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In vivo Anticandidal Activity of *Euphorbia prostrata*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JPD and JRK designed the study. Authors RTT and JPD wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. Authors RTT and JPD managed the analyses of the study. Author RTT managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: *Euphorbia prostrata,* is an annual herbaceous plant reported to possess many biological activities. In Cameroon, it is traditionally used to treat various diseases among which fungal infections.
Aims: This study aimed at evaluating the safety and the therapeutic effectiveness of *E. prostrata* extract in an experimentally induced systemic candidiasis on rats.
Methods: The acute oral toxicity of the *E. prostrata* extract was determined using the Organization for Economic Co-operation and Development (OECD) guideline 425. The *in vitro* antifungal activity was assessed by the broth micro-dilution method against four *Candida* strains. The *in vivo* activity was evaluated in rat model with disseminated candidiasis due to *Candida albicans* by estimating the fungal burden in the kidneys.The side effects associated with antifungal therapy were determined by the assessment of some serum biochemical parameters using commercial kits.
Results: The doses used during acute toxicity study, did not cause any mortality or significant behavioural changes, thus leading to a LD_{50} value greater than 5000 mg/kg. *E. prostrata* extract exhibited good anticandidal activity against the tested yeasts with a MIC value equal to 64 µg/mL,

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obtained against the tested *C. albicans* strain. Oral administration of *E. prostrata* extract at the doses 33.2, and 166 mg/kg of body weight (bw) to artificially infected rats, resulted in a complete recovery of the animals after 15 days of treatment with the number of colony forming units per milliliter of *C. albicans* cells in the kidney equal to zero.The evolution of the serum biochemical parameters during the treatment revealed that *E. prostrata* extract exhibited relative side effects on rats.

Conclusion: The present study demonstrates the therapeutic effectiveness of *E. prostrata* extract on an experimentally induced systemic candidiasis on rat as well as its relative safety, thus justifying its traditional use for the treatment of mycoses.

Keywords: Antifungal; Euphorbia prostrata; yeasts; disseminated candidiasis; side effects.

1. INTRODUCTION

Candida albicans is the main cause of invasive fungal infections [1] and represents a serious public health challenge with increasing medical and economic importance due to the high mortality rates and increased costs of care and duration of hospitalization [2]. The pathogenicity of *Candida* species is attributed to certain virulence factors, like the ability to evade host defenses, biofilm formation, adherence and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin [3]. Nowadays, the incidence of the fungal infections is still increasing due in majority to the increase of factors contributing to the immunosuppression of individuals [4], to poor sanitary measures and the lack of adequate financial means to follow treatment [5].

Although there are many antifungal active principles available, their exploitation for the treatment of invasive fungal infections has also registered a lot of failures over the years due to the development of resistance to antifungal agents already available in the market and their toxicity [6]. For these reasons, there is,an urgent need for novel treatment options with improved features, and herbal medicine is one of the alternative. Medicinal plants have received much attention as a source of new antifungal drugs since they are considered as time-tested, comparatively safe both for human and environmental uses, and are available at a low cost [7].

The procedure for the discovery of new antifungal active principles is long and tedious, this is why there are just a few or no new antifungal molecules over the years [6,8]. The search for new antifungal agents is still therefore relevant. Among the potential sources of new antifungal agents, we have plants like *Euphorbia prostrata*, which is an annual herbaceous plant which belongs to the family Euphobiaceae. It is traditionally used in Cameroon to fight against various diseases among which fungal infections [9]. Previous studies have reported that it possesses many biological activities [10-13] with an extraordinary antifungal potential [14].

