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Research Article

In Vivo Toxicological and Microbiological Activity of *Marrubium vulgare* L. on *Candida albicans* Isolated from Nosocomial Infections

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Abstract

Background: The objective of this study was to test the toxicology and microbiological of methanolic extract of *Marrubium vulgare "MEMV"* in rat and its efficiency in the treatment of systemic candidiasis.

Methods: Forty (40) male rats (Wistar type) were divided into four groups (10 rats /group) where the toxicological, microbiological and histological studies were applied. Later, the infection with 10^7 cells /ml of *Candida albicans* and the curative treatment were applied by the daily administration with a gavage of 800 mg/kg of *MEMV*.

Results: The toxicology studies indicated that the dose of *MEMV* at 800 mg/kg of body weight was not harmful. The microbiological and histological analysis showed that the treatment with *MEMV* of rat colonized with 10^7 cells/ml of *Candida albicans* limits the multiplication of the yeast in the intestine and colon of the colonized rat. The translocation of *Candida albicans* in liver, spleen and lungs in rat which was treated with 800 mg/kg of *MEMV* was significantly lower than in controls to seven post-infection days. However, we did not detect yeast in the kidneys and hearts of the infected treated groups and the infected untreated ones.

Conclusion: Our results suggest that *MEMV* limits the gastrointestinal colonization and dissemination of *Candida albicans* in the other organs.

Introduction

The knowledge of herbal remedies in traditional cultures has widely contributed to the development of the use of herbal medicines, if we take into account their widespread use and since we have 80% of the world population dependent on these medicines for their health care.[1,2] Considering these aspects, natural products, particularly higher plant species, continue to be important sources of medicine and supplementary health products. This represents a challenge to science because of various aspects, including their chemical diversity, synergism to biological activity and variable composition.[1,3] Both the complexity and the chemical diversity with the biological properties of natural products have played an important role in the discovery and development of new compounds. The history of natural products used since ancient times by humans for the relief and cure of diseases has enriched scientific research on new active entities or phytotherapeutic products.[1] All the drugs available for the treatment of fungal infections are not completely effective. That is because they possess a certain degree of toxicity. In addition, mushrooms grow a quick resistance due to their large-scale use. Although doctors have turned to therapies of combination, the miracle drug to prevent fungal infections and to avoid the resistance has not yet been published.[4,5] Among the different possible sources of antifungal compounds, plants retain a great interest, because they offer limitless possibilities for the insulation of new antifungal compounds due to their uneven availability of chemical diversity.[6-8] *Marrubium vulgare L.* is a perennial herb of the Lamiaceae family which is naturalized in North and South America, the latter and Western Asia. It possesses tonic, aromatic, stimulant, expectorant, diaphoretic and diuretic properties. *Marrubium vulgare which is* known as horehound in Europe, is frequently used in folk medicine[9-12] to cure a variety of diseases.[13] In this context, Extensive pharmacological studies have demonstrated that *Marrubium vulgare* displays a suite of activities inclusing antinociceptive,[14] antioxidant,[12,15] cardioprotective,[16] vasorelaxant,[17] gastroprotective,[18] antispasmodic,[19] immunomodulating,[20] antioedematogenic,[21] analgesic,[22]

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antidiabetic[23] and antigenotoxic properties.[24] Other medicinal effects are reported for *Marrubium vulgare* such as insecticidal and antinociceptive action in several models of pain in mice.[21] Earlier phytochemical investigations of *M. vulgare* led to the characterization of several flavonoid glycosides and lactates and several labdane diterpenoids with marrubiin as main component and a small amount of an essential oil.[25] Recently, a large number of their constituents have been investigated for their biological activity, notably antibacterial and antifungal properties.[26-30] The demands for more natural antimicrobials have driven scientists to investigate the effectiveness of inhibitory compounds such as extracts from plants that have promising biological activity to control various fungal diseases.[31,32] This study aims at assessing the toxicological effect of the methanolic extract of *Marrubium vulgare (MEMV)* on the male rats and at evaluating the antifungal activity of the *MEMV* on male rats rendered experimentally candidiasis.

Materials and Methods

Plant material and preparation of the MEMV

The medicinal plant in this study is *"Marrubium vulgare L."*. The spice is collected between the months of Dec and Jan 2015. The crop is found in Khalouia (35°27'34.76" north, 0°17'37.57" east) in the East of Mascara-Algeria. The voucher specimen was deposited at the herbarium-ENSA and identified by Pr. Benhouhou Salima - Professor at the laboratory of Botany, Ecole Nationale Superieure d'Agronomie, Algiers- Algeria. The aerial part (leaves, flowers and stems) of the plant is harvested. Next, it is dried in the shelter of the light and then chopped into powder for later use.

For the extraction of polyphenoles, methanolic solutions are employed according to the method described by Boutlelis.[33] 50 g of dried plant rendered powder placed in a glass container, covered with 1000ml of MeOH aqueous 70% (7:3); the mixture is heated to 70°C over 5mn and then the sample is left macerated during one night (24 hours). After a first filtration on filter paper, the filtrate is evaporated under vacuum to dryness using a Rota steam at the temperature of 50°C. At the end, *MEMV* is sterilized by filtration and stored at 4 ± 1°C.

Identification of polyphenols by HPLC-DAD-ESI-MS

The identification of phenolic compounds was carried out on a Shimadzu system(Prominence i. LC-2030C 3D) equipped with a surveyor UV-Vis photodiode array (PDA) detector, and a LCQ Advantage max ion trap mass spectrometer (all from Thermo Fisher Scientific, Waltham, MA, USA), coupled through an electrospray ionization (ESI) source. The separation was carried out with Ascentis Express C18 column (15 cm X 4.6 mm) id packed with 2.7 µm partially porous particles (Supelco, Bellefonte, PA, USA). The binary mobile phase consisted of water/acetic acid (solvent A) and methanol/acetic acid (solvent B), in a linear gradient mode: 0-5 min: 2% B, 5-120 min: 2-100% B. The mobile phase flow rate was 0.7 ml/min and the chromatogram was recorded at λ = 280 nm. Spectral data were collected in the range of 200–800 nm for all peaks. HPLC-ESI-MS/MS data were acquired under positive and negative ionization modes, using the Xcalibur software.[34]

