Original Article

Anticandidosic Activity and Acute Toxicity of *Quercus Suber L.* Bark Extracts

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A R T I C L E   I N F O

### A B S T R A C T

The cork oak (*Quercus suber* L.) is commonly used in traditional pharmacopoeia. The main objective of this work is to contribute to the valorization of this plant species through the study of the anticandidosic activity of cork oak (*Quercus suber)* bark extracts stemming from decoction, maceration and Soxhlet methods of extraction. The anticandidosic potential of the elaborated extracts was tested on five different *Candida albicans* strains. Our results showed that all tested extracts tested displayed an inhibitive activity against the five strains. The study of the acute toxicity showed that the lethal dose is 1150 mg/kg in mice, which remained moderately toxic according to Hodge and Sterner classification scale. Taken together, these results underline the antifungal potential of cork oak bark extracts against Candida albicans strains, suggesting a prospective use of these extracts for candidiasis treatment.

G R A P H I C A L   A B S T R A C T
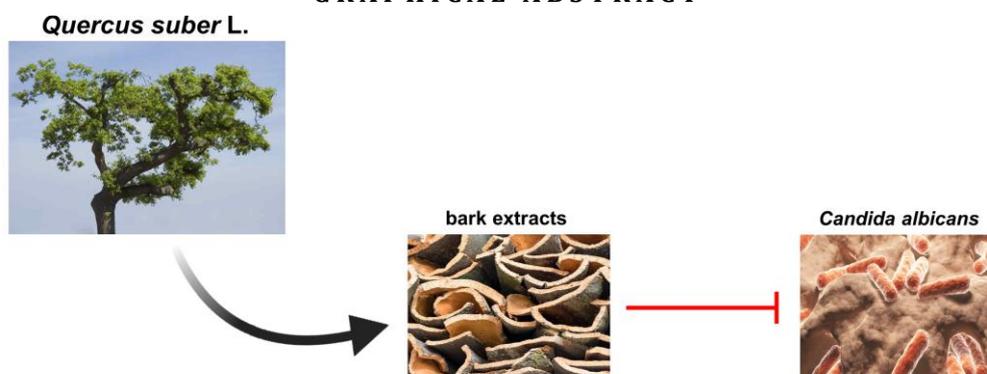
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Introduction

Candida species are among the most common opportunistic pathogens causing fungal infections with high mortality and morbidity [1]. With nearby 150 species, *Candida* species account for many species that are endosymbionts of humans and responsible of serious superficial and invasive infections mainly in immunosuppressed hosts [2]. *Candida* genus comprises yeast pathogenic isolated from skin, mouth, intestinal tract, and vagina [1]. Over the past few decades, candidiasis incidence has substantially increased [3,4]. *Candida albicans* infection represents around 80% of candidiasis infections while the remaining 20% are mainly due to *Candida* non-albicans infections including *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida dubliniensis* [5].

Recently, increased resistance of *Candida* species to antifungal agents such as azoles, due to drug pressure, has been widely reported [6]. Thus, the search and identification of new alternative anticandidosic formulations is crucial to deal with *Candida* infections. Phytotherapy represents a more efficient and less toxic alternative than chemical drugs for anticandidosic treatments [7,8]. Phytotherapy focuses on the use of plant-derived substances or drugs mostly extracted from medicinal plants in disease prevention and treatment [9].

For a long time, medicinal plants have widely been used as drugs to treat several pathogens. According to the World Health Organization (WHO), around 65-80% of the world population in developing countries, mostly depend on traditional herbal medicines for their primary health care, due to poverty and lack of access to modern medicine [10]. Thus, despite the progress of pharmacology and the remarkable advances in synthetic organic chemistry of the twentieth century, more than 25% of drugs prescribed in industrialized countries come directly or indirectly from plants [11–13].

Morocco is known for the abundance and diversity of its flora that constitutes a true phyto-genetic reservoir, with approximately 4500 species and subspecies of vascular plants, of which two-thirds are endemic to Morocco. Aromatic and medicinal plants represent 8,69% and 14,28% of the total Moroccan flora, respectively [14]. Given its large diversity of flora, Morocco occupies a distinctive place among the Mediterranean countries, having a long medical history and a traditional know-how based on medicinal plants [15].

