toxic component examined on larvae of the crustacean *Artemia salina*, while menthone, limonene and the essential oil as a whole were less toxic (Sutil et al., 2006). Nair (2001) reported that pulegone and large amount of menthone (> 200 mg/kg/day) caused cystlike lesions in the cerebellum of rats in short-term and subchronic oral studies. Pulegone was shown to be able to cause severe hepatotoxicity and carcinogenesis through metabolism, generating the glutathione depletory p-cresol (Zhou et al., 2007). In contrast, Franzios et al. (1997) reported that pulegone showed a very weak positive genotoxic activity and menthone was found to be a potent mutagenetic in Wing Somatic Mutation and Recombination Test on *Drosophila melanogaster*. This disagreement could be explained by the fact that essential oils have to be considered as substances acting as a whole, with synergistic/antagonistic phenomena in their constituents. Furthermore, previous studies on the toxicity effect of pennyroyal oil and its main constituents on *D. melanogaster* and *B. oleae* larvae have shown that the toxicity of pulegone was suppressed in the presence of menthone, suggesting antagonist interactions (Pavlidou et al., 2004).

The high non cytotoxic nor genotoxic concentrations of Mv-EO in our study are interesting because previous findings demonstrated that less concentrations of Mv-EO were active against the pathogens *Bacillus subtilis* Cohn, *Staphylococcus aureus* Rosenbach, *Streptococcus faecalis* Orla-Jensen, *Bacillus cereus* Frankland&Frankland var. *micoides*, *Proteus mirabilis* Hauser, *Escherichia coli* Casellani & Chalmers, *Salmonella typhi* and *Herpes virus* (De Feo et al., 1998; Maldonado et al.,

ACCEPTED MANUSCRIPT

2001; Primo et al., 2001). Thus, our data suggests that *M. verticillata* essential oil has selectivity of action against these pathogens. The inhibitory effect of Mv-EO on potential enteropathogenic bacteria (*S. aureus, S. faecalis, B. cereus, E. coli* and *S. typhi*), may explain why peperina is used in folk medicine to treat digestive disorders.

On the other hand, previous studies demonstrated that Mv-EO has repellent properties against the mite *Varroa destructor* and insecticidal activity against the *Musca domestica* L (Ruffinengo et al., 2005; Palacios et al., 2009; Rosy et al., 2012). The lack of toxicity of Mv-EO demonstrated in our study on normal cells, could promote its possible use also as a fumigant.

In conclusion, the results obtained in this study showed that *M. verticillata* essential oil is not cytotoxic *in vitro* nor cyto-genotoxic *in vivo* both at low and high concentrations and therefore appears to be safe as a therapeutic agent. However, due to the toxicity of the major constituents present in *M. verticillata* essential oil after chronic treatment, further *in vivo* studies at non-lethal concentrations should be conducted.

Acknowledgements

This work was supported by grants from CONICET, PICTOR 8/20325 BID 1728/OC-AR, Agencia Córdoba Ciencia-Nación, Resolution #222, years 2007/10, and SeCyT, Resolution #763, years 2009/11, from Universidad Nacional de Río Cuarto. The authors are also grateful to Dr. Margarita Grosso for the taxonomic classification of the plant specimen.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

Abdillahi, H.S., Verschaeve, L., Finnie, J.F., Van Staden, J., 2012. Mutagenicity, antimutagenicity and cytotoxicity evaluation of South African *Podocarpus* species. J. Ethnopharmacol. 139, 728-738.

Amirghofran, Z., Bahmani, M., Azadmehr, A., Javidnia, K., 2007. Immunomodulatory and apoptotic effects of *Stachys obtusicrena* on proliferative lymphocytes. Med. Sci. Mon. 13, 145-150.

Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A., Idaomar, M., 2005. Cytotoxicity and gene induction by some essential oils in the yeast *Saccharomyces cerevisiae*. Mut. Res. 585, 1-13.

Banchio, E., Zygadlo, J., Valladares, G.R., 2005. Effects of mechanical wounding on essential oil composition and emission of volatiles from *Minthostachys mollis*. J. Chem. Ecol. 31, 719-727.

Bandoni, A.L., Mendiondo, M.E., Rondina, R.V.D., Coussio, J.D., 1972. Survey of Argentine medicinal plants. I. Folklore and phytochemical screening. Lloyd. 35, 69-80.