Candida albicans strain

Identification was carried out according to Api *Candida* system (bioMerieux, Marcy l'Etoile, France).[35] The strain of *C. albicans* used in this work are isolated in the Hospital "Mascara-Algeria" from the vaginal secretions of a woman with a vulvo-vaginitis infection. The identification is based on the: Test of Blastese or Filamentation on Serum. The *Candida albicans* isolates produced germ tubes when incubated in sterile tubes that contain a fungal suspension of 0.5 ml and 0.5 ml of a serum. The mixture is incubated 3-4 h at 37°C.[35,36]

From a solid medium SDA + chloramphenicol (Sabouraud Dextrose Agar), a colony is removed with an ensemensor and placed in an erlenmeyer containing 50 ml of medium YEPD liquid. The latter is placed in a water bath shaker at 30°C for 18 hours**.** The chloramophenicol (0.05 %) is added in the middle YEPD liquid to avoid contamination by bacteria.[37] A volume of 3ml of pre-culture is sedimented media by centrifugation at 2000 x g for 10 min at 4°C. The gall is resumed in an equal volume of phosphate buffer (PBS)**.** The cells are thus washed 3 times and resuspended particulates in PBS and then adjusted to optical density = 0.4 corresponds to a concentration of 10^8 cells / ml; a dilution has been made (1:10) to arrive at a concentration of 10^7 cells / ml.[37]

Animal protocol

Our study has been carried out on forty male rats "*Wistar*" whose weight varies from 85 to 120g, distributed into : group A (n=10), group B (n=10), group C (n=10) and group D (n=10). They all present a generation descendant of a race which is not passed by any medical treatment. All experiments were approved by the Animal Ethics Committee of the Mascara University in which the procedures used during these studies were in accordance with the European directive concerning Animal Testing (2010/63/EU).[38] These animals are reared within the pet center of the experimental station of the Faculty of Natural Sciences and Life; Mascara University-Algeria. The rats are housed in cages with free access to food and water. They are put under an ambient temperature with a natural cycle of light and of darkness.[39,40]

Sub-acute toxicity study

Two groups were selected for the toxicological study. Each group is composed of ten male rats (n=10).

Group A "placebo": receiving a cramming orally, 1 ml of a solution of distilled water.[41]

Group B "test": taking a cramming orally, a solution of *MEMV* diluted in 1 ml of distilled water at the rate of 800mg/kg/day for 28 days.[42] This group represents the phantom in this protocol, from what it deems the acute toxicity of the

extract administered. After the daily administration of the *MEMV* orally at a dose of 800 mg/Kg, rats were observed for 28 days to evalute mortality and the morbidity.

In order to test the antifungal activity of the *MEMV*, It was interesting to assess the toxicological effect of the *MEMV* on our animals. Our study is, on one hand, based on the work of Elberry et al,[42] which has demonstrated that a dose of 500 mg /kg/j does not present mortality on diabetic rats treated by the *MEMV*. On the other hand, the work done in our laboratory by testing the toxicity of the *MEMV* on rats wistar with increasing doses of the *MEMV* up to 500 mg/kg has given identical results to those of Elberry et al.[42]

Group B is represented by ten (10) male rats. They receive a dose of 800 mg/kg of the extract. The animals fasted for 24 hours and the various solutions are administered 1 ml taken orally. The administration is carried out by a cramming using a rigid probe for 28 days during which are noted the variations of body weight, the signs of toxicity potential (behavioral changes in and outside cage. The fall of hair, rashes, lachrymose, digestive manifestations and other, as well as the mortality rate are noted.

Histopathological studies

The rats were weighed and then sacrificed using chloroform. The organs were weighed and then fixed with neutral buffered formalin (10%). The fixed organs were dehydrated, embedded in paraffin, sectioned to a thickness of 5 µm. Microscopic observation was performed after staining with hematoxylin and eosin. Light microscopic examination by an anatomopathologist of several tissue sections of each organ of both groups was performed and images representing the typical histological profile were examined.[43,44]

Anti Candida albicans treatment

Three groups of male rats have been constructed to assess the antifungal activity of the *MEMV.*

Group A "placebo": receiving a cramming orally, 1 ml of a solution of distilled water.[41]

Group C "test + infected": receiving 200 µl of an inoculum of 10^7 cells / ml of *Candida albicans* by cramming and then treated with a dose of 800 mg/kg/day by oral route for seven (7) days. The treatment begins 72 hours after the inoculation of the strain of *Candida albicans* and continues seven (7) days.[45]

Group D "infected non-treaty": receiving 200 µl of an inoculum of 10^7 cells/ml on day 0 and remaining under the followed within ten (10) post inoculations.

During the period of experimentation, all rats (witness + test) are daily weighed. After eight (8) hours of fasting, the sacrifice is held by the inhalation of chloroform. The blood is taken by a cardiac puncture**.** An aliquot is recovered a sowing vacuum in the eppendorf tubes sterile for microbiological analysis.

Microbiological analysis

The quantification of the degree of infection and the determination of the effectiveness of the curative treatment has been carried out by a microbiological analysis by the enumeration of *Candida albicans* in the intestine, colon and internal organs. Samples from the intestines "5 cm" and colons "5 cm" have been sampled and cut into pieces, then hashed with a scalpel, diluted in sterile NaCl at 0.9 % and homogenized with ultra-turrax. The evaluation of the fungal load in the internal organs is made by the broyats of organs (liver, kidney, spleen, heart and lung) as well as in the blood after 10 days of post inoculation. The organs have been removed, cut into pieces, chopped with a scalpel, diluted in NaCl at 0.9 % and homogenized with ultra-turax. The enumeration of *C. albicans* per gram of tissue has been calculated by serial dilutions that have been prepared with the aim of finding the appropriate concentrations to count the number of colonies by using sabouraud dextrose agar (SDA), and incubated for 48 hours at 37°C. Then the number of CFU /g of organ is determined according to Cem et al.[46] The blood (100 µl) is transferred into EDTA tubes and then in boxes petri dishes containing SDA + chloramphenicol (0,05%). After incubation 24-48 h to + 37°C, the number of CFU/100 µl is determined.[37]

Statistical analysis

All experiments have been carried out in duplicate. The results are expressed in mean ± Standard deviation. The data were analyzed using R program 3.4.2. The multivariate analysis of variance (ANOVA) was used. The main groups (4), organs and blood (8), individuals (40 rats) and days were treated as fixed factors. $P < 0.05$ was used to define statistical significance. If a significant difference was determined among these averages, a Student unpaired test was used to determine significant difference between standard group and the one modified by *MEMV*. A principal component analysis (PCA) was used followed by multiple Comparisons of averages: Tukey Contrasts to assess the variation of CFU in the different organs in relation to the three groups A, C and D.

