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Acute and Subacute Toxicity Assessment of Ethanolic Extract of *Rosmarinus officinalis* in Female Wistar Rats

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Abstract

ROSMARINUS officinalis (rosemary), an aromatic plant rich in phenolic antioxidant compounds, is widely used in traditional therapy by Moroccan population. The study aims to evaluate the acute and subacute oral toxicity of ethanolic extract of *Rosmarinus officinalis* (EERO) in female Wistar rats. In the acute toxicity study, 30 rats were divided into 5 groups (control, 1000, 2500, 3500, and 5000 mg/kg dose). Similarly, in the subacute toxicity study, 30 rats were divided into 5 groups (control, 200, 300, 500 and 1000mg/kg dose). The sub-acute toxicity study aimed to identify the LD50, through following the same signs of toxicity, haematological parameters, biochemical parameters, markers of oxidative stress, and histological analyses were evaluated. The results showed that no significant differences were observed in acute and sub-acute toxicity parameters (body weight gain, food and water intake), nor acute toxicity was any mortality observed, suggesting that the LD50 is greater than 5000 mg/kg. The sub-acute toxicity study showed no significant differences in haematological, biochemical, and histological parameters, while the extract increased CAT levels and decreased NO in the liver and kidney. The RO extract is non-toxic up to the doses tested and exerts antioxidant activity, manifested by a nitric oxide level decrease and antioxidant enzyme catalase level increase in the liver and kidneys.

Keywords: *Rosmarinus officinalis*, Hematological analysis, Toxicity, Catalase, Nitric oxide, Rats.

Introduction

In recent years, interest in herbal medicine has been on the rise, with more and more people turning to medicinal plants for their health. It should be underscored that the traditional use of a plant for therapeutic effects is no guarantee of its safety [1]. While the therapeutic effects of many plants have been shown in various studies, their toxicity is generally unknown. Assessing the toxicity of herbal preparations is therefore essential in determining the safety of these plants [2].

For a long time, the World Health Organization has been resolutely committed to the revaluation of traditional pharmacopoeia in order to meet the health needs of populations. Currently, natural molecules in plants are still an important reservoir of new drugs. They represent nearly 60% of the drugs we have. The remaining 40%, or synthetic drugs, are often born from the chemical synthesis of substances or parts of natural molecules taken as lead drugs [3,4]. According to the World Health Organization, 80% of the African population uses traditional medicine. However, it is important to note that the use of certain traditional medicines made from plants, due to the complexity of the human body, can have interactions with it that can cause toxic effects. Indeed, studies highlight the short, medium, and long-term toxic effects of plant extracts with established biological characteristics [4].

Rosmarinus officinalis, known as rosemary, is an aromatic plant belonging to the Lamiaceae family. Since ancient times, rosemary has been widely used in culinary, cosmetic, and medicinal products [5]. Several pharmacological studies have investigated the beneficial properties of rosemary and other medicinal plants, particularly their antioxidant effects, anti-inflammatory effects [6], anti-nephrotoxic effects [7,8] and hepatoprotective effects [9].

For a long time, man has used food plants for nutrition, medicinal plants for illness treatment, and toxic plants to use as arrow poisons for hunting or war. Despite a certain eclipse due to the rise of synthetic chemistry from the 19th century, herbal

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medicines are still widely used today, both in developing countries where traditional practitioners play a considerable role and in industrialized countries where they are mainly used for self-medication [10–13]. However, many plants are toxic or even addictive, which is why the idea that everything that is plant-based and natural would be risk-free is dangerous. In Morocco, 5.1% of poisonings observed in hospital emergency rooms and poison control centers are attributed to the ingestion – accidental or voluntary – of toxic plants. Indeed, the absorption of aconite, yew, datura, belladonna, to name only the most common cases, can have lethal or morbid consequences. Other plants cause a state of drug addiction (cannabis resin from *Cannabis sativum*, hallucinogenic cacti such as peyote *Echinocactus williamense*, etc.) or contain addictive molecules that can be extracted or chemically synthesized (cocaine from the leaves of *Erythroxylon coca*, etc.) [14–16].

The main objective is to determine whether the ethanolic extract of rosemary shows significant toxic effects in the short term (acute toxicity) or after repeated exposure over a prolonged period (sub-acute effect) in female Wistar rats. We aim to identify signs of toxicity, functional and structural alterations in key organs, and relevant biological parameters.

In the acute toxicity study, we used female Wistar rats to evaluate the detect acute toxicity at LD50 and observe signs of toxicity (diarrhea, respiratory disorders and changes in body weight, food and water consumption), haematological parameters, biochemical parameters, markers of oxidative stress, and histological analyses were also evaluated.

Material and Methods

In this study turkey′s breast and thigh samples were collected from different supermarkets in Menofia governorate, Egypt. These samples were examined microbiologically for validation of their effects on consumers' health. The microbiological examination focused on the microorganisms which have public health importance and could be used as hygienic quality indicators. The APC, coliforms, *E. coli*, *Salmonella*, *Staphylococcus aureus*, yeast and mould were detected in the collected samples.

Plant preparation

Rosemary (*Rosmarinus officinalis*) was harvested in July 2021 in the Ras Lma region of Taza. After drying the plant, the leaves were carefully recovered for subsequent extraction.

Rosmarinus officinalis leaves were dried at a temperature of (25 ± 3°C), in the open air and protected from light to preserve the integrity of the molecules as much as possible, then finely ground using an electric grinder; crushing was followed directly by sieving (250 to 500µm), which resulted in a fine powder and a better extraction yield.

A weighed quantity (100g) of *Rosmarinus officinalis* air-dried leaf powder was extracted with 95% ethanol in the Soxhlet extractor. Once extraction was completed, the solvent was then separated from the extract using a rotary flash evaporator at 60°C for 30 min at 2000 rpm to obtain a solid, sticky residue.

The various doses (1000, 2500, 3500 and 5000 mg/kg for acute toxicity and 200, 300, 500 and 1000mg/kg for subacute toxicity) of the leaves ethanolic extract of *Rosmarinus officinalis* were administered orally to female Wistar rats according to their body weight.

Rosmarinus officinalis toxicity assessment

Toxicity assessment was carried out based on guidelines of the organization for Economic Cooperation and Development Fuidelines (n 407) (OCDE, 2008).