Bluma, R., Amaiden, M.R., Daghero, J., Etcheverry, M., 2008. Control of *Aspergillus* section *Flavi* growth and aflatoxin accumulation by plant essential oils. J. Appl. Microbiol. 105, 203-214.

Bonzani, N., Ariza Espinar, L., 1993. Estudios anatómicos de tres especies de Lamiaceae usadas en medicina popular. Act. Farm. Bonaerense 12, 113-123.

Borenfreund, E., Puerner, J.A., 1985. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). Method. Cell. Sci. 9, 7-9.

Cariddi, L.N., Panero, A., Demo, M.S., Sabini, L.I., Maldonado, A.M., Grosso, M., Zyglado, J., 2007. Inhibition of immediate-type allergic reaction by *Minthostachys verticillata* (Griseb.) Epling essential oil. J. Essent. Oil Res. 19, 190-196.

Cariddi, L.N., Escobar, F., Moser, M., Panero, A., Alaniz, F., Zygadlo, J., Sabini, L., Maldonado, A., 2011. Monoterpenes isolated from *Minthostachys verticillata* (Griseb.) Epling essential oil modulates immediate-type hypersensitivity responses *in vitro* and *in vivo*. Planta Med. 77, 1687-1694.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

De Feo, V., Ricciardi, A., Biscardi, D., 1998. Chemical composition and antimicrobial screening of the essential oil of *Minthostachys verticillata* (Griseb.) Epl. (Lamiaceae). J. Essent. Oil Res. 10, 61-65.

De Logu, A., Loy, G., Pellerano, M.L., Bonsignore, L., Schivo, M.L., 2000. Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell virus spread by *Santolina insularis* essential oil. Antivir. Res. 48, 177-185.

Fentem, J.H., 1994. The use of human tissues in vitro toxicology. Summary of general discussions. Hum. Exp. Toxicol. 13, 445-449.

Fester, G.A., Martinuzzi, E.A., 1950. La esencia de peperina. 2a comunicación. An. Asoc. Quím. Argent. 38, 255-267.

Fester, G.A., Martinuzzi, E.A.,Retamar, J.A., Ricciardi,A.I.A., 1960. Estudios sobre esencias volátiles argentinas, vol. 29, Revista de la Facultad Ing Quim, Universidad Nacional del Litoral, Santa Fe, Argentina, pp. 21-44.

Franzios, G., Mirotsou, M., Hatziapostolou, E., Oral, J., Scouras, Z.G., Mavragani-Tsipidou P., 1997. Insecticidal and Genotoxic Activities of Mint Essential Oils. J. Agr. Food Chem. 45, 2690-2694.

González Pereyra, M.L., Cariddi, L.N., Ybarra, F., Isola, M.C., Demo, M.S., Sabini, L.I., Maldonado A.M., 2005. Immunomodulating properties of *Minthostachys verticillata* on human lymphocytes and basophils. Rev. Alergia Mex. 52, 105-112.

González, M.J., Marioli, J.M., 2010. Antibacterial activity of water extracts and essential oils of various aromatic plants against *Paenibacillus larvae*, the causative agent of American Foulbrood. J. Invertebr. Pathol. 104, 209-213.

Krishna, G., Hayashi, M., 2000. *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. Mut. Res. 455, 155-166.

Kuttan, G., Pratheeshkumar, P., Manu, K.A., Kuttan, R., 2011. Inhibition of tumor progression by naturally occurring terpenoids. Pharm. Biol. 49, 995-1007.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Lu, X.G., Zhan, L.B., Feng, B.A., Qu, M.Y., Yu, L.H., Xie, J.H., 2004. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. World J. Gastroentero. 10, 2140-2144.

Maldonado, A.M., Calvo, D., Cariddi, L.N., Demo, M., 2001. Efectos linfoproliferativos y antimicrobianos de productos vegetales derivados de *Minthostachys verticillata* y *Achyrocline satureioides*. Archivos Alergia e Inmunología Clínica 32, 82.

Militão, G.C.G., Dantas, I.N.F., Pessoa, C., Falcão, M.J.C., Silveira, E.R., Lima, M.A.S., Curi, R., Lima, T., Moraes, M.O., Costa-Lotufo, L.V., 2006. Induction of apoptosis by pterocarpans from *Platymiscium floribundum* in HL-60 human leukemia cells. Life Sci. 78, 2409-2417.