Results

Extraction and determination of phenolic compounds

The extraction of polyphenols by the method of confrontation by organic solvent from the after crushing of the plant "*Marrubium vulgare*" shows that the extract methanol represents an average of 21 per cent ± 01.98 in relation to the weight of the plant material. Kanyonga et al[47] have obtained a yield of 39.2 % of the *MEMV* whereas the groups of Elberry et al[42] have noted a rate of 12 %. However, there is a difference between the yield obtained with those of the literature, because the efficiency is only relative and depends on the method and the conditions in which the extraction has been carried out on one hand, as well as to the geographical origin of a share and the used part of the plant on the other hand.

The comparison of the retention times of the standards (Table 1) with those recorded in the chromatogram (Figure 1) allows the probable identification of different

Table 1. Retention time of the various standard compounds.

Peak	RT	Lambda max	m/z	Compound
1	3.40	258	137	*p*-Hydroxybenzoic acid
2	8.444	283	193	*trans*-Ferulic acid
3	12.07	279	197	Syringic acid
4	24.015	323	181	hydrocaffèic acid
5	24.730	283	329	Vanillic acid glucoside
6	40.022	272. 335	577	Apigenin 7-O-rutinoside
7	42.326	276. 331	359	Rosmarinic acid
8	45.837	276. 356	609	Rutin
9	63.220	286. 338	267	Apigenin
10	63.629	286. 330	423	Verbascoside
11	79.144	288. 346	579. 285	Luteolin-7-O-glucoside
12	87.404	282. 342	285	Lutéoline
13	89.362	284. 362	741. 285	Kaempferol-3-glucoside
14	96.228	278	359. 197	Syringic acid hexoside

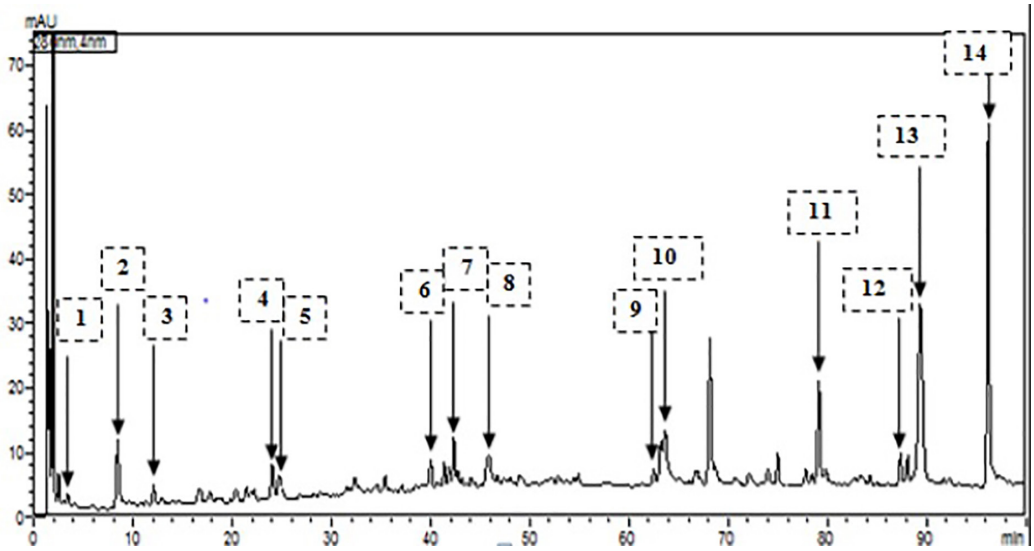

Figure 1. Chromatogram of liquid chromatography under high pressure (HPLC-DAD-ESI-MS) of methanolic extract of *Marrubium vulgare* (MEMV).

compounds in the *MEMV*. The chromatogram of HPLC (Figure 1) of the *MEMV* can conveniently be divided into three different parts: the first from 3.40 to 24.730 minutes is that in which: *p*-Hydroxybenzoic acid, trans-Ferulic acid, Syringic acid, hydrocaffeic acid and Vanillic acid Glucoside elute progressively. The second range between 40.022 and 63.629 minutes; in which Apigenin 7-O-rutinoside (40.022 minutes), Rosmarinic acid (42.326 minutes), Rutin (45.837 minutes), Apigenin (63.220 minutes) and Verbascoside (63.629 minutes) . The third range is that of Luteolin-7-O-glucoside (79.144 minutes), Luteoline (87.404 minutes), Kaempferol-3-glucoside (89.362 minutes) and Syringic acid hexoside (96.228 minutes). In a study about the extract profile of *Marrubium vulgare L.* by HPLC-PDA-ESI-MS procedures, Tlili et al[48] reported 11 phenolic compounds. Moreover, according to our results, we found also the presence of Vanillic acid glucoside, *trans*-Ferulic acid, *p*-Hydroxybenzoic acid, Syringic acid, Apigenin 7-O-rutinoside, Rosmarinic acid, Verbascoside, Kaempferol-3-glucoside and Syringic acid hexoside. Similar study has been conducted to investigate the polyphenolic profile of *M.vulgare* in Tunisia. According to Rezgui et al,[49] HPLC analysis showed that the main phenolic compounds found are sinapic acid, quercetin, ferulic acid, p-coumaric acid, caffeic acid, apigenin and luteolin present in *M.vulgare*. In the same context, comparing with the current interest, the discrepancy between studies could be attributed to chemotype, climatic and environmental condition, extraction and quantification method plant populations. In a study by Kozyra et al,[50] they have found that the antioxidant activity of methanolic extracts of *Marrubium spp* was low and the total phenolic compounds was considered inferior regarding the origin of the plant. In a study by Ghedadba et al,[29] the highest concentration of polyphenols has been measured in the methanolic extract. All the content in phenolic extracts from the *Marrubium vulgare* depends on the type of extract and its polarity.[51] The high solubility of phenols in the polar solvents gives the high concentration of these compounds in the extracts obtained. This difference in performance is justified by Connan et al,[52] they have demonstrated that the same plant pushing in heterogeneous ecological conditions

Table 2. Evolution of the weight of the rats during the Sub-acute toxicity study Weight[a] of the rats (g) ± SD.