Cork oak (*Quercus suber* L.) is an endemic species of the Mediterranean region [16]. In Morocco, Cork oak is frequently used in traditional medicines to treat many diseases. Recent studies have shown that Cork oak displays high antimicrobial activities. For instance, suberin film extracted from cork showed a bactericidal property against *Staphylococcus aureus* and *Escherichia coli* strains [17]. This bactericidal activity was mainly attributed to phenolic compounds [18]. Cork oak bark is rich in polyphenolic substances so it can confer great curative and preventive properties [19]. To prompt the use of natural products for medical purposes and to assess the medicinal potential of the Mâamora forest Cork oak bark, the present work aimed to study the anticandidosic activity and the acute toxicity of methanolic extracts of cork oak (*Quercus suber*) bark powder.

Materials and Methods

Plant material and the extract preparations

The bark of the stem of *Quercus suber,* belonging to the Fagaceae family, was collected from the forest of Mâamora « canton A », Rabat, Morocco. Three different extractions were made for aqueous extract (AE) and the dried powder (5g/500 mL) of the cork oak bark was decocted with distilled water while the methanolic extracts (ME$_M$) were prepared. On the one hand, 2 g of dried powder was macerated with 500 ml of methanol with agitation for 24 h. After filtration through Whatman paper, the residue was re-extracted in 500 ml of methanol for 24 hours; this same operation was repeated two more times (24 hours for each). On the other hand, the second methanolic extract (ME$_S$) was obtained by Soxhlet extraction of 100g of aerial parts for 32 h. The extracts were filtered and concentrated using a rotary evaporator to obtain a dry extract. All extracts were stored at 4∘C.

For each extraction, the extraction yield (yield of extracts) was calculated using the following equation: $Y = W_e / W_i \times 100$ with Y (%): yield of

extract, We: weight of the recovered extract (mg) and Wi: initial weight of dry plant matter (mg).

Antifungal activity

Fungi tested and growth medium
Five *Candida albicans* (Robin) Berkhout strains, opportunistic fungi largely responsible for candidiasis, were tested for antifungal activity including *Candida albicans* L13 (IHEM 15835), *Candida albicans* L2 (IHEM 15824), *Candida albicans* L5 (IHEM 15827), *Candida albicans* L14 (IHEM 15836) and *Candida albicans* (L12) (IHEM 15834).

The strains were obtained from the Moroccan Coordinated Collections of Microorganisms (CCMM), Laboratory of Microbiology and Molecular Biology (LMBM), National Center for Scientific and Technical Research (CNRST), Morocco. The five *Candida albicans* were cultivated on Sabouraud dextrose agar at °C for hours couleur.

Candida albicans colonies count
A range of successive dilutions, going 10^{-1} to 10^{-7}, was carried out. Each dilution required a 9 ml tube of physiological water plus 1 ml of suspension of the five strains. From each dilution, a volume of 1 ml was taken and then added by the streaking technique to the surface of Sabouraud Dextrose Agar medium. Three biological replicates were performed for each dilution. The cultures are then incubated at 30°C, for 48 hours. The counting was based on the concept of CFU (Colony Forming Units). The CFU of each strain was calculated according to the following equation: $N= \Sigma$ *colonies/V* $(1 + 0,1\ n2) \times d$l where: N= Number of CFU per gram or per ml, Σ colonies = Sum of the colonies, V= Volume of solution deposited (1 ml), n1: Number of boxes considered at the first selected dilution; n2: Number of boxes considered at the second selected dilution and dl: Factor of the first retained dilution.

Minimum Inhibitory Concentration and Minimum Fungistatic Concentration
Minimum Inhibitory Concentrations (MICs) were determined in sterile 96 well microplatesaccording to CLSI protocol M27 - A3.