Mongini, C., Waldner, C., 1996. Metodologías para la evaluación de las células inmunocompetentes, in: Margni, R.A. (Ed.), Inmunología e Inmunoquímica. Editorial Médica Panamericana, Argentina, pp. 730-734.

Montaner, B., Navarro, S., Piqué, M., Vilaseca, M., Martinell, M., Giralt, E., Gil, J., Pérez-Tomás, R., 2000. Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. Brit. J. Pharmacol. 131, 585-593.

Nair, B., 2001. Final report on the safety assessment of Mentha Piperita (Peppermint) Oil, Mentha Piperita (Peppermint) Leaf Extract, Mentha Piperita (Peppermint) Leaf, and Mentha Piperita (Peppermint) Leaf Water. Int. J. Toxicol. 20, 61-63.

Nakaizumi, A., Baba, M., Uehara, H., Iishi, H., Tatsuta, M., 1997. d-Limonene inhibits N-nitrosobis(2-oxopropyl)amine induced hamster pancreatic carcinogenesis. Cancer Lett. 117, 99-103.

Núñez, C.M., Cantero, J.J., 2000. Las plantas medicinales del Sur de la provincia de Córdoba. (Ed.), De la Fundación Universidad Nacional de Río Cuarto, Argentina, pp. 1-144.

Ojeda, M., Coirini, R., Cosiansi, J., Zapata, R., Zygadlo, J., 2001. Evaluation of variability in natural populations of peperina (*Minthostachys mollis* (Kunth.) Griseb.), an aromatic species from Argentina**.** Plant Genet. Resour. Newsl. 126, 27-30.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Palacios, S.M., Bertoni, A., Rossi, Y., Santander, R., Urzúa, A, 2009. Insecticidal activity of essential oils from native medicinal plants of Central Argentina against the house fly, *Musca domestica* (L). Parasitol. Res. 106, 207-212.

Pavlidou, V., Karpouhtsis, I., Franzios, G., Zambetaki, A., Scouras, Z., Mavragani-Tsipidou, P., 2004. Insecticidal and genotoxic effects of essential oils of Greek sage, *Salvia fruticosa*, and Mint, *Mentha pulegium*, on *Drosophila melanogaster* and *Bactrocera oleae* (Diptera: Tephritidae). J. Agr. Urban Entomol. 21, 39-49.

Primo, V., Rovera, M., Zanon, S., Oliva, M., Demo, M., Daghero, J., Sabini, L., 2001. Determination of the antibacterial and antiviral activity of the essential oil from Minthostachys verticillata (Griseb.) Epling. Revi. Argent. Microbiol. 33, 113-117.

Rabi, T., Bishayee, A., 2009. d-Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J. Carcinog. 8, art. no. 9.

Rossi, Y.E., Canavoso, L., Palacios, S.M., 2012. Molecular response of *Musca domestica* L. to *Mintostachys verticillata* essential oil, (4R)(+)-pulegone and menthone. Fitoterapia. 83, 336-342.

Ruffinengo, S., Eguaras, M., Floris, I., Faverin, C., Bailac, P., Ponzi, M, 2005. LD_{50} and repellent effects of essential oils from Argentinean wild plant species on *Varroa destructor*. J. Econ. Entomol. 98, 651-655.

Schmid, W., 1975. The micronucleus test. Mut. Res. 31, 9-15.

Schmidt-Lebuhn, A.N., 2008. Ethnobotany, biochemistry and pharmacology of *Minthostachys* (Lamiaceae). J. Ethnopharmacol. 118, 343-353.

Sun, J., 2007. D-Limonene: Safety and clinical applications. Alternat. Med. Rev. 12, 259-264.

Sutil, S.B., Astesano, A., Vogt, V., Torres, C.V., Zanon, S.M., Sabini, L.I., 2006. *Minthostachys verticillata:* toxicity of its essential oil and major constituents to *Artemia salina* and cell lines. Mol. Med. Chem. 10, 41-42.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Zhou, S.F., Xue, C.C., Yu, X.Q., Wang, G., 2007. Metabolic activation of herbal and dietary constituents and its clinical and toxicological implications: an update. Curr. Drug Metab. 8, 526-553.