	Day 1	Day 7	Day 14	Day 21	Day 28
Group A	107.01 ± 10.38	142.24 ± 11.38	174.61 ± 12.29	194.28 ± 10.47	223.06 ± 13.74
Group B	104.87 ± 9.55	136.67 ± 13.47	180.67 ± 14.66	227.76 ± 11.88	256.31 ± 11.47

[a] $P < 0.05$

accumulates a secondary metabolites with different characteristics. The geographical variations especially the altitude, climatic factors, the nutrient content of the soil as well as the various environmental stresses are the origin of the variations of quantitative and qualitative registered in the various secondary metabolites. These factors influence on the biosynthesis of phenolic compounds of the plant and consequently on the proportion of quantitative and qualitative polyphenols.

Identification of Candida albicans

The current methods of clinical laboratory *C.albicans* identification depend on the germ tube (GT) test and the ability of the yeast to utilize sugars. The same result was found by Dealler,[53] Ramani et al,[54] Madhavan et al,[55] in this context and according to which shows the expected results of the different biochemical reactions visually examined at 37°C, based on the presence or absence of turbidity in the carbohydrate wells. A seven-digit biocode was generated on the basis of these observations by assigning a weighted score to positive reactions. These codes were then compared to those listed in the API 20C Analytical Profile Index which confirms that the strain is *Candida albicans* (7112).[54]

Toxicity study

The toxicity study showed the non-toxic effect of *MEMV*. Rats treated with 800 mg / kg of *M. vulgare* showed no evidence of drug-induced physical toxicity throughout the study period and no deaths were recorded.

Morphological and histopathological analysis

The morphological and histopathological analysis of the internal organs is a fundamental axis to study the manifestations of diseases. In the course of the experimental period (28 days), the weight of rats (placebo and test) is noted daily at the same time. We have found that the treatment affects significantly the body weight of rats ($P < 0.05$). For the two groups, the weight of the animals has been 223.06 g (group A) and 256.31 g (group B). It is interesting to note that the weight of rats in group B, which receives the *MEMV* is significantly higher than that of the control which is probably due to the daily exposure of rats to the *MEMV* characterized as well by an increase of the gain of the body weight (Table 2). In our study, the toxicological study was determined by the administration of the *MEMV* at 800 mg/kg in rats and their observation for 28 days, has shown no symptoms of toxicity characterized by the two parameters (mortality and morbidity) and based on the external signs such as the decrease of the body weight, change of behavior, the posture, the erasures, the aspect and the fall of the hairs, the presence of diarrhea, the aggressiveness and rectal temperature.[56] The internal organs of groups A and B are weighed after the sacrifice. Following the administration of the *MEMV* at 800 mg/kg, the weight of different organs (intestine, colon, liver, spleen, kidney, heart and lungs) has dramatically increased in relation to groups. The difference is significantly higher, especially at the liver of group B. Our results show that the *MEMV* tested at a dose of 800 mg/kg has significantly affected ($P < 0.05$) the weight of the different organs (liver, intestine, colon, kidney, spleen, heart and lung). There has been a noteworthy increase at the level of the liver of rats of the group B in relation to group A as well as a slight increase has been detected in the kidneys of the group B which is probably due to the *MEMV*. The tissues of the intestine and colon have a similar profile of response to the dose after the administration of the *MEMV.* Our results show that no rat of group B receiving the *MEMV* at 800 mg/kg is dead. In our study, pathological examination (Figures 2-8) of the organs of male rats treated with *MEMV* showed no difference compared to untreated groups. The histopathological study revealed no detectable abnormalities and concluded that the *MEMV* had no adverse effects on the internal organs. Gross macroscopic and microscopic examination of the liver, kidneys, heart, lungs, spleen, intestine and colon shows that the shape, size, cells of the organs of control together and treated rats are not deformed. *MEMV* did not cause degeneration of organ cells.

Search and enumeration of C. albicans in the intestine, colon and its translocation in the internal organs

The weight of the animals inoculated by the strain of *Candida albicans* (group D) has been reduced to 132.40 g compared to the control group: 155.02 g (Table 3). In our work, the intestine and the colon show a notable presence of the opportunistic agent of *C. albicans* among the groups C and D. In group D, the small intestine and the colon show the highest burden of *C. albicans* with a rate of $\log_{10}$1.23 CFU /g of organ and $\log_{10}$1.11 CFU / g of organ respectively at 10 days post-infection .When it has detected $\log_{10}$0.90 and $\log_{10}$0.84 CFU / g respectively of component for the group C treaty by the *MEMV*. There is a significant reduction ($P<0.001$) of the fungal load (Intestine and Colon), in all treated groups compared to the group of infected rats not being treated. In the two groups C and D, according to (Table 4), a significant difference has been reported with a curve of growth disrupting the group C in relation to the group D which is probably due to the suffering of animals following the infection caused by *C. albicans*. It is noteworthy that the infection by *C.*

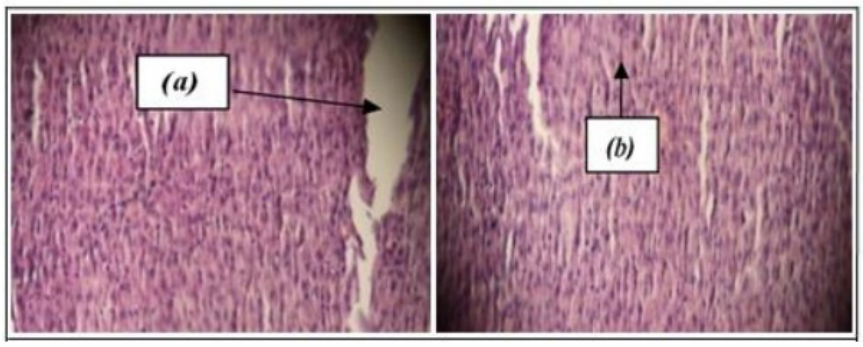

Figure 2. Histological section of Lung tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (sections were stained with H&E, X100). (a): Alveoli, (b): Alveolar epithelium