In acute toxicity, Wistar female rats in good health were distributed into 5 groups (n = 6/group) according to weight homogeneity. One group of these animals (control group) received oral distilled water, while the other 4 groups received an ethanolic extract of *Rosmarinus officinalis* at doses of 1000, 2500, 3500 and 5000 mg/kg by oral gavage during 14 days. Whereas in the subacute toxicity protocol, female Wistar rats received distilled water orally or the ethanolic extract of *Rosmarinus officinalis* at doses of 200, 300, 500, 1000 mg/kg/day administered for 28 consecutive days before the food. The volume of solution administered to the different groups of rats was constant (1ml). Body weight was measured weekly, and food and water consumption were monitored every midday. Rats were observed for any sign of abnormality (salivation, diarrhea, tremor, etc.) throughout the experiment.

At the end of the treatment, the rats spent the night fasting but with free access to water. They were then anesthetized with chloral hydrate 7g/100 ml distilled water (0.5ml/100g rat body weight) by intraperitoneal injection, then blood samples were taken for hematological and biochemical studies, with and without anticoagulant ethylene-diamine-tetraacetic acid (EDTA) and for the study of histological sections for target organs.

Acute toxicity

Acute toxicity tests assess the toxic effects that appear within a short time (1 to 14 days) after oral administration of *Rosmarinus officinalis* ethanolic extract.

Acute toxicity was conducted using the method recommended in OECD article no. 425 (2008).

- Animals and protocol

The study was conducted within the Faculty of Science at Ibn Tofail University in Kénitra.

The study protocol respected the recommendation of the Institutional Animal Ethics Committee.

Female Wistar rats (150-200g) of around 8 weeks of age were used since they were considered to be slightly more sensitive (Lipnick et al., 1995). They are kept at a temperature of 25±2°C and a relative humidity of 45-55%, under a light-dark cycle (12h light :12h dark). The animals had free access to water ingestion and food consumption throughout the study. All experiments were performed between 9:00 and 16:00.

In this study, the rats were left to fast for approximately 16 hours, then divided according to weight homogeneity into 5 cages of 6 rats each. The ethanolic extract of Rosemary was administered orally in a single dose. Let's remember that the individual volume of solution administered to each group of rats is constant (1ml).

The rats were distributed into 5 groups:

Group 1: Control group: these rats received only distilled water.
Group 2: 1000 mg/kg of ethanolic extract of *Rosmarinus officinalis*.
Group 3: 2500 mg/kg of ethanolic extract of *Rosmarinus officinalis*.
Group 4: 3500 mg/kg of ethanolic extract of *Rosmarinus officinalis*.
Group 5: 5000 mg/kg of ethanolic extract of *Rosmarinus officinalis*.

- Clinical characteristics

Body weight measure, food consumption, water intake.

Subacute toxicity

Animals and protocol

Group 1: Control group: received only distilled water
Group 2: 200 mg/kg of ethanolic extract of *Rosmarinus officinalis*.
Group 3: 300 mg/kg of ethanolic extract of *Rosmarinus officinalis*.
Group 4: 500 mg/kg of ethanolic extract of *Rosmarinus officinalis*.
Group 5: 1000 mg/kg of ethanolic extract of *Rosmarinus officinalis*.

- Body weight, food and water intake, biochemical and hematological parameters were measured.

An automatic hematological analyzer was used for measuring red blood cell (RBC) and white blood cell (WBC) counts, neutrophils (N%), eosinophils (E%), basophils (B%), lymphocytes (L%), monocytes (M%), hematocrit (Hct), hemoglobin (Hb), platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin content (TGMH).

The biochemistry of serum was performed using a spectrophotometer (J.P. SELECTA, s.a. Autovia., Abrera, Spain) for the following parameters: Cholesterol, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), creatinine (Cr), glucose, total cholesterol (CHO), triglycerides (TG), urea.

Determination of oxidative stress markers

Nitric oxide

The assessment of nitric oxide (NO) production from organ homogenates (liver and kidneys) is based on determining the concentrations of the end products of NO synthesis, namely nitrates and nitrites, as described by Bryan and Grisham in 2007. This determination is carried out using the Griess reagent, consisting of two solutions (A : 0.1% of dichlorohydrate naphthylethylene diamine diluted in water and B: 1% sulfanilamide diluted in 5% H_3PO_4). The experimental procedure involves mixing 100 µL of the Griess reagent and 100 µL of nitrite-containing sample in a spectrophotometer cuvette. Incubation of the mixture is carried out for 30 minutes at room temperature, followed by measurement of optical Density at 548 nm, following the method of Chao et al., 1992. NO levels are expressed in µmol/g tissue [17].

Catalase activity

The catalase activity (CAT) in organ homogenates was determined following the method developed by Aebi in 1984. This technique is based on the measurement of optical Density changes resulting from H_2O_2 decomposition. For each sample, a quantity of 60 µl (tissue extract or phosphate buffer per sample) was combined with 2340 µl of phosphate buffer (0.05 mM, pH 7.4) in a quartz cuvette [18]. Initiation of the reaction was achieved by adding 600 µl of H_2O_2 (1 M), and the decrease in absorbance was recorded over 2 minutes (with readings every 30 seconds) at a wavelength of 240 nm. Catalase activity is expressed in international units per minute per gram of tissue (IU/min/g tissue or µmoles of H_2O_2 destroyed per minute per gram of tissue at 25°C [19].

Histological examination

Liver and kidney samples from each treatment group were subjected to histopathological examination. After fixation in 10% formalin, tissues were dehydrated and embedded in in paraffin. Sections 3 to 5 µ thick were cut and stained with hematoxylin and eosin (H&E). Slides prepared were examined under a light microscope [20].

Statistical analysis

Data analyses were performed using the software GraphPad. All parameters are presented as mean ± standard error of the mean (mean ± SEM) and were compared using one-way analysis of variance (ANOVA) with Tukey-Kramer test. Differences between treated and control rats are considered significant at $P<0.05$.

Results

Acute toxicity

No signs of toxicity were observed in the treated groups, only diarrhea: the higher the dose, the more diarrhea appeared.

Table 1 shows the effect of exposure to ethanolic extract of *Rosmarinus officinalis* on rat mortality.