Zygadlo, J., Maestri, D., Lamarque, A., Guzmán, C., Velasco-Negueruela, A., Pérez-Alonso, M., García-Vallejos, M., Grosso, N., 1996. Essential oil variability of *Minthostachys verticillata*. Biochem. Syst. Ecol. 24, 319-323.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figures

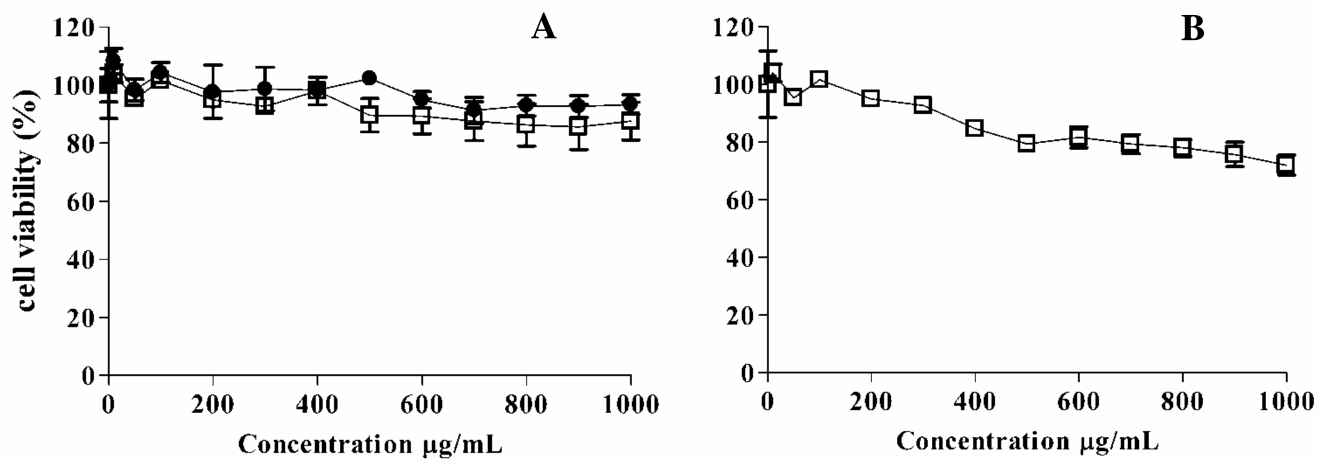


Figure 1. Viability of two cell types exposed to different concentrations of *Minthosthachys verticillata* essential oil determined by trypan blue dye exclusion ( ) and NRU (●) assays. A) Vero cells, B) human PBMCs. The results are presented as percentage (mean ± SD).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