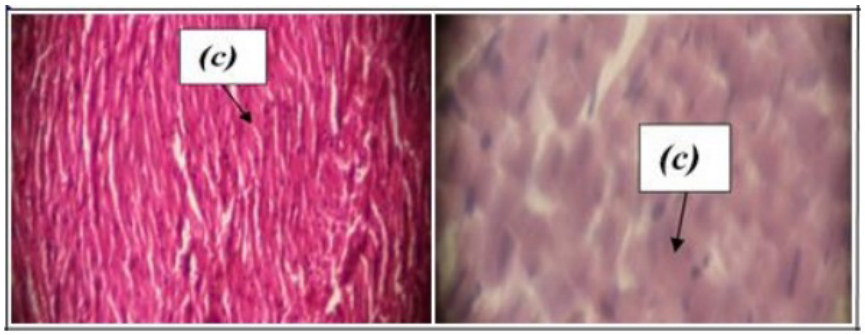

Figure 3. Histological section of Heart tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (sections were stained with H&E, X100). (c): Striated muscles cells

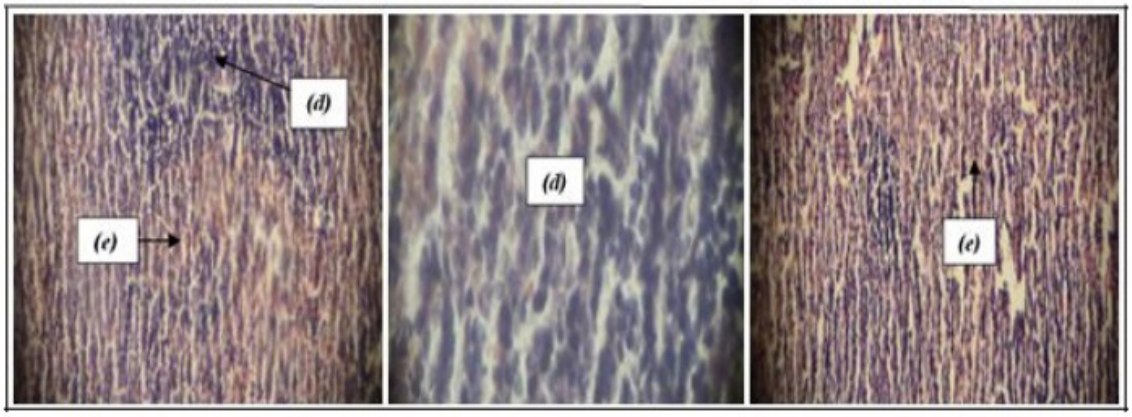

Figure 4. Histological section of Spleen tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (sections were stained with H&E, X100 and X400). (d): Red pulp; (e): White pulp

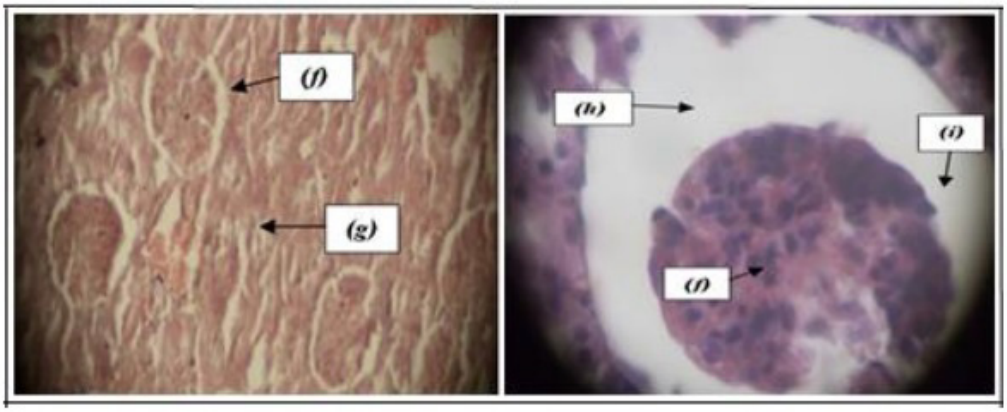

Figure 5. Histological section of Kidney tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (sections were stained with H&E, X100 and X400). (f): Glomeruli; (g): Interstitial tissue; (h): Glomerular filtrate; (i): Bowman's capsule.

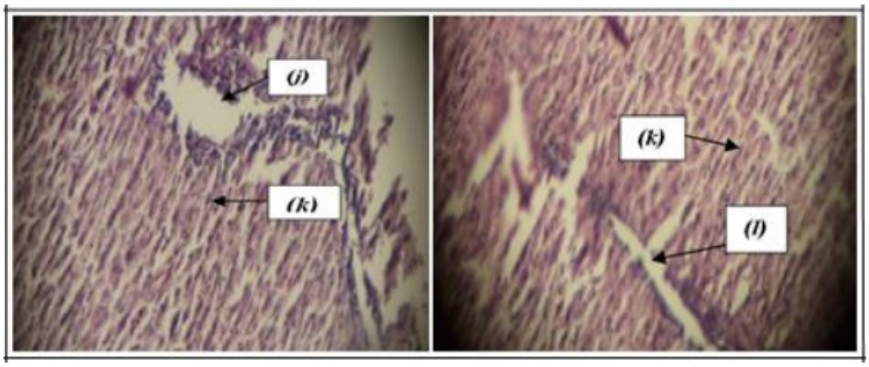

Figure 6. Histological section of Liver tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (Sections were stained with H&E, X100). (j): Central vein ; (k): Centrilobular hepatocytes ; (l): Portal vein