The chemical analysis of *E. prostrata* revealed that it contains phenolic compounds (gallic acid, ellagic acid, 3, 5, 7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one and 2-hydroxycinnamic acid), flavonoids (apigenin and luteolin), carbohydrates, sterols, glycosides, saponins, tannins which are known to be responsible of various biological activities [14-18]. Works related to the safety and the therapeutic effectiveness of methanolic extract of *E. prostrata* has not been reported. The main objective of this work is to verify the therapeutic effectiveness of methanol extract of *E. prostrata* against an experimentally induced systemic candidiasis in rats. In order to be sure that the doses used *in vivo* would not be toxic and to evaluate the side effects related to the treatment, an oral acute toxicityas well as the follow-up of some biochemical parameters.

2. MATERIALS AND METHODS

2.1 Ethics statement

Animal experiments were carried out in this study according to the guidelines set for the care and use of laboratory animals and with the rules formulated under the Animal Welfare Act by the United States Department of Agriculture (USDA) and by adopting ARRIVE guidelines [19].

2.2 Plant material and extraction

The *E. prostrata* whole plant was collected in January 2014 at the Baham Subdivision during an ethnopharmacological survey investigated in order to find out the plants used in this locality to treat fungal infections, and identified at the

Cameroonian National Herbarium, Yaoundé [9]. The extraction of *E. prostrata* crude extract was doneby soaking 100 g of dried and ground plant material in 600 mL of methanol for 48 h with intermittent shaking. After decantation, the organic solvent was separated from plant material by the use of Whatman No. 1 filter paper. The filtrate was then evaporated under vacuum at 40ºC to obtain the crude extract. This plant extraction yield to 20 g of crude extracts which was kept at 4ºC until further use.

2.3 Fungal strains and culture media

The microorganisms used during this study were *Candida albicans* ATCC 9002, *Candida parapsilosis* ATCC 22019 (American Type Culture Collection) and two clinical isolates namely *Candida krusei* and *Candida albicans*. Sabouraud dextrose agar (SDA) (Liofilchem Laboratories) was used for the maintenance and culture of fungal strains while Sabouraud dextrose broth (SDB) was used for the determination of the minimum inhibitory concentrations (MICs) and the minimum fungicidal concentrations (MFCs).

2.4 Oral acute toxicity of the crude extract of *Euphorbia prostrata*

In order to be sure that the different doses used *in vivo* will not be toxic, the acute oral toxicity of the methanol extract of *E. prostrata* was determined by using the Organization for Economic Co-operation and Development (OECD) guideline 425 [20]. In brief, prior to dosing, the rats were fasted overnight before oral administration of a single dose of the test samples. Doses of 175, 550, 1750 and 5000 mg/kg of body weight of the test samples were given using oral gavage to rats of Group I to Group IV respectively. All the rats were observed for general behavioral changes; symptoms of toxicity and mortality after treatment for the first four (critical) hours, then over a period of 24 h, thereafter daily for 14 days.

2.5 Antifungal assay

2.5.1 MIC and MFC determination

MICs and MFCs of *E. prostrata* extract were determined using the broth microdilution method as previously described by Dzoyem et al. [21]. All the experiments were carried out in triplicates and ketoconazole was used as positive control.

2.5.2 Effect of *E. prostrata* extract on an experimentally induced disseminated candidiasis in rats

The *in vivo* antifungal assay was performed as described by Richard and Emma [22] and Polak [23] with slight modifications.

2.5.2.1 Experimental animals

Seventy eight pathogen-free female albino Wistar rats (6–8 weeks old) weighing between 100 to 150 g were used. These animals were bred in the animal house of the Department of Biochemistry, Faculty of Science of the University of Dschang. The rats were placed in stainless steel cages, which were maintained at 25ºC in a 12 h dark–light cycle and provided with food and water *ad libitum*.

2.5.2.2 Induction of systemic infection with C. albicans in rats

Disseminated candidiasis infection was induced by inoculation of 0.2 mL of a 10^6 CFU/mL inoculum, prepared in sterile saline from a fresh 48 h *C. albicans* culture to rats via the tail vein. Twenty-four hours after infection, three animals were sacrificed to check the effectiveness of the infection by assessing the fungal load in the kidneys. To be sure that the animals were not having a disseminated candidiasis before the infection, three animals were also sacrificed to check the absence of *C. albicans* in the kidneys. This *in vivo* study was conformed to rules formulated by the ARRIVE guidelines.