Briefly, overnight liquid cultures of *Candida albicans* strains grown on Sabouraud liquid medium were enumerated according to Gassaaja, (2002) [20]. A series of concentrations varying from 25 to 50 mg/mL were prepared. 200 μL of Sabouraud liquid medium and extract (1:1;v/v) was distributed in all test wells, then 10 μl of yeast suspensions was added to each well. The plates were incubated at 37°C for 48 hours and the MIC was defined as the lowest concentration that prevented visible growth. Fungizone (1μg/ml) was used as a positive control.

To conclude, the Minimum Fungistatic Concentration (MFC) value, 100 (unity) of broth from the no-growth wells, was inoculated and incubated at 37°C for 24 hours. The minimum fungistatic concentration (MFC) was determined as the lowest concentration of cork oak bark extract that allow the development of yeast strain.

Acute toxicity test

Experimental animals
Balb/c type mice aged between 3 and 4 months old and weighting 20 – 30 g, obtained from the Faculty of Sciences of Rabat – Animal Physiology Lab, Morocco, were used for the study. Housed individually in cages, maintained under 12 hours' light/ dark cycle at a temperature 23 ± 3°C and relative humidity range of 50 - 60%, the animals were fed with standard diet and water through the whole experiments time.

Toxicity test of methanolic extract
The oral acute toxicity (LD$_{50)}$ of methanolic extract of cork bark was evaluated in animals described above. Indeed, mice were divided into three groups (n=4 for each lot): Animals of the first group (control), which received a volume of 20 ml/mg IP of physiological water NaCl 0.9% **intraperitoneal (IP)**. The second group received a dose of 300 mg/kg of the methanolic extract of cork oak bark in a volume of 20 ml/kg IP. The mice of the third group received a dose of 2000 mg/kg of methanolic extract of *Quercus suber* in a volume of 20 ml/kg IP for 15 days.

An animal was picked at a time, weighted, and dosed with the equivalent volume of extract. The extract was administered orally using gastric feeding tube. During this period, for each group, a

number of parameters were observed including the body weight, signs of toxicity and the number of deaths. The LD50 was calculated using the method of Dragstedt and Lang (1944) [23], using the following equation:

$$LD50 = \frac{50\,(X2 - X1)\ +\ X1Y2 - Y1X2}{Y2 - Y1}$$

With Y1: Percentage of mortality corresponding to X1, Y2: Percentage of mortality corresponding to X2, X1: lower dose surrounding the LD and X2: higher dose surrounding the LD.

Statistical analysis
All tests were done in triplicates. Values of each experiment were expressed as mean ± standard deviation (SD). Statistical comparisons were performed using a one-way ANOVA, followed by a Dunnett's-t-test for comparison against a single group. The statistical analysis was performed using Graph Pad Prism software (Version 8).

Differences (between groups) were considered as statistically significant at $p < 0.05$.

Results and Disscusions

Extraction yield
Quercus Suber extracts were obtained using water and methanol as solvents. As summarized in Table 1, the aqueous extract (AE) showed the highest extraction yield (19.2 %) than the methanolic extracts. A slight difference was recorded between the two methanolic extracts in term of the extraction yield. Indeed, we found that by macerating 200g of plant material in methanol, the yield of extract was by 16 %. Meanwhile, with half quantity of cork oak bark material, the yield of extract reached 17.5 %. This difference, mostly linked to the extraction method, underlined the efficiency of Soxhlet over maceration, which allowed the depletion of plant material in only few cycles, resulting in a better yield.

Table 1: Cork oak bark extracts yields obtained using maceration of Soxhlet extraction techniques. The yield of each extract was expressed in percentage. Unpaired Student t-test was used to calculate P values

	Decoction (Extract 1)	Maceration (Extract 2)	Soxhlet (Extract 3)
Plant material weight (g)	5	200	100
Crude extract weight (g)	0.96	32	17.5
Extraction yield (%)	19.2	16	17.5