On constate from the results obtained (Table 1) that the doses of *Rosmarinus officinalis* ethanolic extract administered to the rats for 14 days (1000mg/kg, 2500mg/kg, 3500mg/kg, and 5000mg/kg) were well tolerated, and no mortality related to the plant extract was reported. Due to the absence of mortality, based on this result, LD50 of the extract is greater than 5000 mg/kg.

Table 2 shows the signs of toxicity observed in groups of rats treated with different doses of the ethanolic extract of *Rosmarinus officinalis* during the study of acute toxicity.

Results showed that ethanolic extract of *Rosmarinus officinalis* administered at doses of 1000 mg/kg, 2500 mg/kg, 3500 mg/kg, and 5000 mg/kg to female rats caused no abnormal signs (changes in behavior or general physical appearance) except diarrhea, throughout the 14-day observation period.

The table 3 presents the clinical effects of *Rosmarinus officinalis* extract in rats of different groups. The result obtained shows the administration of the extract at single doses of 1000, 2500, 3500, and 5000 mg/kg, causes a diarrhea in all treated groups; the greater the dose presented, the more diarrhea appeared. Diarrhea was greatest in the highest dose group (5000 mg/kg), where it occurred daily in over 50% of rats during the first week of the study, and to a lesser degree during the second week.

Body weight

After the 7th and 14th days of administration of *Rosmarinus officinalis* ethanolic extract, body weight increased slightly in rats treated with 1000, 2500, 3500, and 5000 mg/Kg, but not significantly compared with the control group (Fig. 1). An elevation of body weight was observed in rats treated with 1000, 2500, 3500, and 5000 mg/Kg of *Rosmarinus officinalis* in a non-significant way compared to the control group (Table 4). the body weight increase even with diarrhea can be explained by the carnosic acid effect [14].

The table 5 show the effect of EERO on food and water consumption during acute toxicity. It was found that after the 7th and 14th day of administration of the ethanolic extract of *Rosmarinus Officinalis*, during acute toxicity, a slight increase in food and water consumption in rats treated with 1000mg/Kg, 2500mg/Kg, 3500mg/Kg and 5000mg/Kg *Rosmarinus officinalis* compared with the control group.

Subacute toxicity

Toxicity signs

No mortality was recorded, and no signs of toxicity were observed in the groups treated with doses of 200, 300, 500, and 1000 mg/kg compared with the control group, only diarrhea: the higher the dose, the more diarrhea appeared. The results show that oral administration of EERO for 28 days at different doses caused a non-significant increase in body weight in all treated rat groups compared to the control group, where the values were lower than those of the control group. Similarly, a slight reduction in weight gain was observed in the treated group, but this was not statistically significant compared to the control group (Fig. 1).

Figures 2 and 3 describe the effect of the ethanolic extract of *Rosmarinus officinalis* on food consumption (g) and water intake (ml) during the subacute toxicity. After daily oral administration of the ethanolic extract of *Rosmarinus officinalis* at doses of 200, 300, 500, and 1000 mg/kg for 28 days, a non-significant increase in food consumption and water intake was observed in all treated rat groups compared to the control group.

Relative mass of organs

Table 6 shows that daily *Rosmarinus officinalis* administration has no significant effect on organ weights (liver, right kidney, left kidney) in the groups treated with doses of 200, 300, 500 and 1000mg/kg compared with the control group ($p>0.05$), indicating that liver and kidneys did not experience any adverse effects during the entire course of treatment.

Hematological parameters

Table 7 describes the effect of daily oral administration of *Rosmarinus officinalis* extract for 28 days on hematological parameters. Assessment of hematological parameters is essential for determining an individual's state of health. The results show that there were no significant hemolytic changes in RBCs, WBCs, Hb, TGMH, CGMH, MCV(VGM), granulocytes, and leukocytes.

Table 8 describes the effect of EERO on biochemical parameters. creatinine, electrolytes, urea, and uric acid constitute an indicator of kidney damage [21]. No significant difference in the levels of creatinine, urea or uric acid, glucose, and

triglyceride in the four dose groups compared with the control group.

Nitric oxide assay

The Fig. 4 describes the effects of EERO on the nitric oxide (NO) parameter (umol/g tissue). The ethanolic extract of *Rosmarinus officinalis* affects NO levels in liver tissue, decreasing nitrite/nitrate (nitric oxide; NO) levels in the 200 mg/kg, 300 mg/kg, and 500 mg/kg rat groups compared with the control group ($p < 0.01$, $p < 0.001$ and $p < 0.05$ respectively) and non-significantly in the 1000 mg/kg group compared with the control group. In the kidneys. (Fig. 5), It is observed that the EERO affects the levels of NO in the left kidney tissue. It decreases the levels of nitrite/nitrate (NO) in the groups of rats treated with doses of 200mg/kg, 300mg/kg, and 500mg/kg compared to the control group, while there is a non-statistically significant increase in the group of rats treated with a dose of 1000mg/kg compared to the control group.

Catalase activity

Fig. 6 presents the effect of Rosmarinus officinalis ethanolic extract on the catalase parameter (CAT) (expressed in mmol/g tissue). The ethanolic extract of Rosmarinus officinalis was found to affect CAT levels in liver tissue. It increased catalase levels was the groups of rats treated with different doses of Rosmarinus officinalis ethanolic extract.

In the kidneys (Fig. 7), the ethanolic extract of Rosmarinus officinalis was found to have an effect on CAT left in right kidney tissue. It increases catalase levels in rat groups treated with different doses of the ethanolic extract of Rosmarinus officinalis, except for the 1000 mg/kg dose group, there was a decrease in catalase levels compared to the control group. A statistically very significant difference was observed in the group of rats treated with a dose of 200 mg/kg, likewise a significant difference was observed in the group of rats treated with a dose of 300 mg/kg and no statistically significant difference was noted in the group of rats treated with the 500 mg/kg body weight dose compared with the control group, whereas a non-significant decrease was observed in the group of rats treated with 1000 mg/kg ethanolic extract of Rosmarinus officinalis, compared with the control group.

Histopathological examination

Histological studies serve as a reference for detecting pathological changes linked to chemical toxicity (plant components) in tissues and organs, particularly the liver and kidneys, which are considered to be the most affected and sensitive to this substance. Histological examination of the liver and kidneys revealed no pathology in the cellular structure (Fig. 8).