2.5.2.3 Antifungal treatment

Infected rats distributed into five groups of 15 animals each were housed in cages and had access to food and water ad libitum. Extract at the doses of 33.2, and 166 mg/kg of bw, were administered orally once per day over 15 consecutive days, starting 24 h after infection. Two control groups were used; untreated control received distilled water and a positive control group treated with reference antifungal drug ketoconazole at 2.6 mg/kg of bw.

2.5.2.4 Determination of viable yeasts in the kidney

After every three days of treatment (at days 3, 6, 9, 12 and 15 of infection) three animals in each group were anaesthetized and sacrificed by cervical decapitation; blood and kidneys were collected from each rat. Kidney tissues were homogenized in 5 mL of sterile saline, and then

serially diluted. 0.1 mL of each dilution was plated onto SDA containing chloramphenicol, incubated for 24 h at 37ºC and the number of fungal colonies were determined. The results were expressed as log of the mean number of CFU/g of kidney per three animals.

2.5.3 Determination of side effects associated to the antifungal treatment

For biochemical analysis purposes, the blood samples were collected into test tubes without anticoagulant and allowed to clot after centrifugation at 2 500 r/min for 15 min to obtain serum and stored at -20ºC until assayed for biochemical estimation. The side effects associated with antifungal therapy were determined by the assessment of some serum biochemical parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, urea and creatinine level using diagnostic kits. Reference ranges for comparison are contingent on the method of analysis, animal species used and other experimental factors. Thus, in this study, the values obtained for the control group were considered as the reference values; and statistical analysis was conducted against the control group.

2.6 Statistical Analysis

One way analysis of variance was used to analyze the fungal load in animal kidneys and the biochemical parameters between each treatment group and the control group. When there were differences between groups, the means were compared using the Student-Newman-Keuls test at a 5%. Results were expressed as mean ± standard deviation. All data were analyzed using GraphPad Prism 5.

3. RESULTS

3.1 Oral acute toxicity studies

In the toxicity study, oral administration of the *E. prostrata* extract at 175, 550 and 1750 mg/kg of body weight did not produce any deaths and clinical signs of toxicity in rats (Table 1). However, in animals treated with 5000 mg/kg of body weight, we observed drowsiness, and a softening of the stool, although they survived after 14 days of oral administration of the extract. As there were few clinical signs of toxicity and no mortality in all the tested doses, LD_{50} value of the methanolic extract of *E. prostrata* was found to be greater than 5000 mg/kg [from calculations using Acute Oral Toxicity (Guideline 425) Statistical Program (Version: 1.0)].

3.2 Antifungal assay

3.2.1 *In vitro* antifungal activity

The *E. prostrata* extract presented variable anticandidal activity against the tested yeasts with MICvalues ranging from 32 to 64 µg/mL while the MICs values of ketoconazole were ranging from 0.25 to 2 µg/mL. The same MIC and MFC values (MIC= 64 µg/mL and

Table 1. Behavior of female rats after administration of single doses of the methanol extract of *E. prostrata*

Study parameters	Doses (mg/kg of body weight)			
	175	550	1750	5000
Reaction to noise	N	N	N	N
Pinch reaction	N	N	N	N
Locomotion	N	N	N	N
State of the tail	N	N	N	N
Stool appearance	G	G	G	S
Tooth grinding	N	N	N	N
Convulsions	Ab	Ab	Ab	Ab
Sleep	Ab	Ab	Ab	D
Aggressiveness	Ab	Ab	Ab	Ab
Salivation	Ab	Ab	Ab	Ab
Mortality after 24 hours	Nrm	Nrm	Nrm	Nrm
Mortality after 14 days	Nrm	Nrm	Nrm	Nrm
Number of female rats used	1	1	1	3

N = Normal; G = granular; S = soft; Ab = absent; D = drowsiness; Nrm = no recorded mortality.