Antifungal activity of extracts against Candida albicans
 Colonies enumeration was performed to determine the concentration of yeast present in the initial preparation. For this purpose, the number of colonies forming units (CFU) was determined using the dilution.
Figure 1 illustrates the CFU for the five *Candida albicans* strains. The CFU determination revealed a large variation between the different strains. For instance, we noticed that L2 and L12 strains

reached the greatest CFU number; 11.10^7 and 14.10^6 CFU/ml, respectively, followed by L14 and L13 strains, with 17.10^5 and 16.10^5 CFU/ml, respectively. However, the lowest CFU value of 8.10^4 CFU/ml was recorded in L5 strain (Figure 1). Given the fact that the five tested strains with different origins belong to the same species (*Candida albicans*) and were cultivated under the same culture conditions, the huge difference in CFU values can be attributed to the difference in the original substrate of each strain.
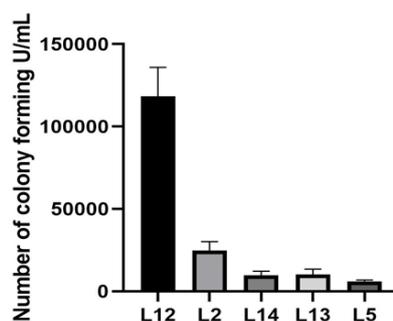

Figure 1: Colony forming units of *Candida albicans* strains isolated from fingernail (L2, L5); oral swab (L13); umbilical catheter (L12) and toenail (L14). Values are mean ± SD of three biological replicates

The antifungal activities of extracts were estimated by the absence or the presence of staining at wells' level (Figure 2). Our results showed that all the tested strains are sensitive to the three different extracts with varying degrees. L14 strain, isolated from a sample of toenail, was shown to be more sensitive than the others. L2 and L5 strains from fingernail infections showed the same degree of sensitivity to AE and the ME$_M$. Moreover, we noticed a difference in extract efficiency depending on the dilutions of each extract and the method of extraction from which it is derived. Yet, an absence of staining was recorded at the first dilution for the five tested strains while no strain was able to develop in the presence of highest concentrations (50 mg/ml and 25 mg/ml) for both methanolic extracts. At a concentration of 3,12 mg/ml, all the tested extracts were found to be inactive on all the studied strains while ME$_S$ was inactive on L14 strain at a concentration of 1,56 mg/ml. Also, L12 and L13 strains from neonatal infection and from oral sample, respectively, were sensitive to both methanolic extracts. However, the AE was less efficient regarding all the tested strains. An inhibition of *Candida albicans* strains growth was recorded at the MIC 50 mg/ml for L12 strain, at 25 mg/ml for L2, L5 and L13 strains and at 12,5 mg/ml for L14 strain.
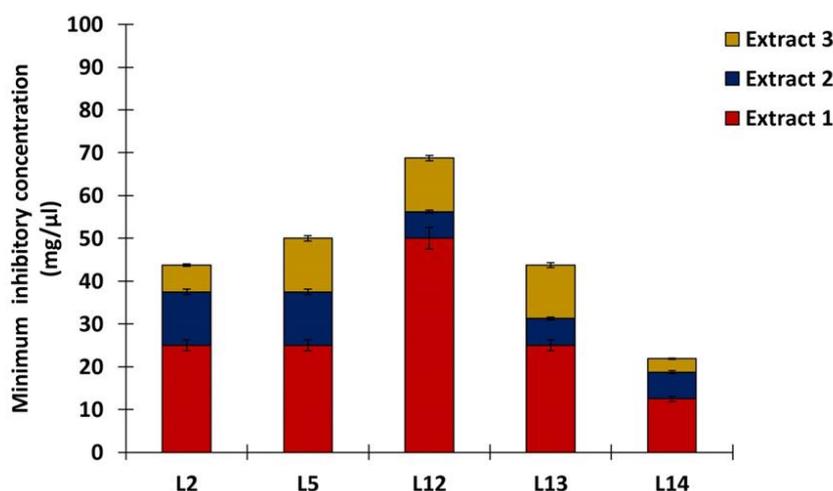

Figure 2: Minimum inhibitory concentrations MIC of the extracts of *Quercus suber* L. tested according to the five strains of *Candida albicans*. L2: Strain taken from fingernail; L13: Oral swab strain; L5: Strain take from fingernail; L12: Sample strain from umbilical catheter; L14: Toenail sample strain. Values are mean ± SD of three biological replicates

The Fungizone efficiency to inhibit *Candida albicans* growth was also tested. Our results showed that this product was not able to inhibit L13 strain growth (Table 2). However, it was more efficient on L2, L5, L12 and L14 strains, suggesting that L13 strain was resistant to fungizone. Thus, given the fact that all the tested strains were sensitive to the three different extracts, we can conclude that these extracts can be used for *Candida albicans* treatment.