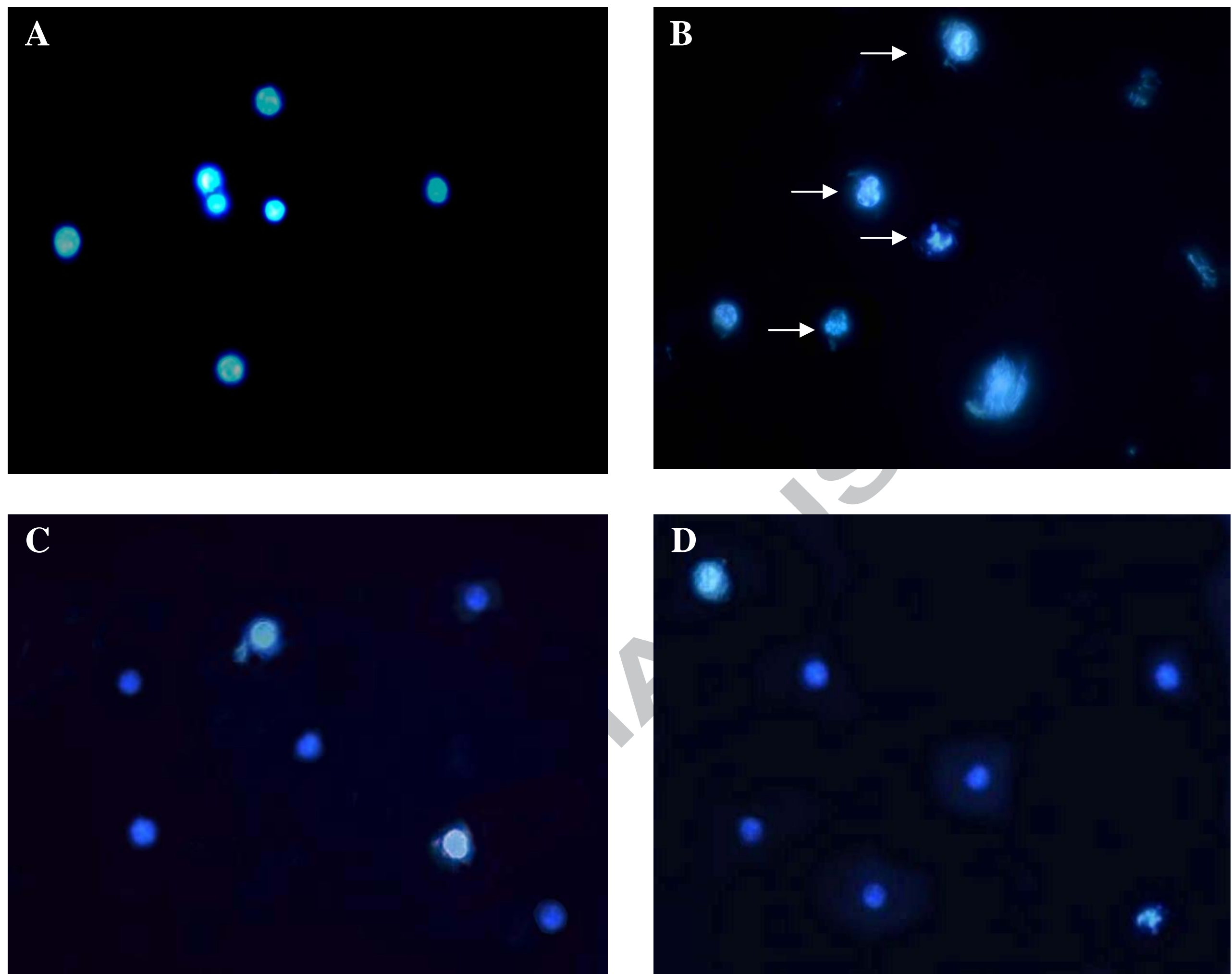


Figure 2. Photomicrographs of the nuclear morphology of normal human PBMCs stained with Hoechst 33258 after 18-24 h treatment (100 X). A) Untreated cells (control), B) treated with hydrogen peroxide (1 mmol/L), C) treated with Mv-EO (10 μg/mL), and D) treated with Mv-EO (100 μg/mL). Arrows show apoptotic cells. These cells were identified by characteristic features of apoptosis (e.g. nuclear condensation, formation of membrane blebs and apoptotic bodies)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Tables

Table **1**: Major compounds of *Minthosthachys verticillata* essential oil

Compounds	% (v/v)
Pulegone	60.5
Menthone	18.2
Limonene	3.76
β- pinene	0.29
α- pinene	0.16
1,8- cineole	0.12
Not determined	16.97

Table **2**. Mean of micronucleated polychromatic erythrocytes observed in the bone marrow cells of Balb/c mice treated with *Minthosthachys verticillata* essential oil, and respective controls.

Groups	Times of cell collection	Number of MNPCE per animal						% MNPCE (mean ± SD)	PCE/NCE (mean ± SD)
		M_1	M_2	M_3	F_4	F_5	F_6		
Negative control (saline)	24 h	2	3	3	2	2	2	2.33 ± 0.5	3.06 ± 0.82
	48 h	0	3	4	1	2	1	1.83 ± 1.4	2.87 ± 1.03
Vehicle (Tween-80 (0.5% v/v)	24 h	3	1	3	2	3	3	2.50 ± 0.8	2.82 ± 1.12
	48 h	0	1	2	2	3	4	2.00 ± 1.4	2.79 ± 1.00
M. verticillata EO 25 mg/kg b.w.	24 h	3	4	4	5	3	4	2.33 ± 0.5	3.06 ± 0.82
	48 h	3	2	5	2	2	1	2.50 ± 1.4	3.02 ± 0.88
M. verticillata EO 50 mg/kg b.w.	24 h	4	5	5	3	3	4	4.00 ± 0.9	2.66 ± 1.49
	48 h	3	3	2	2	1	5	2.67 ± 1.4	2.85 ± 1.30
M. verticillata EO 100 mg/kg b.w.	24 h	5	4	4	5	5	3	4.50 ± 1.0	3.12 ± 1.20
	48 h	4	5	3	2	5	2	3.50 ± 1.4	2.96 ± 0.93
M. verticillata EO 250 mg/kg	24 h	5	6	5	4	3	3	4.50 ± 1.4	3.48 ± 0.71
	48 h	2	3	2	4	4	5	3.33 ± 1.2	2.77 ± 1.25