MFC= 512 µg/mL) was obtained from the two *C. albicans* tested (strain and isolate). During this assay, *C. parapsilosis* was the most sensitive strain (MIC= 32 µg/mL and MFC= 1024 µg/mL) while *C. krusei* was the most resistant (MIC= 256 µg/mL and MFC= 1024 µg/mL).

3.2.2 *In vivo* antifungal activity

The *in vivo* activity of *E. prostrata* extract was evaluated on an experimentally induced disseminated candidiasis with *C. albicans* in rats, by estimating the fungal burden in the kidney. The results on the estimated number of viable *C. albicans* cells in kidneys from animals that were infected intravenously with 1×10^6CFU/mL and treated for fifteen consecutive days with saline, extract (33.2 and 166 mg/kg of bw) and ketoconazole (2.6 mg/kg of bw), are shown in Fig 1. The administration of the extract caused a significant reduction of the fungal load after 3 days in the kidneys of infected rats compared to untreated control. This decrease was greater with ketoconazole. In addition, the treatments (with extract and ketoconazole) led to a significant decrease in the fungal load in the kidneys compared to that of the untreated control, throughout the duration of the treatment. The animals treated with the extract at the different doses showed a complete recovery after 15 days of treatment while those treated with ketoconazole recovered earlier (12 days of treatment). At the highest dose (166 mg/kg), reduction in the log of CFU/g in the kidneys was more pronounced compared to the lower dose.

3.2.3 Side effects associated with the antifungal treatment

The effect of oral administration of *E. prostrata* extract at different doses (33.2 and 166 mg/kg of bw) during the treatment of the serum biochemical parameters are presented in Fig 2. The serum urea, creatinine, and protein levels as well as serum alkaline phosphatase (ALP) and transaminase (alanine transaminase, ALT and aspartate transaminase, AST) activities of treated animals (with extract and ketoconazole), during treatment increased to a maximum then gradually decreased between the twelfth and fifteenth days of treatment to give values significantly similar compared to those of healthy animals. The evolution of all the serum biochemical parameters evaluated during treatment was less important in the treated animals than in untreated control, except at the sixth day of treatment. The serum protein and urea levels and the serum ALP and AST activity during treatment were higher in ketoconazole-treated rats compared with extract-treated rats, although the differences were significant only on the sixth day of treatment. In addition, ketoconazole-treated animals showed a lower increase in serum creatinine levels compared to that of extract-treated animals, whereas the ALT activities were significantly similar. The increases in serum creatinine and protein levels, as well as the serum ALP and AST activities during treatment, was not dose-dependent, while serum urea levels and serum ALT activities appeared to be dose-dependent.

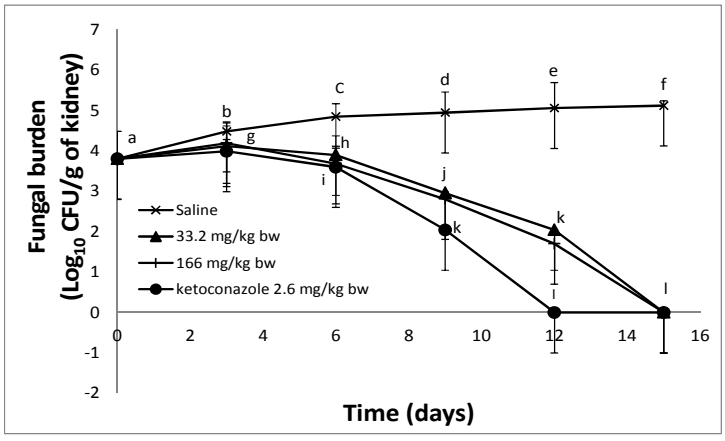

Fig 1. Variation of fungal burden of kidneys in rat model of disseminated candidiasis at various time intervals upon treatment with *E. prostrata* extract and ketoconazole *Animals were infected intravenously with 10^6 cells/mL (C. albicans) and were treated for fifteen consecutive days with saline, extract (33.2 and 166 mg/kg of bw) and ketoconazole (2.6 mg/kg of bw). The fungal burden was assessed by determining the number of CFU. Values are expressed as mean ± SD values of three determinants.Values carrying the same superscripts letter are not significantly different at p ≤ 0.05 (Student-Newman-Keuls test)*.