Liver parenchyma showing normal cells with no obvious signs. Inflammation (red dot hepatic lobule, black dot centrilobular vein), 200mg/kg; Liver parenchyma showing normal cells with no obvious sign of inflammation (blue: hepatocyte trabeculae with binucleated hepatocyte red: hepatic sinusoid, black: centrolobular vein), 300mg/kg; Normal-appearing liver parenchyma showing no signs of inflammatory abnormality or signs of malignancy, 500mg/kg; Normal-appearing liver parenchyma showing no obvious signs of inflammatory abnormality or signs of malignancy, 1000mg/kg; Normal-appearing liver parenchyma (blue: portal space and red centrilobular vein).

In control rat kidneys, normal renal parenchyma with no obvious signs of abnormality (glomeruli green, microtubule blue, bowman's capsule red),200mg/kg; normal renal parenchyma showing no signs of inflammatory or malignant abnormality (glomeruli green, microtubule blue, bowman's capsule red), the same at doses of 300mg/kg, 500mg/kg and 1000 mg/kg normal renal parenchyma showing no signs of inflammatory or malignant abnormality.

Discussion

A study of the acute and subacute toxicity of *Rosmarinus officinalis* ethanolic extract was carried out in accordance with OECD guideline 407. The respective doses (1000, 2500, 3500 and 5000 mg/kg) of ethanolic extract of plant leaves were administered once only by oral gavage during 14 days of acute toxicity and 28 days of subacute toxicity.

According to the OECD, the LD50 of this extract is greater than 5000 mg/kg, and it is therefore classified in category 5 of the Harmonized System of Classification (GHS) as non-toxic by the oral route. These results concur with those of Kabubii in 2015 showing that administration of *Rosmarinus officinalis* aqueous extract at single doses of 1000 mg/kg and 5000 mg/kg to rats over the 14 days, resulted in no mortality, and no signs of toxicity were recorded; the LD50 is greater than 5000 mg/kg [22]. Similarly, another study showed that the LD50 is greater than 8500 mg/kg of ethanolic extract administered to rats [22].

According to the Hodge and Sterner toxicity classification scale in laboratory rats (Hodge & Sterner, 1949), [23], an oral LD50 ≥ 5000 mg/kg (5000< DL50 <15000mg/ kg) means that the substance is almost non-toxic, confirming that the extract tested in this study is almost non-toxic. The administration of different types of *Rosmarinus officinalis* extracts at doses of 2000 mg/kg for 14 days to rats, were well tolerated, no signs of toxicity or mortality were observed, and the LD50 of *Rosmarinus officinalis* was greater than 2000 mg/kg [24,25]. Rosmarinus officinalis was marginally safe

according to OECD guidelines with an LD50 > 5,000 mg/kg [24].

The 1000, 2500, and 3500 mg/kg rat groups were less affected by diarrhea, with prevalence generally not exceeding 50%. According to the 2012 carnosic acid toxicity study, on the toxicity of carnosic acid, where they demonstrated that administration of this acid caused diarrhea in rats treated with different doses [26]. *Rosmarinus officinalis* is characterized by a high carnosic acid content [27]. This suggests that this acid is the cause of the diarrhea observed in this study.

The subacute toxicity study of ethanolic extract of *Rosmarinus officinalis* (EERO) was carried out following OECD guideline 407 (OCDE, 2008). The respective doses (200, 300, 500, and 1000 mg/kg) of ethanolic extract of *Rosmarinus officinalis* leaves were administered for 28 successive days.

The daily administration of EERO at different doses during 28 days induces no statistically significant reduction of body weight gain in all treated female Wistar rats in comparison to normal rats (Fig. 2). Previous studies have shown that oral administration of *Corrigiola telephiifolia Pourr*. and *Herniaria glabra* extracts have no effect on animal body weight in a dose lower than 2000 mg/kg [28,29]. Weight change is used as a general indicator of the adverse effects of chemical compounds [30]. Kabubii in 2015 found that administration of *Rosmarinus officinalis* extracts for 28 days at doses of 500mg/kg, 1500mg/kg, and 3000mg/kg to the rat groups did not cause a significant difference in the body weight of rats compared to the control group. *Rosmarinus Officinalis* is characterized by a high carnosic acid content [1].

After daily oral administration of the ethanolic extract of Rosmarinus officinalis at doses of 200, 300, 500, and 1000 mg/kg for 28 days, a non-significant increase in food consumption and water intake was observed in all treated rat groups compared to the control group. Similarly, this increase was lower than that of the control group. These results are consistent with the study by Salokhe et al. who found that daily administration of *Rosmarinus officinalis* hydroalcoholic extract at doses of 300, 500, and 1000 mg/kg to Wistar rat groups for 28 days did not result in significant changes in food and water consumption compared to the control group [20].

The mean values of the increase in body weight in all groups of treated rats were lower than in the control group. However, compared with control values, terminal body weights, and body weight gains were not statistically reduced throughout the 14-day study period. Even diarrhea was recorded in all treated rats. The body weight increase can be explained be the positive effect of rosemary extract, which contains carnosic acid., the molecule responsible for diarrhea. These results are in line with previous studies, which demonstrated that administration of *Rosmarinus officinalis* aqueous extract at a single dose of 1000 and 5000 mg/kg to rats resulted in no significant difference in body weight in treated rat groups compared to the control group over the 14-day experimental period [9,20,24]. Similarly, other studies have shown that the administration of different types of *Rosmarinus officinalis* extracts to rats at doses of 2000 mg/kg *Rosmarinus officinalis* extract provoked no significant change in the body weight of the treated rat groups compared to the control group [31,32]. This increase is less than that of the control group. Note that this difference is not statistically significant. This result concurs with that of Anadón et al, who demonstrated that the administration of two Rosemary extracts (A and B), each at a single oral dose of 2000 mg/kg body weight during acute toxicity, recorded no significant difference in food consumption and water intake compared with the control group [33].

After the 7th and 14th day of administration of the ethanolic extract of Rosmarinus Officinalis, during acute toxicity, a slight increase in food and water consumption in rats treated with 1000mg/Kg, 2500mg/Kg, 3500mg/Kg and 5000mg/Kg Rosmarinus officinalis compared with the control group. This increase is less than that of the control group. Note that this difference is not statistically significant. This result concurs with that of Anadón et al, who demonstrated that the administration of two rosemary extracts (A and B), each at a single oral dose of 2000 mg/kg body weight during acute toxicity, recorded no significant difference in food consumption and water intake compared with the control group [33].

