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Hepatotoxicity evaluation of an aqueous extract of *Clinopodium vimineum* (L.) Kuntze in female *Wistar* rats

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Abstract

Clinopodium vimineum has been used to relieve gastrointestinal symptoms due to its carminative and antispasmodic properties. Besides, it has been associated with antimicrobial, sedative, analgesic, anti-inflammatory, and healing effects. Nevertheless, its essential oil has hepatotoxic compounds such as pulegone, so the plant's possible hepatotoxic effect on aqueous extract was evaluated with a female *Wistar* rats model. An aqueous extract of the vegetal material was prepared, and an assay was carried out with three experimental groups of five subjects each. The first consisted of a control group that did not receive the extract; the second was treated for four days with the extract, receiving a loading dose on the first day. The third was similar to the previous one, except for not receiving the indicated loading dose. Each animal was evaluated for some hepatic function parameters (alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase). Likewise, histopathological analysis of the liver was performed. With the information obtained, it was impossible to establish that the aqueous extract of *C. vimineum* has a hepatotoxic effect in female *Wistar* rats.

Keywords: *Clinopodium vimineum* (L.) Kuntze; Aqueous extract; *Wistar* rats; Hepatic function parameters; Histopathological analysis.

1. Introduction

Clinopodium vimineum (known in Costa Rica as *menta de palo*) [1] is an aromatic shrub native to Central America and the Caribbean. It is described as a plant species with woody stems of vertical growth and multiple branches. The height is calculated between 15 and 20 cm. Its leaves have oval and opposite shapes, with toothed margins, a lime green upper side, engraved veins, and covered by hairiness. Furthermore, its flowers are small, tubular, and with white or pink/purple tones [2, 3].

It has been traditionally utilized to relieve gastrointestinal tract symptoms, as it has carminative and antispasmodic properties. Other described assets are a healing agent, antimicrobial, sedative, analgesic, and anti-inflammatory [2, 3].

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Approximately 40 chemical compounds have been described in *C. vimineum*. The essential oil of its leaves includes monoterpenes such as p-menth-3-en-8-ol (40.0 %), pulegone (35.3 %), and p-mentha-3,8-diene (5.2 %) and some sesquiterpenes such as β-caryophyllene (3.6 %), α-humulene (1.3 %), limonene (1.0 %), and terpinolene (0.9 %) [4]. Attracts attention to pulegone, which has reported concentrations between 10 and 40 % of the total essential oil, with potential hepatotoxic effect [5]. Therefore, the consumption of this plant can represent a health risk.

Given this situation, this work aimed to evaluate the possible hepatotoxic effect of an aqueous extract of *C. vimineum* in a female *Wistar* rats model.

2. Material and methods

2.1. Vegetal material

Flowers, buds, and stems of *C. vimineum* were collected at the School of Agriculture of the Humid Tropical Region (EARTH University) (10°13'09" N 83°35'33" W) located in Guácimo, Limón. The sample was identified and deposited in the Dr. Luis A. Fournier Origgi Herbarium of the School of Biology of the Universidad de Costa Rica (UCR, for its Spanish acronym). The material was dried at room temperature and pulverized until achieving an acceptable material for its preservation.

2.2. Preparation of the plant's aqueous extract

An infusion was produced with 45 g of the powdered plant material, adding boiling water. The mixture was left to rest for 30 minutes in a container with a lid. Later, it was diminished by reduced pressure at 50 °C, using a Büchi® R-134 rotary evaporator (Büchi® B-480 water bath and Büchi® B-721 vacuum controller). The extract was lyophilized for four days (LABCONCO® FreeZone 6) to facilitate its administration. The final product was stored at -80 °C.

2.3. Experimental animals

Adult *Wistar* female rats (HsdBrlHan: WIST) with 207.80 ± 3.33 g of body weight, between eight and nine weeks of age, from the Laboratory of Biological Assays (LEBi, for its Spanish acronym) of the UCR were employed.

Fifteen subjects were considered. They were kept in polycarbonate boxes in the laboratory for *in vivo* work of the Phytopharmacology and Pharmaceutical and Cosmetic Technology Laboratory (LAFITEC, for its Spanish acronym) of the Instituto de Investigaciones Farmacéuticas (INIFAR, for its Spanish acronym) under the following conditions: temperature: 23.2 °C; relative humidity: 73 %; and periods of light and darkness: 12 hours. Bottled drinking water (Members Selection®) and rodent food (Aguilar & Solís®) were provided *ad libitum*. They were at the laboratory for seven days without manipulation to allow their adaptation.

2.4. Experimental design

Random block modeling was established with a total of three groups of five experimental subjects each: group 1 (control, which was administered water by gastric cannulation instead of the extract), group 2, which received extract for four days, starting with a loading dose of 300 mg/kg/day on the first day and then continuing with a dose of 150 mg/kg/day for three more days by intragastric cannula; and group 3, whose subjects received a daily dose of 150 mg/kg/day for four days by the same route mentioned above.