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

M. verticillata EO 500 mg/kg b.w.	24 h	4	2	3	4	6	6	4.16 ± 1.6	3.82 ± 1.28
	48 h	3	4	5	3	3	2	3.33 ± 1.0	3.02 ± 1.21
Cyclophosphamide 30 mg/kg b.w.	24 h	11	15	10	13	9	12	11.67 ± 2.2*	3.03 ± 0.87
	48 h	9	8	7	10	11	10	9.16 ±1.5*	2.80 ± 0.84

For each period (24 and 48 h), 1000 polychromatic erythrocytes were analyzed. SD = standard deviation; PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes; M = male; F = female. *Significantly different from negative control ($p < 0.001$).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Highlights

Toxicity and chemical composition of *M. verticillata* essential oil were studied.

The main compounds were pulegone, menthone and limonene.

The essential oil not induced cytotoxic effect both on Vero cell and human PBMCs.

The oil not induced apoptosis on human PBMCs nor genotoxicity on erythrocytes of mice.

The results suggest this species appears to be a safe therapeutic agent.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Elsevier Editorial System(tm) for Food and Chemical Toxicology
Manuscript Draft

Manuscript Number: FCT-D-12-00791R1

Title: Lack of cytotoxic and genotoxic effects of Minthostachys verticillata essential oil: studies in vitro and in vivo

Article Type: Full Length Article

Keywords: Minthostachys verticillata, essential oil, gas chromatography, cytotoxicity, apoptosis, genotoxicity

Corresponding Author: Microbiologist Franco M Escobar, Dr

Corresponding Author's Institution: Universidad Nacional de Río Cuarto, Argentina

First Author: Franco M Escobar, Dr

Order of Authors: Franco M Escobar, Dr; Laura N Cariddi, Dr; María C Sabini; Sonia B Sutil, MSc; Cristina V Torres, MSc; Silvia M Zanon, Dr; Liliana I Sabini, Dr

Abstract: Minthostachys verticillata (peperina) is an aromatic and medicinal plant with several uses and ethnobotanical properties. Numerous studies have demonstrated that its essential oil (Mv-EO) presents antimicrobial capacity and shows immunomodulating and anti-allergic properties in human cell lines. Thus, the goal of this study was to investigate the main chemical composition, analyzed by GC-FID, and the cyto-genotoxic effects of Mv-EO, using Vero cells, human PBMCs and mice bone marrow cells. The Mv-EO was rich in pulegone 60.5% and menthone 18.2%. Our results clearly show that Mv-EO is not cyto-genotoxic in vitro nor in vivo. It not induced cytotoxic effects, as indicated by trypan blue dye exclusion and NRU assays both in Vero cells and human PBMCs. In addition, Mv-EO (100-1000 μg/mL) not induced apoptotic effects on human PBMCs, as indicated by Hoechst staining and DNA fragmentation analysis by agarose gel electrophoresis. The in vivo assay showed that Mv-EO (25-500 mg/kg) not increased the frequency of micronucleus in bone marrow cells of mice. Further, the ratio of polychromatic/normochromatic erythrocytes was not modified. These findings suggest that Mv-EO appears to be safe as a therapeutic agent.

Response to Reviewers: Reviewers' comments:
Reviewer #1: This is a significantly improved version of the previously rejected manuscript.
I have only a few minor comments and suggestions:

Comment N°1. Authors must explain why they have used un-traditional solvents - emulsion with DMSO for in vitro studies and emulsion with Tween 80 for in vivo studies? Usually, for in vivo studies ethanol or acetone is used as a solvent, and for in vivo studies - oil (corn, olive, etc.).
Response to Reviewer comment N°1: Both DMSO and Tween 80 are widely used in in vitro and in vivo assays (see the following references: Lima et al., 2004, Toxicol In vitro 18: 457-465; Saddi et al., 2007, Ann Clin Microbiol Antimicrob. 6: 10; Cariddi et al., 2011, Planta Med. 77, 1687-1694; Castro et al., 1995, Pharmacol Biochem Behav. 50: 521-526; González-Trujano et al., 2009, Epilepsy Behav. 16: 590-595; Apryani et al., 2010, J Ethnopharmacol. 129: 357-360. We chose these solvents on the basis of the above references and because we observed the best disolution of the oil in them.