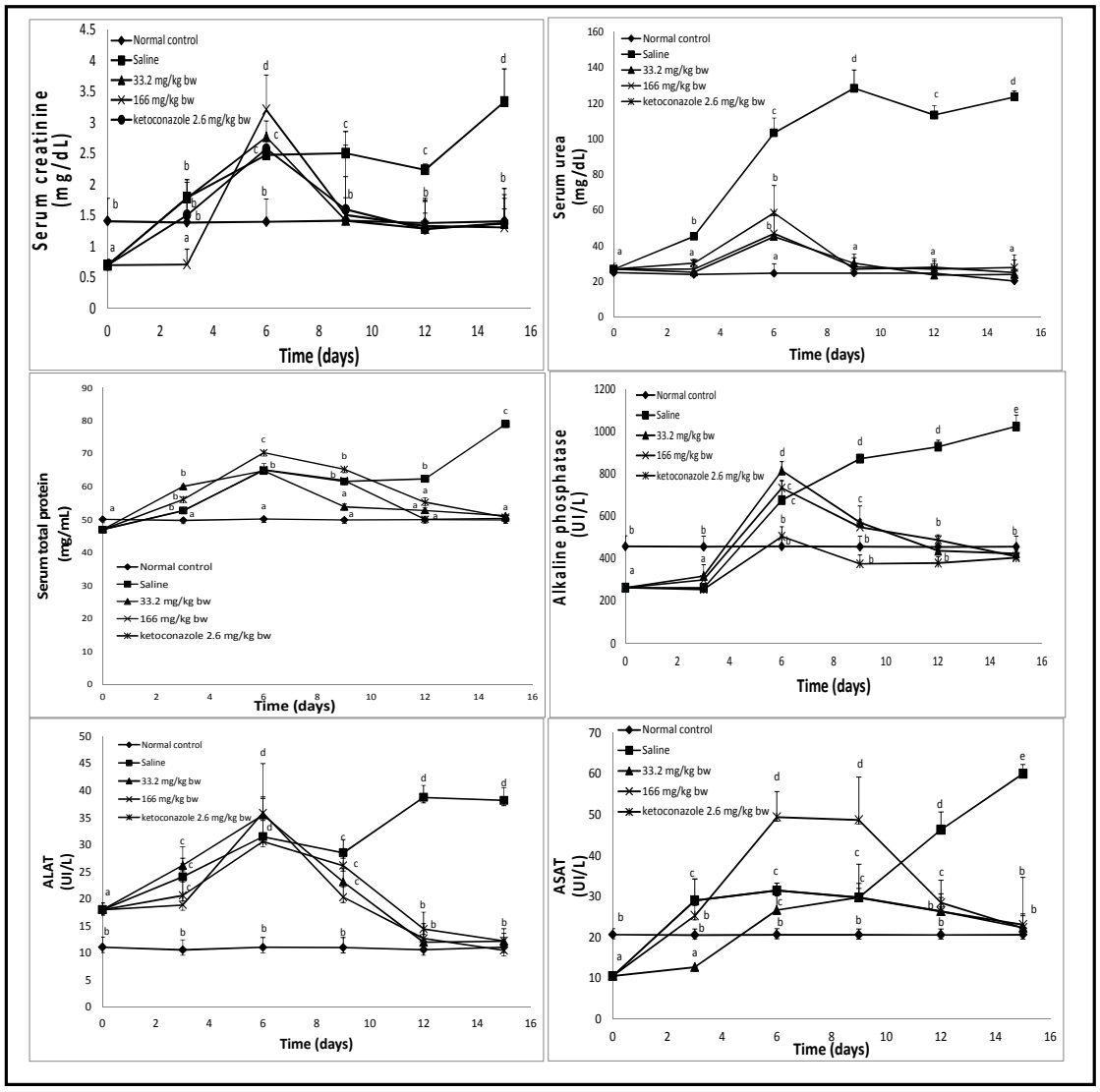

Fig. 2. Variation of serum creatinine (A), serum urea (B), serum total protein (C) concentrations and alkaline phosphatase (D), ALT (E) and AST (F) activity in rat model of disseminated candidiasis at various time intervals upon treatment with *E. prostrata* extract and ketoconazole

Animals were infected intravenously with 10^6CFU/mL (C. albicans) and were treated for fifteen consecutive days with saline, extract (33.2 and 166 g/kg of bw) and ketoconazole (2.6 mg/kg of bw). These parameters were assessed by using commercial kits. Values are expressed as mean ± SD values of three determinants. In each figure, values carrying the same superscripts letter are not significantly different at p ≤ 0.05 (Student-Newman-Keuls test).

4. DISCUSSION

Due to the increase in the use of medicinal plants for their pharmacological properties, it is crucial to insure their safety and effectiveness by experimental screening of their toxicity. However, there is a lack of proven scientific studies on the toxicity and side effects of these remedies [24]. Therefore, acute oral toxicity study is vitally needed not only to identify the range of doses that could be used during further investigations but also to reveal the possible clinical signs elicited by the substances under study. The inconvenience of acute toxicity studies is that it does not detect effects on vital functions like the cardiovascular, central nervous, and respiratory

systems which are not usually assessed during the study and that should be evaluated prior to human exposure [25].

In this study, the rats in the control and treated groups were administrated with vehicles and crude extracts, respectively. The rats were monitored daily for fourteen days to check any sign of toxicity and mortality. During the acute toxicity evaluation period, there were no observable symptoms of toxicity or death apart from drowsiness of animals, and a softening of their stools after the administration of *E. prostrata* extract