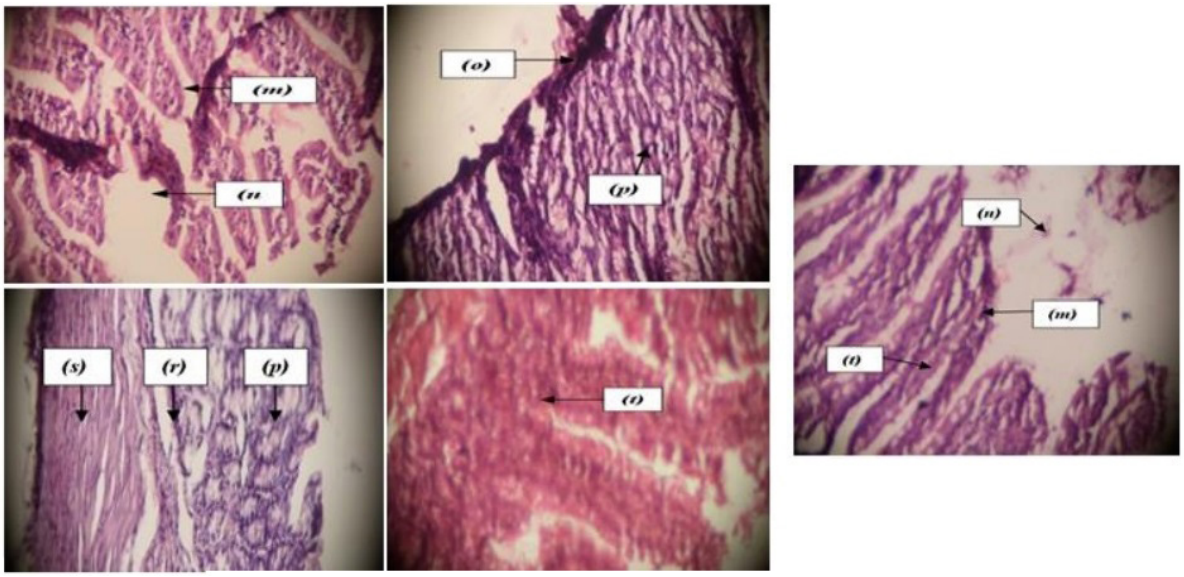

Figure 7. Histological section of Intestine tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (Sections were stained with H&E, X100 and X400). (m): Intestinal vili; (n): Intestinal lumen ; (o) : Surface epithelium ; (p) : Mucous ; (r) : Submucosa ; (s) : Muscularis mucosae ; (t) : Smooth muscle cells.

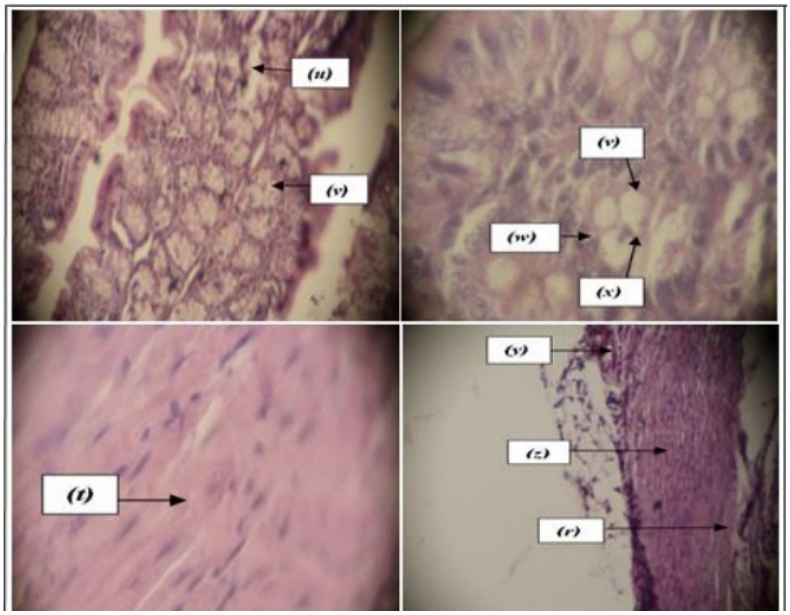

Figure 8. Histological section of Colon tissue of control group male rat, male rat treated with 800 mg/kg body weight dose (Sections were stained with H&E, X100 and X400). (u): Goblet cell; (v): Gland of Lieberkuhn ; (w): Chorion; (x): Lumen of the gland; (y): Serous; (z); Muscularis.

Table 3. Evolution of the weight of the rats during the anti *Candida albicans* treatment Weight[a] of the rats (g) ± SD.

Days	Group A	Group C	Group D
1	107.1 ± 11.74	141.72 ± 13.18	129.69 ± 8.35
2	110.74 ± 10.45	140.46 ± 12.99	137.95 ± 9.97
3	114.04 ± 10.29	137.99 ± 13.52	145.47 ± 12.26
4	118.62 ± 13.42	142.24 ± 11.90	122.83 ± 9.79
5	125.52 ± 11.85	149.04 ± 14.52	118.46 ± 21.21
6	137.98 ± 11.74	156.17 ± 14.12	133.48 ± 14.42
7	142.24 ± 9.58	154.09 ± 12.78	131.88 ± 9.65
8	148.79 ± 11.18	145.33 ± 13.56	133.40 ± 14.81
9	150.03 ± 13.37	156.58 ± 15.52	140.33 ± 14.52
10	155.02 ± 13.57	159.05 ± 13.82	132.40 ± 11.54

[a] $P < 0.05$ (ANOVA); $P < 0.05$ groups; C–D; $P < 0.01$ groups; A–C (Multiple Comparisons of Means: Tukey Contrasts).

Table 4. Evolution of the weight of the internal organs in rats Weight of the internal organs (g) ± SD.

	No. of rats	Intestine[a]	Colon[b]	Liver[c]	Spleen[d]	Kidney[e]	Heart[f]	Lung[g]
Group A	10	8.70 ± 1.18	3.79 ± 0.88	6.99 ± 0.7	0.5 ± 0.11	0.83 ± 0.11	0.73 ± 0.06	1.16 ± 0.2
Group B	10	10.38 ± 1.63	3.64 ± 0.49	10.5 ± 0.46	0.65 ± 0.17	1.18 ± 0.13	0.79 ± 0.05	1.52 ± 0.16
Group C	10	8.24 ± 0.53	5.14 ± 0.47	7.2 ± 1.13	0.59 ± 0.06	0.63 ± 0.07	0.55 ± 0.06	1.4 ± 0.15
Group D	10	8.54 ± 0.83	4.36 ± 0.34	5.9 ± 0.42	0.43 ± 0.14	0.67 ± 0.05	0.51 ± 0.04	1.2 ± 0.21