Table 2: Fungicide effect of fungizone on *Candida albicans* strains growth

	L2	L5	L12	L13	L14
Fungizone (1µg/ml)	-	-	-	+	-

L2: Strain taken from fingernail; L13: Oral swab strain; L5: Strain take from fingernail; L12: Sample strain from umbilical catheter; L14: Toenail sample strain. (+) Positive culture; (-) negative culture

The fungicide effect of extracts was evaluated for all the different extracts obtained from the three different extraction methods. The results revealed that the methanolic extract (ME$_S$) obtained by Soxhlet has a fungicidal effect on all the five tested strains of *Candida albicans* at different

concentrations, and a fungistatic effect on L14 strain at 3,12 mg/ml (Table 3). The other methanolic extract (ME$_M$) (obtained by maceration) displayed fungistatic activity on all strains and fungicidal activity on L12 strain at 50 mg/ml. As for the aqueous extract, all five strains exhibited a MIC which it's equal to the minimum fungistatic concentration.

Table 3: Fungicidal (MFC) and fungistatic (CFS) effect of cork oak bark extracts (Aqueous and methanolic extracts) on the five strains of *C. albicans*.

	L2		L5		L12		L13		L14	
	MFC	**CFS**	**MFC**	**CFS**	**MFC**	**CFS**	**MFC**	**CFS**	**MFC**	**CFS**
Extract 1 (decoction)	-	25	-	25	-	50	-	25	-	12.5
Extract 2 (maceration)	-	12.5	-	12.5	50	6.25	-	6.25	-	3.12
Extract 3 (Soxhlet)	6.25	-	12.5	-	12.5	-	12.5	-	6.25	3.12

MFC: Minimum fungicidal concentration; CFS: Minimum fungistatic concentration; L2 and L5: Strains taken from the fingernail; L12: Sample strain taken from the umbilical catheter; L13: Oral swab strain; L14: Toenail sample strain

Sub-acute and acute toxicity test of the methanolic extract of cork oak bark

The sub-acute toxicity test revealed that the intraperitoneal administration of 300 mg/kg dose of methanolic extract (ME$_S$) disturbed at short time mice behavior. Indeed, within the extract injection, a slight depression and sedation along with a decrease in mice reflex have been recorded (Table 4). Nonetheless, the animals recovered quickly and resumed their normal habits after 5 - 10 minutes. The acute toxicity test performed with the administration of a single 2000 mg/kg dose of the same extract negatively affected mice physical activity and usual behavior. A decrease in mice mobility, sedation, breathing difficulties, loss of appetite leading to refusal to eat as well as paralysis of the hind limbs and reduced sensitivity to pain and noise were detected in treated mice.

Table 4: Clinical signs observed during the first 24 hours following the treatment application. + indicated a positive response, - indicated a negative response

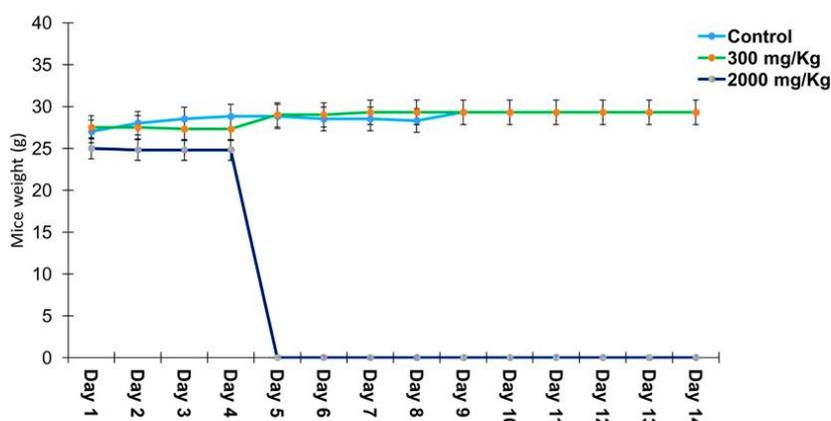|	**Control**	**300 mg/Kg**	**2000 mg/Kg**
Mice grouping after 5 minutes	+	-	-
Sedation effects after 5 minutes	-	+	+
Immobility	-	+	+
Feeding	+	+	-
Accelerated breathing	-	-	+
Hind limb paralysis	-	-	+