After the macroscopic analysis of the organs, where we found no change in the colour and volume of these organs (liver, right kidney, left kidney), the weight of the organs is one of the parameters that helped us determine the degree of toxicity of the extract studied.

The kidney and liver are the two organs involved in the detoxification of toxic substances and are generally the most likely to be affected by xenobiotics [34]. The kidneys and livers of treated female Wistar rats showed neither hypotrophy nor hypertrophy compared to the control group, further confirming that the doses administered of the extract are non-toxic.

Increased WBC release is a notable biomarker of stress and also helps defend the body against certain inflammatory conditions, such as bacterial infections, leukaemia, and haemorrhage. The results of this study revealed that Rosmarinus officinalis extract did not induce significant changes in the number of WBCs or their subtypes, including neutrophils,

lymphocytes, monocytes, and eosinophils, at any dose, compared with the control group. This suggests that EERO is not toxic. Our results revealed that there was no significant difference in the levels of creatinine, urea or uric acid, glucose, and triglyceride in the four dose groups compared with the control group. Any alteration in AST and ALT parameters is a sign of liver toxicity [20], but the results of the present study showed no alteration in these parameters. It can therefore be concluded that Rosmarinus officinalis ethanolic extract produced no signs of liver toxicity when administered orally for 28 days.

There was a very significant difference between the group of rats treated with a dose of 200 mg/kg and a highly significant difference in the group of rats treated with a dose of 300 mg/kg compared to the control group. Similarly, a significant difference was observed in the group treated with a dose of 500 mg/kg compared to the control group, whereas no difference was observed between the group of rats treated with a dose of 1000mg/kg of the ethanolic extract of Rosmarinus officinalis and the control group.

It is observed that the ethanolic extract of Rosmarinus officinalis has an effect on the levels of NO (nitric oxide) in the right kidney tissue. It decreases the nitrite/nitrate levels in all groups of rats treated with doses of 200, 300, 500 and 1000 mg/kg compared to control group. A statistically highly significant difference was observed between the rats treated with 200 mg/kg and control rats ($p<0.001$), very significant difference between rats treated with 300 mg/kg and controls($p<0.01$) and no difference was observed between the rats treated with 500 and 1000 mg/kg of the ethanolic extract of Rosmarinus officinalis and control rats ($p>0.05$).

Reactive oxygen species (ROS) result from oxidative metabolism in mitochondria; they present a broad class of molecules to give rise to reactive free radicals as hydrogen peroxide, hydroxyl radicals, and superoxide. Free radicals participate in oxidation reactions causing organic substrate damage, such as lipids, proteins, and DNA in organisms, resulting in a harmful biological state known as oxidative stress [35]. The oxidative stress biological damages are harmful at the cellular level. The plants contain some antioxidants which neutralize the negative effects of oxidative stress [11]. The main antioxidant components of *Rosmarinus officinalis* (rosemary) leaf extract are the phenolic diterpenes carnosol and carnosic acid [9]. One study showed that administration of Rosmarinus officinalis extract by oral gavage at a dose of 400 mg/kg and rosmarinic acid at a dose of 10 mg/kg intraperitoneally daily for 28 weeks resulted in an increase in oxidative stress biomarkers and a decrease in inflammatory biomarker levels [36]. Carnosol and carnosic acid, the main diterpenoid phenolic compounds of rosemary, inhibit the production of nitric oxide (Yu et al., 2013). This result agrees with our study, which shows that ethanolic extract administered by oral gavage for 28 days at doses of 200, 300, and 500 mg/kg causes a statistically significant decrease in nitric oxide (NO) levels in the liver respectively ($p< 0.01$; $p < 0.01$ $p< 0.05$), while the dose of 1000mg/kg body weight is not statistically significant ($p< 0.05$) compared with the control group. Similarly, doses of 200mg/kg and 300mg/kg caused a statically significant decrease in nitric oxide (NO) levels in the right and left kidneys respectively ($p< 0.01$; $p < 0.01$), but doses of 500mg/kg and 1000mg/kg body weight were not statistically significant ($p < 0.05$) compared with the control group.

A statistically very significant difference was observed in the group of rats treated with a dose of 200 mg/kg, likewise, a highly significant difference was observed in the group of rats treated with a dose of 300 mg/kg, and a statistically significant difference was noted in the group of rats treated with the 500 mg/kg body weight dose compared with the control group. No difference was observed in the rat group treated with a 1000 mg/kg ethanolic extract of Rosmarinus officinalis compared with the control group.

The ethanolic extract of Rosmarinus officinalis affects CAT level in the right kidney tissue. It increases catalase levels in rat groups treated with different doses of EERO. A statistically very significant difference was observed in the group of rats treated with a dose of 200 mg/kg, likewise, a significant difference was observed in the group of rats treated with a dose of 300 mg/kg, and no statistically significant difference was noted in the group of rats treated with the 500 mg/kg and 1000mg/kg body weight dose compared with the control group.

Oxidative stress is characterized not only by increased production of free radicals but also by a reduction in antioxidants [19]. Plants contain a wide variety of antioxidants that are classified as natural antioxidants and neutralize the damaging effects of oxidative stress [37]. Several studies have shown that rosemary is rich in various phenolic compounds and flavonoids [38–40]. Afonso et al indicate that rosemary phenolics improve antioxidant defense in different tissues and attenuate oxidative stress in rats [41,42]. A study demonstrated that administration of a 10 mg/kg dose of the essential oil of Rosmarinus officinalis in Wistar rats for 7 consecutive days induced a significant increase in the level of catalase in the liver compared to the control group and also significantly altered most biomarkers of oxidative stress and improved the oxidative status of the liver, hence reduced oxidative stress in rats [43,44].

This result is in accord with our study, which demonstrated that ethanolic extract administered by

oral gavage for 28 days at doses of 200mg/kg, 300mg/kg, and 500mg/kg caused a statistically significant increase in catalase (CAT) levels in the liver respectively ($p< 0.01$; $p < 0.001$ $p< 0.05$), while the 1000mg/kg body weight dose was not statistically significant ($p< 0.05$) compared with the control group. Similarly, doses of 200mg/kg and 300mg/kg caused a statically significant increase in catalase (CAT) levels in the right and left kidneys respectively ($p< 0.01$; $p < 0.05$), but doses of 500mg/kg and 1000mg/kg body weight were not statically significant ($p< 0.05$) compared with the control group. Wang et al. (2017) reported that intake of rosemary extract increases SOD and CAT activities and decreases malondialdehyde levels significantly [46]. A study carried out on other animals, notably chickens, demonstrated that administration of the ethanolic extract of Rosmarinus officinalis at doses of 100mg/kg and 200 mg/kg for 42 days increased the level of the enzyme catalase ($P < 0.001$) and significantly reduced oxidative stress parameters ($P < 0.01$) compared with the control group [45,46].