at the dose 5000 mg/kg of bw. Drowsiness may reflect a depressive or sedative effect of the crude extract [26]. The latter would have caused an attack on the nervous system, either at the level of the nerve receptor cells or at the level of the efferent neurons [27]. The appearance of the stool was indicating the beginning of diarrhea, suggesting that at high doses, the extract can produce an irritant effect on the smooth muscle of the intestinal wall, thus causing changes in fluid and electrolyte permeability [28]. This indicates that the administration of this crude extracts below the dose 5000 mg/kg of body weight has negligible toxic effects on the animals. In principle, the main test method is not intended to be used for determining a precise LD_{50} value, but it serves as a suggestion to classify the crude extracts based on the expectation on dose at which the animals are expected to survive [29]. According to the chemical labeling and classification of acute systemic toxicity recommended by OECD, the crude extracts of *E. prostrata* was assigned (LD_{50}> 5000 mg/kg) as belonging to the lowest toxicity class. Similar results were found during oral acute toxicity of aqueous extract of *E. prostrata* [30].

E. prostrata extract presented significant anticandidal activity with MIC values ranging from 32 to 64 µg/mL against the tested yeasts. These good antifungal activities could be due to the chemical composition of *E. prostrata* [14,18] as it is reported that the secondary metabolites of plants have many biological properties including antimicrobial properties [17]. These results corroborate with those of Uzair [14] who in Pakistan, demonstrated the antifungal activity of *E. prostrata* using solid medium diffusion method. From these results, it is clear that in our case, the origin as well as the climatic conditions in which the plants have grown, factors that are known to affect the chemical composition of a plant, have not affected its antifungal properties [31].