Figure 3: Weight evolution of 4 mice after injection of 300 mg/Kg and 2000 mg/Kg dose of methanolic extract of cork oak bark (*Quercus suber* L.). Values are mean ± SD of three biological replicates

The Lethal Dose 50 of the methanolic extract of cork oak bark (*Quercus suber* L.) was then calculated according to Dragstedt and Lang (1944). A linear regression was observed between the methanolic extract's doses and the lethality percentage, as shown in Figure 4. The LD50 was estimated to be around 1150 mg/Kg, which can be slightly toxic to mice according to Hodge and Sterner Toxicity Scale.
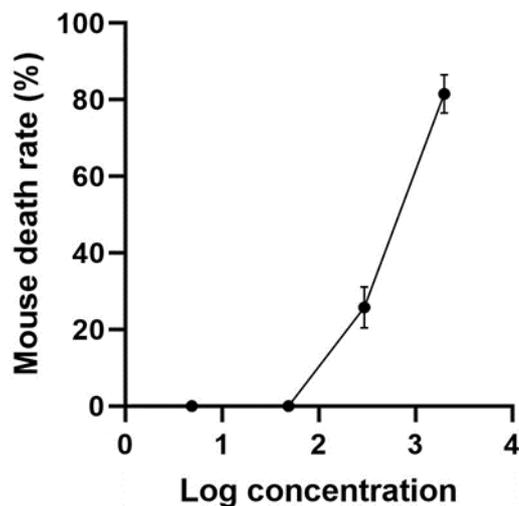

Figure 4: Acute toxicity curve of the methanolic extract of Quercus suber L. (obtained by Soxhlet) on mice, according to the method of Dragstedt and Lang (1944). Values are mean ± SD of three biological replicates

Candidiasis is an infection by *Candida albicans* strains that can affect intestinal walls, mouth, vagina, and skin. The current study aimed to evaluate the anticandidal effectiveness *Quercus suber* extracts. Water and methanol solvents, maceration, decoction and Soxhlet extractions techniques were used to investigate their effects on the yield and bioactive extracted compounds. Yield of extract calculation revealed that the AE obtained by decoction displayed the highest yield as compared with methanolic extracts. Similar findings were reported by Dhanini *et al.* (2017) [20]. These results may be attributed to water polarity and temperature parameters [21]. Vergara-Salinas *et al.* (2012) showed that the increment of solvent temperature improves plant particles wetting by decreasing surface tension that ultimately results in higher extraction yield [22]. The two methanolic extraction obtained by maceration and Soxhlet also showed a good extraction yield percentage. A yield of 16% and 17.6% was recorded with maceration and Soxhlet, respectively. In previous work, cork oak bark extracts were only obtained with the Soxhlet. Thus, for the first time, we were able to perform successful extraction using maceration. Moreover,

by using Soxhlet, the yield of extract was by 17.6%, which was significantly higher than that of reported by Castola *et al.* (2005) [23]. The use of Soxhlet although gave the best yield (17.6%) for a period of 32 hours and with less consumption of plant material and solvent. Extractions by maceration gave an almost similar yield, but it requires more time, more plant material and solvent. Thus, Soxhlet can be considered as more efficient compared with the maceration.