Histological analysis of the liver and kidneys revealed (showed) no abnormalities (pathology) in the cellular architecture (structure) of these organs [47,48]. The histopathological findings are also corroborated (confirmed) by the results of the haematological examination and the biochemical estimation of biomarkers of liver and kidney damage, which were normal in all groups treated with different doses of the extract, Similarly, corroborated with the results of the oxidative braiding biomarker assessment which showed that the extract decreases nitric oxide levels and increases catalase levels in the liver and kidneys, thus exerting antioxidant effects and improving the oxidative status of these organs [49-51].

The histopathological results were not significant compared with the control group. Based on the histopathological results, we can conclude that the plant extract produced no pathologically significant abnormalities.

The results show that ethanolic extract of Rosmarinus officinalis is safe at dose of 1000mg/kg during 28 days oral administration.

Conclusion

Oral administration of ethanolic extract of RO at different doses showed no significant differences ($p > 0.05$) in body weight, relative organ weights, food and water consumption. Similarly, no signs of mortality were recorded, indicating that the LD50 is greater than 5000 mg/kg. In the subacute toxicity study, no significant differences ($p > 0.05$) were found in body weight, food, and water consumption, similarly, none of the haematological, or biochemical parameters showed any significant difference and histological examination showed no macroscopic abnormalities compared with the control, suggesting that the extract of Rosmarinus officinalis administered is not toxic. Similarly, in the sub-acute study, the results of biomarkers of oxidative stress showed that the plant extract exerts antioxidant activity by decreasing the levels of nitric oxide and increasing the levels of the catalase enzyme in the liver and kidneys.

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Funding statement

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Faculty of Sciences, Ibn Tofail University, Kenitra, Morocco (ethics approval number; 05/2022).

TABLE 1. Mortality after a single dose of *Rosmarinus officinalis* ethanolic extract.

Groups	Dose (g/Kg)	Mortality
Control	0 mg/kg	0/6
Group1	1000mg/kg ethanolic extract of *R. officinalis*	0/6
Group 2	2500mg/kg ethanolic extract of *R.* officinalis	0/6
Group 3	3500mg/kg ethanolic extract of *R. officinalis*	0/6
Group 4	5000mg/kg ethanolic extract of *R. officinalis*	0/6

TABLE 2. Assessment of clinical signs in rat groups treated with different doses of ethanolic extract of Rosmarinus officinalis during acute toxicity study.

	Control		1000mg/kg		2500mg/kg		3500mg/kg		5000mg/kg	
Clinical signs	**First 4H**	**Daily**	**First 4H**	**Daily**	**First 4H**	**Daily**	**First 4H**	**Daily**	**First 4H**	**Daily**
Trembling	-	-	-	-	-	-	-	-	-	-
Convulsion	-	-	-	-	-	-	-	-	-	-
Salivation	-	-	-	-	-	-	-	-	-	-
Diarrhea	-	-	+	+	+	+	+	+	+	+
Coma	-	-	-	-	-	-	-	-	-	-
Aggressive behavior	-	-	-	-	-	-	-	-	-	-
Respiratory problems	N	N	N	N	N	N	N	N	N	N
Changes in hair, eyes, mucus	-	-	-	-	-	-	-	-	-	-

+ : Presence of sign, - : No sign, N: Normal.

TABLE 3. Diarrhea acute effect of *Rosmarinus officinalis* extract in rats of different groups.

Groups	Control	1000 mg/kg	2500 mg/kg	3500 mg/kg	5000 mg/kg
1st 4 Hours	-	1/6	2/6	2/6	3/6
1st day	-	1/6	2/6	2/6	3/6
2nd day	-	1/6	2/6	2/6	3/6
3rd day	-	2/6	3/6	3/6	4/6
4th day	-	2/6	1/6	2/6	4/6
5th day	-	3/6	2/6	2/6	5/6
6th day	-	1/6	3/6	3/6	4/6
7th day	-	2/6	2/6	3/6	3/6
8th day	-	2/6	2/6	2/6	2/6
9th day	-	1/6	1/6	2/6	3/6
10th day	-	2/6	2/6	2/6	2/6
11th day	-	1/6	1/6	1/6	2/6
12th day	-	1/6	2/6	3/6	3/6
13th day	-	1/6	1/6	2/6	2/6
14th day	-	-	1/6	2/6	2/6

- : No diarrhea, …/6 number of rats with diarrhea

TABLE 4. Effect of *Rosmarinus officinalis* (RO) ethanolic extract on rat body weight (g).

Groups	Body weight (mean ± SEM)		
	Day 1	**Day 7**	**Day 14**
Control	176.33±1.56	180.17±1.54	184.00±1.46
Group 1: 1000mg/kg ethanolic extract of *RO*	175.50±1.38	179.00±1.26	181.83±1.14
Group 2: 2500mg/kg ethanolic extract of *RO*	174.17±0.75	178.50±0.92	181.33±0.92
Group 3: 3500mg/kg ethanolic extract of *RO*	173.50±0.76	176.83±0.79	180.00±0.82
Group 4: 5000mg/kg ethanolic extract of *RO*	172.33± 0.95	176.00±0.86	179.83±0.75

TABLE 5. Effect of ethanolic extract of *Rosmarinus officinalis* (EERO) on food consumption and water intake during acute treatment over 14 days.

