
Research Article

The therapeutic effects of *Juglans regia* Linn (Walnut) extracts on oral *Candida* infection

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Abstract

The difficulties associated with oral *Candida* infection management necessitate the development of novel antifungal medicines in order to widen the spectrum of activity against *Candida albicans*. The aim of this work is to study the oral anticandidal activity of *Juglans regia* L. cultivated in Algeria. This plant was chosen due to its traditional use for the treatment of oral infections. Methanolic, ethanolic, butanolic, ethylic acetate and acetonc extracts of the bark of *J. regia* L. were extracted in a Soxhlet device and screened for in vitro activity against *C. albicans*. Plant preparations were screened for antifungal activity using a standard agar well diffusion assay. Following a study of the antimicrobial activity of plant extracts, their minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values were determined using a broth microdilution assay. Among *J. regia* L. extracts, ethylic acetate extract had potent antifungal activity against *Candida* strain with diameters of inhibition ranging from 15.16±0.76 to 20.83±0.76 mm, followed by acetonc extract with diameters of inhibition ranging from 14.83±0.76 to 19.33±0.28 mm. The *J. regia* L. demonstrated MIC values ranging from 0.46875 to 3.75 mg mL⁻¹ and the lowest MIC was recorded for ethylic acetate, acetonc extracts and AMB at 0.46875 mg mL⁻¹. The ethylic acetate extract showed the lowest MFC value (0.9375 mg mL⁻¹), followed by butanolic extract (3.125 mg mL⁻¹). These results indicate that *J. regia* L. bark extracts can contain compounds with therapeutic potential against oral *C. albicans* and, hence, their possible use as therapeutic agents for oral candidiasis.

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1. Introduction

The oral cavity is widely acknowledged as the mirror of total body health (Kulkarni, 2023). While bacteria constitute the majority of the oral microbiota, it is impossible to ignore the fungus that make up a small portion of the oral microbiome. The most frequent type of this fungus is *Candida*, which is a common invader in the mouth and has become habituated to reside as commensals in 30 to 60% of healthy persons (Patel, 2022; Rapala-Kozik et al., 2023). When there is any imbalance in this relationship or the presence of other predisposing factors, they turn into the pathogen.

Francois Veilleux, a pediatrician, initially reported oral candidiasis in 1838. It is a superficial opportunistic oral cavity infection brought on by *C. albicans* (Al-Kolaibe et al., 2023). Particularly in the early and late stages of life, the most prevalent fungus infection in humans. It manifests as tongue, buccal, and palatal mucosal irritation. This disease develops as a result of interactions between the immune system, oral tissues, oral environment and microbiological variables (Feller et al., 2014).

Unfortunately, antifungal therapy is very limited; in addition; to prolong use of antifungal treatment will produce problems of side effects, for example hematological, hepatic, and/or renal toxicity and may be lead to the emergence of strain resistant (Kadhim, 2018). Study of natural compounds that are effective against *Candida* species. Considering their pharmacological efficacy and cost-effectiveness, a variety of plant extracts have garnered significant interest as supplementary and a substitute chemical antimicrobials and as therapeutic agents for oral diseases. This is especially the case with the *J. regia* species of tree, which is a member of the Juglandaceae family (Rébufa et al., 2022). Because of its tough, fibrous structure, the bark has been applied to mechanically clean teeth. Juglone is its primary and most significant ingredient (Khattak et al., 2022).

Despite *J. regia* L. (walnut) is widely used, it has not been extensively researched or given much attention. Consequently, the current work is a research on the antifungal properties of five distinct extracts of *J. regia* L. bark grown in Algeria against *C. albicans* strain isolated from patient cases with oral candidiasis infection diagnosed by a dentist at a clinic in Bechar, Algeria. Additionally, to distinguish between plant extracts produced with various polarity solvents (diluted acetone, methanol, 1-butanol, ethyl acetate, and ethanol) based on the polarity index, according to Snyder. Seeking to promote the usage among these substances from plants as alternative compounds for fungus control, well diffusion and microdilution assays are being applied.

2. Materials and methods

2.1. Fungal isolates

Isolates of *Candida* strain had been obtained from patients' oral cavities with oral candidiasis in a dental clinic by the swabbing method (Kadhim, 2018); no patient was under antifungal drugs. A sterile cotton swab was cultivated instantly on Sabouraud chloramphenicol agar to generate an isolated sample, which was aerobically cultivated at 37 °C for a period of 24 to 48 hours (Khadka et al., 2017). Colonies of *C. albicans* strain purified and diagnosed according to morphological characteristics.