E. prostrata extract (MIC = 64 µg/mL) against *C. albicans* was used to treat systemic candidiasis induced in the rat. Given the fact that *E. prostrata* extract possesses many bioactive secondary metabolites [9,14,18], it could have *in vivo,* directly suppressed *C. albicans* in the body by killing it or by acting on the immune system by stimulating (directly or indirectly) the production of nitric oxide by granulocytes and monocytes which will destroy *C. albicans* [32]. In fact, according to Khan et al. [32], the pathway of *Candida* destruction in the immunocompromised rat depends on nitric oxide (NO). NO is responsible for the defense against pathogens that live and proliferate in the cellular environment of several somatic cells [33]. According to Polak [23], during systemic candidiasis, *C. albicans* can colonize all organs and even the bones, but it is only in the kidneys that progressive growth of *C. albicans* can be observed. This is why the fungal burden during the treatment has been monitored in the kidney. Also, the fungal cells in the kidneys are lodged at the level of the glomerulus and the distal convoluted tubule, immune to inflammatory mechanisms and phagocytosis [34]. The significant decrease and even eradication of *C. albicans* in animals treated with *E. prostrata* extract is, therefore, an indication that this plant can effectively fight against a systemic candidiasis. The results obtained from this study could be considered as significant, bearing in mind that *C. albicans* survival in the kidney plays a primary role in mortality in patients with disseminated candidiasis [35]. Our results revealed that the extract from *E. prostrata* displayed an inhibitory effect on fungal proliferation *in vivo* although this activity was lower than that of ketoconazole which is a pure product. To the best of our knowledge, the *in vivo* antifungal properties of *E. prostrata* are herein reported for the first time.

The analysis of blood parameters during drug treatments provides information on side effects that may affect specific tissues, specifically the kidneys and the liver and therefore the average lifespan of experimental animals as well as that of humans by extrapolation [36]. In addition, this blood parameters analysis also provides useful information regarding the mechanisms of toxicity of the therapeutic agent [37]. All the organs of the body including the kidneys and the liver are attacked during a systemic candidiasis and this causes their damage, thus impairing their function. Creatinine and urea levels are some markers of renal function assessment [38] while

the activities of liver enzymes like ALT and AST are that of liver function assessment [26]. An increase in serum creatinine level indicates renal impairment [39]. In addition, liver is the main organ of xenobiotic metabolism and then in a case of hepatic damage, serum activities of liver enzymes like ALT and AST increase [26].

In this study, all the serum parameters analyzed, increased in animals treated at different doses and then decreased to stabilize at levels significantly similar to that of infected controls. This significant increase observed on the sixth day mean that the *E. prostrata* extract has slightly exacerbated the alteration of the renal and liver tissues caused by *C. albicans*. According to Aliyu et al. [40], each marker of nephrotoxicity (creatinine or urea) increases significantly in serum if 75% of the nephrons are affected and the same thing happens for liver enzymes (ALT and AST) when the liver is severely damaged [26]. Urea is the result of metabolic processes in the ornithine cycle and it is the main metabolic pathway for excretion of excess body nitrogen. Its serum level reflects the balance between its production and its excretion [41]. Serum urea levels increased significantly in treated animals because its production rate exceeded that of its clearance, which was disrupted by the damage of the kidneys by *C. albicans* [42]. Generally, an increase in serum protein reflects a cellular injury, so the evolution of the serum protein level is in agreement with the damage suffered by the liver cells [43]. In addition, the increase in ALP activity is a hallmark of cholestasis characterized by insufficient excretion of bile due to some drugs or microorganisms [44]. This increase in the activity of the ALP would be therefore due to an impairment of the hepatobiliary function caused by *C. albicans*and certain compounds present in the extract [44].This is why the elimination of *C. albicans*, main cause of kidney and liver damage by the extract resulted in a gradual recovery of their function.

5. CONCLUSION

The present study demonstrates the therapeutic effectiveness of the methanol extract of *E. prostrata* on an experimentally induced systemic candidiasis on rat as well as its relative safety, thus justifying the traditional use of this plant for the treatment of mycoses.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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