Groups	Day 1		Day 7		Day 14	
	Food	**water**	**Food**	**Water**	**Food intake**	**Water intake**
Control	11.16±0.10	17.93±0.16	12.31±0.10	19.18±0.16	13.34±0.11	20.96±0.17
Group 1: 1000mg/kg ethanolic extract of *RO*	11.06±0.09	17.83±0.14	12.21±0.09	19.03±0.13	13.18±.0.08	20.69±0.13
Group 2: 2500mg/kg ethanolic extract of *RO*	10.96±0.05	17.66±0.08	12.17±0.06	18.95±0.10	13.14±0.07	20.63±0.10
Group 3: 3500mg/kg ethanolic extract of *RO*	10.96±0.05	17.59±0.08	12.06±0.05	18.77±0.08	13.04±0.06	20.48±0.09
Group 4: 5000mg/kg ethanolic extract of *RO*	10.90±0.06	17.47±0.10	12.00±0.06	18.71±0.08	13.03±0.06	20.49±0.07

TABLE 6. Impact of *Rosmarinus officinalis* extract on relative weight of liver and kidney in each group.

Groups	Control	200mg/kg	300mg/kg	500 mg/kg	1000 mg/kg
Liver	4.97±0.70	5.37±0.28	4.05±0.29	4.56±0.80	5.92±0.49
Kidney	0.44±0.04	0.47±0.05	0.49±0.03	0.44±0.05	0.56±0.02

TABLE 7. Effect of ethanolic extract of Rosmarinus officinalis on hematological parameters.

Groups	Control	200mg/kg	300mg/kg	500 mg/kg	1000 mg/kg
RBCs (M/mm3)	6.14±0.93	7.04±0.09	7.89±0.59	7.24±0.45	7.48±0.70
Hb (g/dl)	12.00±1.30	13.25±0.05	14.55±1.35	13.30±1.00	13.20±1.40
Hematocrit (%)	35.30±4.30	39.90±0.20	45.25±3.25	40.70±2.80	40.55±4.45
VGM (u3)	58.00±2.00	56.50±0.50	57.50±0.50	56.50±0.50	54.00±1.00
TGMH (pg)	20.00±1.00	19.00±0.00	18.50±0.50	18.50±0.50	17.50±0.50
CGMH (%)	34.50±0.50	33.50±0.50	32.00±1.00	32.50±0.50	32.50±0.50
WBC (Milles/mm3)	4800±1500	6400±2400	3050±1050	3339±3261	2550±1450
Lymphocytes (L%)	84.50±4.50	83.50±1.50	82.00±3.00	84.00±6.00	83.50±3.50
Monocytes (M%)	4.00±0.00	4.50±0.50	4.50±0.50	5.00±0.00	4.00±1.00
PLT (Milles/mm3)	746±35	799±16	822±162	844±75	1009±54

TABLE 8. Effect of Rosmarinus officinalis extract on hematological parameters.

Groups	Control	200mg/kg	300mg/kg	500 mg/kg	1000 mg/kg
Cholesterol (mmol/L)	5.40±0.90	4.79±0.34	5.25±0.47	5.35±0.69	5.93±0.49
Creatinine (mg/dl)	42.13±1.33	41.45±1.67	40.33±2.11	43.24±1.75	45.56±1.16
Urea (mmol/L)	2.72±0.56	2.33±0.63	0.77±0.51	1.86±0.70	1.36±0.47
Glucose (mmol/L)	5.26±0.34	5.79±0.71	5.70±0.50	5.31±0.74	5.58±0.69
Triglyceride (mg/dl)	2.46±0.28	3.72±0.67	3.34±0.66	3.16±0.38	3.12±0.51
ALT (U/L)	22.70±2.67	15.70±6.15	11.06±4.07	15.71±6.13	7.57±3.24
AST (U/L)	12.22±4.84	28.89±8.68	11.11±1.11	18.89±2.94	14.44±4.44

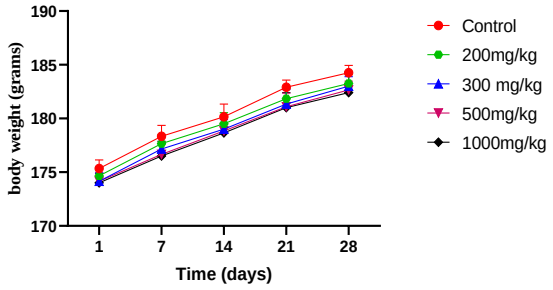

Fig. 1. Effect of *Rosmarinus officinalis* ethanolic extract on body weight (g) during subacute toxicity. Results are expressed as mean ± SEM. (n=6). *p<0.05.

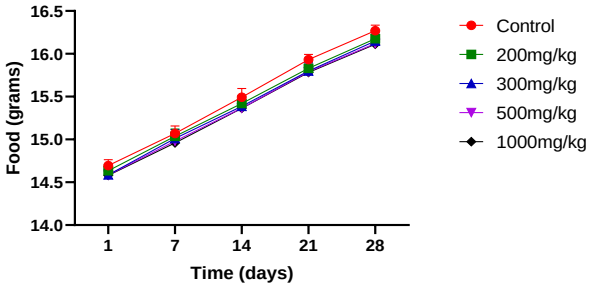

Fig. 2. Effect of *Rosmarinus officinalis* ethanolic extract on food consumption (g) during subacute toxicity.

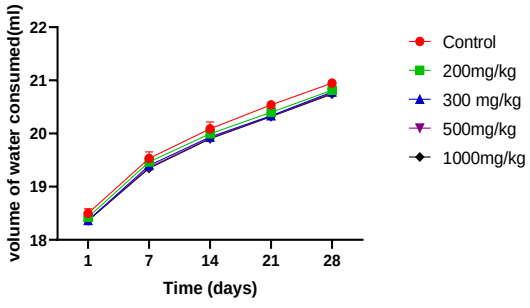

Fig. 3. Effect of *Rosmarinus officinalis* on water consumption (ml) during subacute toxicity.

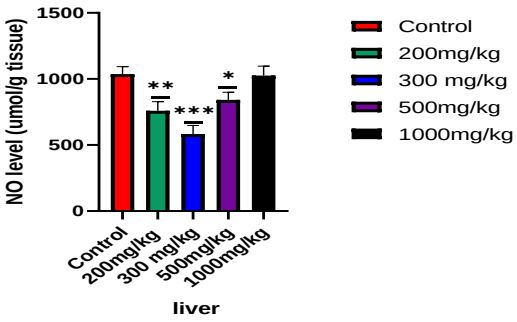

Fig. 4. The effect of *Rosmarinus officinalis* ethanolic extract on the nitric oxide (NO) parameter (umol/g tissue) in the liver.