The antifungal activities study revealed that plant extracts exert an important anticandidal effect against *Candida albicans* tested with a variable degree. This inhibitory property varies depending on the extract nature and the tested strain. For instance, methanolic extracts obtained by maceration and Soxhlet displayed an inhibitory effect against *Candida* strains with a MIC reaching 6.25 and 3.12 mg/ml, respectively. Previous studies have underlined a high antimicrobial activity of cork oak against *Staphylococcus aureus* and *Escherichia coli* strains [24]. Akroum (2017) showed that acetone extract of the powder of *Quercus suber* has an active effect on *Candida albicans* [25]. Güllüce et al. (2004) reported an antimicrobial activity of methanolic extract of

Quercus ilex L. leaves against *Candida albicans* L. due to the fact the MIC (125 µg/ml) was lower than the MIC of the conventional drug (500 µg/ml) [26]. This antifungal activity against *Candida* genus was also reported with Methanolic extracts of the leaves of *Q. suber, Q. cerris, Q. pubescens* and *Q. coccifera* [27,28].

Minimum fungicidal concentrations are among various *in vitro* microbiological parameters used to determine the fungicidal activity of antimicrobial agents. The fungicidal profile of the three extracts was variable depending on the extraction method. The best fungicidal effect against the five *Candida* strains was recorded with the methanolic extract obtained by Soxhlet. This extract also displayed a great fungistatic effect on L14. The other methanolic extract (obtained by maceration) displayed a fungistatic effect on the five tested strains and a fungicidal activity against L12 strain at 50 mg/ml. The fungistatic effect of the aqueous extract and the other methanolic extract demonstrated a temporary action of these extracts on the pathogens development without eliminating them completely [29]. These differences in the fungicidal profile between the three different extracts can be due to the difference in the chemical composition of the extracts.

The acute toxicity of ME did not show any significant difference in mice weight injected with 300 mg/kg of the methanolic extract while, a significant decrease in weight was recorded in mice treated with 2000 mg/kg of methanolic extract. The lethal dose LD50 was determined based on the logarithm of Soxhlet extract doses and the percentage of lethality. This parameter was estimated to be around 1150 mg/kg in male mice when administrated intraperitoneally, which can be considered as slightly dangerous based on previous report [30]. The LD50 value in our work was lower than that advanced by Milrzaei and al, in 2013, which shows that the LD50 dose of the *Quercus suber* fruit powder aqueous extract administrated intraperitoneally to mice was clearly greater than 5000 mg/kg [31]. This difference can be linked to the plant material (part) used, which suggest that the *Quercus suber* fruit extracts are less toxic than those obtained from the bark [32]. Also, the LD50 value of *Quercus suber* remains higher than those found in *Anogeissus leiocarpus* and *Mansonia altissima*, whose LD50s were 290,81 and 186,5 mg/kg of body weight, respectively [33].

Conclusion

This work has therefore contributed to the discovery of the antimicrobial potential of cork oak bark extracts against *Candida* genus. By exploring the antifungal activity of the aqueous and the two methanolic extracts through the determination of the MIC and the MFC, we found that the extracts displayed an anticandidosic activity against the five *Candida albicans* tested strains, which remained different depending on the method of extraction (decoction, maceration and Soxhlet) and the nature of the solvent used for the extraction (water or methanol). The Soxhlet extract proved to be the best, offering in addition to a strong anticandidosis activity even at high concentrations, a good yield in a short time and with less consumption of plant material and solvent. This process could therefore be extended at an industrial scale and can be proposed as an alternative solution to synthetic pharmaceutical products that are today against fungal infections caused by *Candida albicans*. Moreover, the identification of the active molecules of the methanolic extracts would also be promising to explain the anticandidosis activity of cork oak bark extracts. Further analysis should be conducted to identify the biologically active compounds present in the methanolic extracts of cork oak bark responsible for the anticandidosic activity that has been demonstrated in this work.

Authors' contributions

QLA, RL, RH, JB and NG designed the research. QLA, RL, RH, YZ and SB performed the research and statistical analyses. QLA, YZ and SB analyzed the data. All authors contributed to writing the paper.

Data Availability Statement

All relevant data are within the paper and its Supporting Information files.

Conflict of Interest

We have no conflicts of interest to disclose.

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