[a] $P < 0.05$ (ANOVA); $P < 0.05$ groups; A–B; $P < 0.01$ groups; B–D; $P < 0.001$ groups; B–C (Multiple Comparisons of Means: Tukey Contrasts). [b] $P < 0.05$ (ANOVA); $P < 0.05$ groups; B–D, C–D; $P < 0.01$ groups; A–C; $P < 0.001$ groups; B–C (Multiple Comparisons of Means: Tukey Contrasts). [c] $P < 0.05$ (ANOVA); $P < 0.05$ groups; A–D; $P < 0.01$ groups; C–D; $P < 0.001$ groups; A–B, B–C, B–D (Multiple Comparisons of Means: Tukey Contrasts). [d] $P < 0.05$ (ANOVA); $P < 0.05$ groups; A–B, C–D; $P < 0.01$ groups; B–D (Multiple Comparisons of Means: Tukey Contrasts). [e] $P < 0.05$ (ANOVA); $P < 0.01$ groups; B–C; $P < 0.001$ groups; A–B, A–C, A–D, B–D (Multiple Comparisons of Means: Tukey Contrasts). [f] $P < 0.05$ (ANOVA); $P < 0.001$ groups; A–C, A–D, B–C, B–D (Multiple Comparisons of Means: Tukey Contrasts). [g] $P < 0.05$ (ANOVA); $P < 0.05$ groups; A–C; $P < 0.01$ groups; B–D; $P < 0.001$ groups; A–B (Multiple Comparisons of Means: Tukey Contrasts).

Table 5. *C. albicans* mean colony counts $\log_{10}$CFU/g of tissue ± SD , $\log_{10}$CFU/100µl of blood ± SD

	No. of rats	Intestine[a]	Colon[b]	Liver[c]	Spleen[d]	Kidney	Heart	Lung[e]	Blood[f]
Group A	10	0.47 ± 0.06	0.3 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Group C	10	0.9 ± 0.26	0.84 ± 0.12	0.69 ± 0.30	0.47 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.3 ± 0.02	0.3 ± 0.02
Group D	10	1.23 ± 0.37	1.11 ± 0.41	0.77 ± 0.43	0.47 ± 0.31	0.00 ± 0.00	0.00 ± 0.00	0.47 ± 0.19	0.47 ± 0.17

[a] $P < 0.05$ Principal Component Analysis (PCA); $P < 0.001$ groups; A–C, A–D, C–D (Multiple Comparisons of Means: Tukey Contrasts). [b] $P < 0.05$ Principal Component Analysis (PCA); $P < 0.001$ groups; A–C, A–D, C–D (Multiple Comparisons of Means: Tukey Contrasts). [c] $P < 0.05$ Principal Component Analysis (PCA); $P < 0.001$ groups; A–C, A–D (Multiple Comparisons of Means: Tukey Contrasts). [d] $P < 0.05$ Principal Component Analysis (PCA); $P < 0.001$ groups; A–C, A–D (Multiple Comparisons of Means: Tukey Contrasts). [e] $P < 0.05$ Principal Component Analysis (PCA); $P < 0.001$ groups; A–C, A–D (Multiple Comparisons of Means: Tukey Contrasts). [f] $P < 0.05$ Principal Component Analysis (PCA); $P < 0.001$ groups; A–C, A–D (Multiple Comparisons of Means: Tukey Contrasts).

albicans significantly affects the body weight of rats.

The effectiveness of the extract has been observed in the lung tissue, but there has been no significant difference (P = 0.119) of the fungal load between the groups C and D. This is probably due to the variability in the response of the immune system innate to the infection in the lung tissue.[57] In the same idea, there is no similarity in the bodies of the spleen. This observation has been reported and can be explained by the difference in the exposure of tissues to the *MEMV* following the therapeutic doses. The liver, the lung and the spleen of group A are less contaminated by *C. albicans* compared to groups C and D after the post inoculation period. On the contrary, homogenates of kidneys and the heart remained sterile (Table 5). *C. albicans* was detected also in the blood of the group C and D ($\log_{10}$0.3 and $\log_{10}$0.47 CFU / 100 µl respectively).

Discussion

In order to contribute to the highlighting efficiency of extracts of *Marrubium vulgare* as an antifungal treatment, we have demonstrated their toxic effects. Our work is therefore devoted to the *in vivo* study which aims at determining the toxicological effect of the methanol extract of *Marrubium vulgare* in rats Wistar, and subsequently allow or not the recommendation for clinical trials in the treatment of infections of fungal origins. In a study by Jaouhari et al, had conducted an acute toxicity of the aqueous extract of *Marrubium vulgare* on albino swiss mice . The preparation tested has been an infusion of 1 g prepared and dried in 50 ml of distilled water. The mice treated with the extract have shown a loss of appetite 03 hours after the ingestion of the infusion but no histopathological changes have been observed.[58] In a study of female rats of acute oral toxicity with a single dose of 2000 mg/kg of extract of *Marrubium vulgare* for a period of 14 days,[18] no change has been detected on the skin, eyes, mucous membranes and the nervous system. The data suggests that the toxic dose of the methanol extract of *Marrubium vulgare* is greater than

2000 mg/kg. It would be classified in category 05 of the toxicity according to the criteria of the HMPC.[58]

Our work presents the first study *in vivo* on the anti *Candida albicans* treatment of *Marrubium vulgare* with an assessment of its toxicity. We have only worked with male rats by eliminating the females because they have a physiological and hormonal disturbance that can disrupt our results. The oral efficacy in *vivo* of the *MEMV* has been tested against a strain of *C. albicans.* Consequently, the main variable that would affect the antifungal activity of the extract would be the activity of the extract itself and its therapeutic levels at the target site. Toxicity studies are conducted to characterize the potential toxic effects the drug can produce. In this study we conducted sub-chronic toxicity study to evaluate the safety of *MEMV* in rat. The literature on the toxic effects of *M. vulgare* is scarce. Clinical pathology is a key tool in detection and interpretation of toxicological studies.[59]

In a study by Abdussalam et al,[60] the physical signs of toxicity observed within the first four hours of ethanol leaf extract of *Marrubium vulgare* administration are restlessness, hyperventilation and later reduced physical activity. They have implied that the extract is slightly toxic to the mice. The current study showed that the rats administered with *MEMV* exhibited no intoxication symptoms. It revealed no microscopic alterations in the organs (lungs, kidneys, heart, spleen and liver), since the kidneys are the main organs in the body susceptible to the toxic effects of drugs.[61] Our result is in agreement with findings of Elberry et al,[42] who detected that the *MEMV* has a role of a protector. This effect may be attributed on one hand, to the antioxidant activities of these extracts and according to Youcefi et al[62] in addition to marrubiin, some compounds isolated from the aerial parts of *M. vulgare* such as glycosidic phenylpropanoid esters have been an anti-inflammatory potential and cardioprotective roles in cardiovascular diseases on the other hand.

