Regulatory Toxicology and Pharmacology 71 (2015) 1–7

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

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

Franco Matías Escobar [a,*], María Carola Sabini [a], Laura Noelia Cariddi [a], Liliana Inés Sabini [a], Fernando Mañas [b], Andrea Cristofolini [c], Guillermo Bagnis [c], Mauro Nicolas Gallucci [d], Lilia Renée Cavaglieri [a]

[a] Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, Río Cuarto, CP 5800 Córdoba, Argentina
[b] Departamento de Clínica Animal, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, Río Cuarto, CP 5800 Córdoba, Argentina
[c] Departamento de Patología Animal, Universidad Nacional de Río Cuarto, Ruta 36 Km 601, Río Cuarto, CP 5800 Córdoba, Argentina
[d] Centro de Investigación y Transferencia de Santiago del Estero (CITSE), CP G4200AQF, Argentina

ARTICLE INFO

Article history:
Received 18 September 2014
Available online 11 November 2014

Keywords:
Minthostachys verticillata
Essential oil
90-Day subchronic toxicity
Genotoxicity

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.

* Corresponding author. Fax: +54 0358 4676231.
E-mail address: fescobar@exa.unrc.edu.ar (F.M. Escobar).

http://dx.doi.org/10.1016/j.yrtph.2014.11.001
0273-2300/

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)