The trial lasted five days, ensuring the animals had access to food and drinking water *ad libitum*. On the fifth day, each animal was euthanized using the decapitation technique to get whole blood, and a general necropsy was performed to collect liver tissue. Plasma samples were obtained by centrifugation at 3000 rpm for 10 minutes (HettichZentrigugen® Universal 320 R model) at LAFITEC of the Faculty of Pharmacy of the UCR.

2.5. Analysis of biochemical liver function parameters

The serum samples were sent to the Clinical Analysis Laboratory at the Faculty of Microbiology of the UCR, and the hepatic enzymes analysis was done (Roche Cobas® c111 equipment). The enzymes analyzed were alkaline phosphatase (AP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

2.6. Hepatic histopathological analysis

The collected organs were fixed with 10 % buffered formalin. Tissues were then dehydrated with increasing concentrations of ethanol, cleared with xylene, and embedded in paraffin. A rotary microtome (Leica® RM2245) was

employed to make 4 to 5 μm thick sections stained with hematoxylin-eosin. Through a Zeiss® AxioLab A1 microscope, morphological and structural changes in hepatocytes were analyzed, along with the physical characterization of sinusoids and vascular structures of said organ (hepatic portal vein, renal artery, and bile ducts).

2.7. Statistical analysis

The results were expressed as the mean together with its relative standard deviation. Unifactorial statistical analysis (ANOVA) was contemplated to settle group differences. The comparison between the means of the groups was elaborated through Tukey's multiple comparison analysis with a significance level of 95 % ($p < 0.05$), utilizing the software GraphPadPrism® version 6.01 for Windows.

3. Results and discussion

3.1. Analysis of biochemical liver function parameters

AST and ALT are the most sensitive cell necrosis or hepatocellular injury indicators [6]. Damage to the hepatocyte membrane provokes an elevation in the transaminases serum levels above the typical values because of their escape into circulation [7]. The quantification usually has high sensitivity but low specificity [8]. ALT is a more specific indicator. Both are at high levels in the liver cytosol. However, AST is also found in the liver mitochondria and has activities in muscle, brain, pancreas, lungs, kidneys, erythrocytes, and leukocytes [9].

As a complement, FA is the best indicator parameter of biliary obstruction. Nonetheless, this enzyme does not differentiate between intrahepatic or extrahepatic cholestasis clinical pictures. Given this, the enzyme is not a conclusive parameter to determine the existence of liver damage [10].

For the *in vivo* model, the changes in the serum levels of AST, ALT, and FA were jointly considered biomarkers of cell damage in hepatocytes. **Table 1** shows the results achieved for the serum parameters of liver function. For FA, there was a significant reduction in groups 2 and 3 compared to the control group ($p < 0.05$).

Table 1 Biochemical parameters of the liver function obtained at the end of the *in vivo* model with an aqueous extract of *C. vimineum*. Data were expressed as the mean along with their respective relative standard error. Concentrations were compared to the control, n = 5 animals per group, * $p < 0.05$.

Experimental group	FA (U/l)	AST (U/l)	ALT (U/l)
Control	86.85 ± 7.00	198.60 ± 17.14	42.28 ± 0.73
Treatment with *C. vimineum* administering load dose	52.90 ± 6.40*	248.50 ± 13.91	41.03 ± 3.05
Treatment with *C. vimineum* without administering load dose	58.00 ± 2.41*	287.70 ± 23.12*	50.08 ± 6.08

Regarding AST, a significant increase was appreciated in the group without a loading dose, compared to the control ($p < 0.05$), rising 1.4 times in five days. Though, this enzyme is not tissue-specific [9].

Finally, there were no appreciable differences between the groups for ALT. Since these results were not conclusive, the hepatic histopathological analysis of each experimental group was made.

3.2. Hepatic histopathological analysis

For the histopathological analysis (**Figure 1**), there were no relevant structural or morphological alterations in the hepatic tissue when comparing the animals exposed to the extract. The main morphological alterations located were the isolated presence of infiltrate with macrophages (Kuffer cells) [11], a slight increase in the size of the bile ducts [12], the widening of the sinusoids oriented towards the hepatic portal vein [13], and the pyknotic nuclei presence with concentrated chromatin [14]. These findings suggest some particular infectious processes.