Body weight gains were recorded in the group of rat treated at the end of the study period. This is in disagreement with Chakir et al[63] who indicated that STZ-induced diabetic rats treated with *MEMV* showed an increase in body weight as compared to diabetic control rats. At a dose of 800 mg/kg, the histology doesn't show disorganization of the hepatic parenchyma, necrosis, hyperemia, and proximity of the centrilobular vein. In addition, the kidney doesn't present enlargement and cellular hypertrophy of convoluted tubules. The lungs were unaffected. There were no macroscopic observations related with the treatment. No gross abnormalities were attributed to the remedial treatment with the *MEMV* for sub-acute toxicities.

Invasive Candidiasis has been established in rats by using a single dose and high of the fungus (10^7 cells / animal). The infection has been established and translated and recovered from homogenized organs. Siems and Allen[64] have recommended that the limit point in a model of disease (chronic infection by systemic *Candida albicans*) is defined as the point at which the animals lose more than 20 % of their weight or when their body temperature drop to more than 4 °C. As an opportunistic pathogen, in some situations, usually related to the immune system, *C. albicans* causes infection limited to the mucosa or in serious cases: the systemic infections death.[65] Several mechanisms of virulence are known to contribute to the ability of this fungus to colonize and invade the cells, the expression of the molecules of recognition of the host or of adhesion molecules, the secretion of hydrolytic enzymes such as lipases, phospholipases and aspartyl proteases.[66]

In this present work and subsequently to the infection, the experimental rats have shown a significant difference ($P<0.001$) to their gastro-intestinal colonization by *C. albicans* where the treatment by the *MEMV* has significantly reduced the burden of fungal tissue of "intestine and colon" of the group C compared with those of the rats of the group D "infected non-treaty" ($P<0.001$). The translocation in the internal organs such as in the liver and the lung has been considerably reduced after the treatment but not with significant difference ($P=0.492$ and $P=0.119$) respectively. In this point of view, these natural substances, which inhibit biofilm formation, may be potentially useful for the treatment of biofilm related *Candida* infections. In a study by Zida et al,[67] they have found that natural substances may act by depolarization or formation of pores in the fungal bilayer membrane; display inhibitory effect on the *C. albicans* yeast-hyphal transition, inhibit biofilm formation and decrease preformed biofilms. Their results indicated that seeds of plants exert its antifungal activity not only through the damage of plasma membrane but also with mitochondrial dysfunction. The powerful antimicrobial agent of *Marrubium vulgare* is essentially due to these active substances such as essential oils, flavonoids and the tannins. In our rat model, we found that *MEMV* was effective in reducing tissue titers of *C. albicans* because this extract works via different mechanisms than the others antifungal agents. In fact, the antifungal activity of the *Marrubium vulgare* extract is apparently related to their high phenolic content on one hand and to the alteration of efflux pump activity by certain plant constituents on the other hand.[68]

Our study contributes to enrich the literature data on polyphenolic profile and biological properties of *Marrubium vulgare* extract. Several studies reported the presence of phenolic compounds and flavonoids in *Marrubium vulgare* extract. Their amount can vary depending on extraction methods, indeed, the methanolic extracts seem to be richer than the extracts obtained by other solvents.[69] In the same context, strong activity has been observed with methanol extracts of *Marrubium vulgare*.[68] In other studies, the antimicrobial activity is determined on raw extracts: methanol, acetone, ethanol, and the water. According to them, the antifungal activity of extracts from plants is due to the different chemical substances present in these extracts. As well, the optimal effectiveness of an extract will be obtained by the combined action (synergy) of different compounds of this extract.[25]

The difference on the number of *Candida albicans* in internal organs between the Group C and D is justified by the mode of action of *MEMV* on the fungal strain as it explains by Bouterfas et al.[28] Our results are in agreement with those found by Kanyonga et al[47] who demonstrated the anti-inflammatory, antinociceptive and antimicrobial potential of *MEMV*. Ajedi et al[70] have reported that the treatment with *M. vulgare* extract at a dose of 500 mg/kg as cure agent in BALB/C mice infected with *Salmonella typhimurium* showed no significant effect on the relative number of CD8+CD62L+ but treatment with same dose of *M. vulgare* extract as protective agent revealed significant increase on the relative number of T cytotoxic cells expressing CD62L. From a pharmaceutical point of view and according to Tlili et al,[48] it is advantageous when antimicrobial drugs display selective toxicity to the microbe and are non toxic to eukaryotic cells. It is interesting to note that *C. albicans* is an opportunistic agent, a normal commensurable digestive tube, in conditions favorable to its growth. It may exercise the mechanisms of serious virulence. As a proposition: another study should be necessary to assess the synergetic interactions between *MEMV* and the other antifungal metabolites such as plant extracts, fluconazole or amphotericin B.

Conclusion

In conclusion, the main purpose of this study has been to assess the toxicological effect of the *MEMV* on male rats wistar and to assess the antifungal activity (curative treatment) of the *MEMV* in male rats rendered experimentally candidiasis. In our study, the oral administration of the *MEMV* at 800 mg/kg in rats for 28 days does not misled at some symptoms of toxicity characterized by two parameters: mortality and morbidity. It can be concluded that *MEMV* does not produce histopathological changes in the internal organs. The effectiveness of oral *MEMV* is challenged against the strain of *C. albicans* (at a dose of 10^7 cells/animal). Consequently, the main variable that would affect the antifungal activity of the extract would be the activity of the extract itself and its therapeutic levels at the target site. The reduction of the translocation of *C. albicans* by the curative treatment of the *MEMV* suggests a therapeutic benefit against the spread of the candidiasis.

From these results, it is clear that the employment of the *MEMV* could be recommended at a dose of 800 mg/kg for clinical trials and opens a new avenue of approach to the use of natural bioactive substances to antifungal treatments.

Ethical Issues

This study protocol was approved by the Local Ethical Comity of the University, based on adequately performed laboratory and animal experimentation according to the Helsinki Declaration (1964).

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Conflict of Interest

The authors claim that there is no conflict of interest.

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