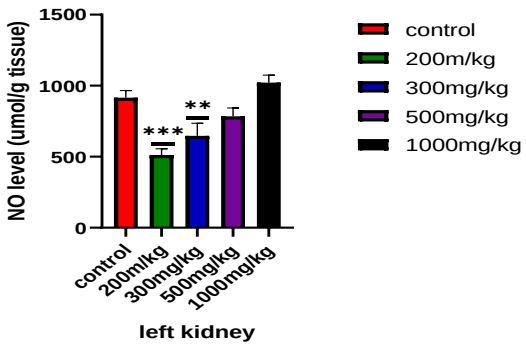

Fig. 5. Effect of *Rosmarinus officinalis* ethanolic extract on nitric oxide (NO) parameter (umol/g tissue) in the kidney.

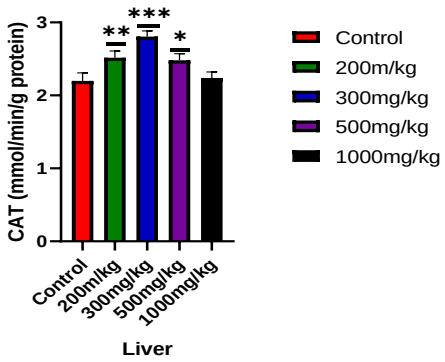

Fig. 6. Effect of *Rosmarinus officinalis* ethanolic extract on catalase (CAT) activity (expressed in mmol/g tissue) in the liver of female rats after 28 days of treatment.

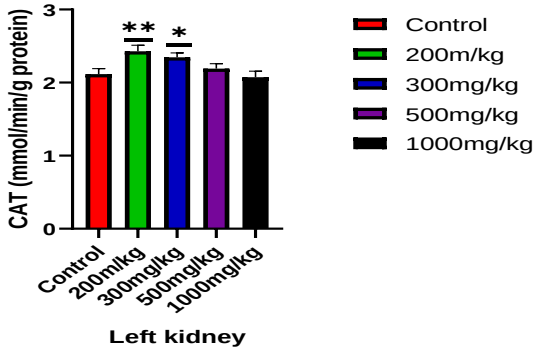

Fig. 7. Effect of *Rosmarinus officinalis* ethanolic extract on catalase (CAT) activity (expressed in mmol/g tissue) in the left kidney of female rats after 28 days of treatment.

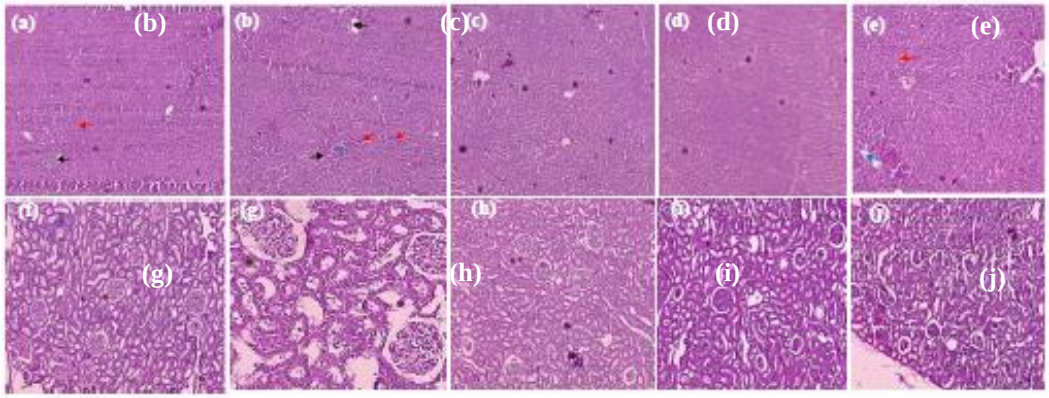

Fig. 8. Histological examinations included liver (a: control, b: 200mg/Kg, c: 300mg, d: 500mg, e: 1000mg) and kidney (f: control, g: 200mg, h: 300mg, i: 500mg, j: 1000mg) sections (10 X, H & E).

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تقييم السمية الحادة وشبه الحادة للمستخلص الإيثانولي لنبات إكليل الجبل عند إناث جرذان ويستار

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الملخص

إكليل الجبل (*Rosmarinus officinalis*) ، وهو نبات عطري غني بمركبات الفينول المضادة للأكسدة، يستخدم على نطاق واسع في العلاج التقليدي من قبل السكان المغاربة. تهدف الدراسة إلى تقييم السمية الفموية الحادة وشبه الحادة للمستخلص الإيثانولي لنبات إكليل الجبل (EERO) في جرذان ويستار. وفي دراسة السمية الحادة، تم تقسيم 30 جرذ إلى 5 مجموعات (مجموعة التحكم، وجرعة 1000، و2500، و3500، و5000 ملغم/كغم). وبالمثل، في دراسة السمية شبه الحادة، تم تقسيم 30 جرذ إلى 5 مجموعات (مجموعة التحكم، وجرعة 200، و300، و500، و1000 ملغم/كغم). هدفت دراسة السمية شبه الحادة إلى تحديد جرعةNOAEL ، من خلال متابعة نفس علامات السمية، والمعايير الدموية، والمعايير الكيميائية الحيوية، وعلامات الإجهاد التأكسدي، والتحليلات النسيجية التي تم تقييمها. وأظهرت النتائج عدم ملاحظة أي فروق كبيرة في معايير السمية الحادة وشبه الحادة (وزن الجسم، الغذاء، الماء)، كما لم يتم ملاحظة أي وفيات بسبب السمية الحادة، مما يشير إلى أن الجرعة المميتة LD50 أكبر من 5000 ملغم / كغم. أظهرت دراسة السمية شبه الحادة عدم وجود فروق كبيرة في المعايير الدموية والكيميائية الحيوية والنسيجية، في حين أدى المستخلص إلى زيادة مستويات CAT وخفض NO في الكبد والكلى. مستخلص RO غير سام حتى الجرعات التي تم اختبارها ويمارس نشاطًا مضادًا للأكسدة، يتجلى في انخفاض مستويات أكسيد النيتريك وزيادة مستويات إنزيم الكاتالاز المضاد للأكسدة في الكبد والكلى.

الكلمات الدالة: إكليل الجبل *Rosmarinus officinalis* ، التحليل الدموي، السمية، الكاتالاز، أكسيد النيتريك، الجرذان.