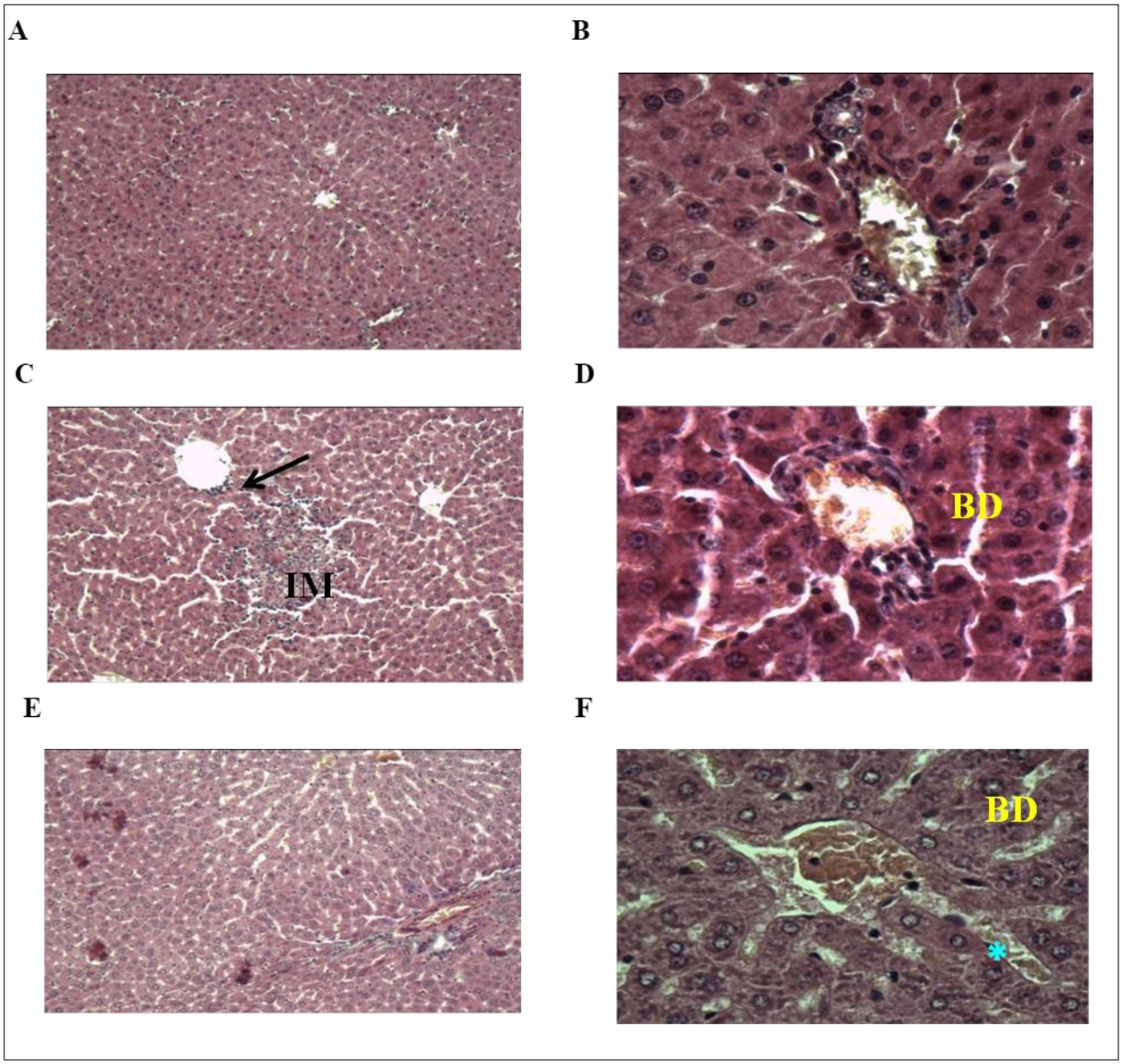

Figure 1 Sagittal sections of livers taken from female *Wistar* rats after the *in vivo* model. **A and B**. Control group. **C and D**. Group exposed to the extract without a loading dose. **E and F**. Group exposed to the extract with a loading dose. IM: infiltration of macrophages. BD: opening of bile ducts oriented towards the central hepatic vein. Arrow: leukocyte infiltration oriented towards the central hepatic vein. Asterisk: bile aggregation in bile ducts. Hematoxylin and eosin staining. Objectives: 10X and 40X.

With the histopathological analysis of the liver tissue and the changes in the serum parameters of liver function (FA, AST, and ALT), it can be assured that after the administration of the aqueous plant extract, there was no liver toxicity induced by characteristic metabolites of the genus *Clinopodium*, such as the pulegone. No indications of cell damage in the liver sections or significant alteration in the transaminase values were found.

4. Conclusion

The evaluation of the serum to study hepatic function parameters showed an appreciable but non-specific increase in AST values. No alterations or considerable changes were found for ALT and FA, which did not suggest liver damage because of the aqueous plant extract. Moreover, there were no structural or morphological alterations in the histopathological analysis for the liver sections that would indicate toxicity in said organ due to the treatment administration. Therefore, the information acquired reveals that the aqueous extract of *C. vimineum* did not generate a hepatotoxic effect in female *Wistar* rats.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The management and care of the animals were made under the provisions of the Animal Welfare Law (No. 7451) and the guidelines stipulated in the Guide for the Care and Use of Laboratory Animals (Decree No. 26668). The experimental design of the model was reviewed and approved by the Institutional Committee for the Care and Use of Animals (CICUA, for its Spanish acronym) of the UCR, document CICUA-018-16.

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