2.2 Morphological identification

Simple gram staining by crystal violet was done for the screening of yeast-like cells and to confirm the purity of the culture. Speciation of *C. albicans* isolate was done by development at 45 °C on SDA for 10 days, the germ tube test in human serum, and the chlamydospore production test on Corn meal agar.

2.3 Plant material

The most frequent species in Algeria is the common walnut, which was brought over a century ago and is today grown in small plots in the Aures massif, as well as in the regions of Setif, Khenchela, Batna, Annaba, Tlemcen, Ain-Sefra, Skikda, and Grande Kabily. It can be cultivated in rows along fields and roadsides, as individual trees, or at various densities in orchards. Walnut farming is not included in Algeria's agricultural statistics. It is managed in a traditional way, with low-yielding single-variety orchards sown with unselected and ungrafted seedlings, with limited agro-botanical performance, and fruiting late (seventh or ninth year after planting) (Bonev, 1973; Ounis et al., 2003).

A sample of the dried bark of *J. regia* L., which was grown in Setif, Algeria, was used in our study. It was collected from one of Bechar's marketplaces.

2.4 Plant extracts

Five different solvents were utilized for the extraction process: 1-butanol ($C_4H_{10}O$), ethanol (C_2H_6O), ethyl acetate ($C_4H_8O_2$), methanol (CH_3OH), and diluted acetone (acetone: water 80:20; v/v) use a Soxhlet extractor in an ascending sequence of polarity while staying below the solvent's boiling point. The process was run until the decolourization of the solvent. After passing through Whatman filter paper (No. 1), the extracts were concentrated in a vacuum at 40 °C utilizing a rotary evaporator. The resulting residues were kept frozen until more tests (DeliBaş & Kiray, 2023).

2.5 Preparation of concentration

The extracts obtained were suspended in dimethyl sulfoxide (DMSO) to create a range of concentrations for antifungal activity screening, from 200 to 15 mg mL⁻¹.

2.6 Antifungal activity

Well diffusion method

Using the agar-well diffusion method, the anti-*Candida* spp. activity was realized. A *Candida* strain was cultivated on Sabouraud dextrose agar and incubated at 37 °C for eighteen hours. The cell density was adjusted to 10⁶ cells/mL (OD_{540 nm} = 0.5) after the yeast culture had been gathered and suspended in sterile saline (0.8 % NaCl) (Noumi et al., 2014).

The agar well diffusion method, as outlined by Perez et al. (1990), was used to search for the antifungal activity of the *J. regia* extract. Fungal organisms were suspended in saline solution (0.8% NaCl) and adjusted to a standard inoculum size of 1-2 x 10⁶ CFU/mL after growing on Sabouraud dextrose broth and being incubated at 37 °C for 18 hours. SDA petriplates were inoculated with 0.1 ml of fungal suspension using a wooden applicator and a sterile, non-toxic cotton swab. Punched six-millimeter-diameter agar wells were each filled with 20 µl of *J. regia* extract. They added 20 µL of DMSO as a negative control. For 24 to 48 hours, the treated plates containing *J. regia* and the control were incubated at 37 °C. Following the incubation period, the treated plates were examined to look for an inhibitory zone encircling the wells. The inhibitory zone was measured and reported in millimeters (mm). Every experiment was conducted three times, and the average (mean ± SD) values were obtained.

Microdilution method for the determination of minimal inhibition concentration (MIC)

Colorimetric microdilution method in microplate

This method is based on inhibiting fungal growth by diluting the respective extracts (active substances) in a liquid medium, as evidenced by the absence of growth in the wells using the redox indicator 2,3,5-triphenyltetrazolium chloride (TTC) as a marker of live fungi. The value of the minimal inhibitory concentration (MIC) for each extract against the examined strain was determined using the microdilution technique in a 96-well microplate according to the [Clinical and Laboratory Standards Institute \(CLSI\) broth microdilution methods \(2019\)](#), with some modifications; in order to quantify the antimicrobial activity of *Juglans regia* L. extracts.

Pipetting into the first row of the plate was a volume of 100 μL of Sabouraud dextrose broth and 100 μL of each extract diluted in 2% (v/v) of DMSO, derived from a stock solution of 15 mg mL^{-1} . Consequently, serial dilutions were carried out so that the test material was present in each well at progressively lower serial concentrations, ranging from 15 to 0.007 mg mL^{-1} . Subsequently, each well received 20 μL of fungal solution (106 CFU/mL). Under the same circumstances, the minimum inhibitory concentrations (MIC) of the positive standards fluconazole (FLC) and amphotericin B (AMB) were calculated at concentrations ranging from 15 to 0.007 mg mL^{-1} . In place of the test chemical, two columns containing all solutions other than the fungal solution and SD broth were used. Three identical plates were produced, and they were put in an incubator at 37 °C for 24 hours. As a measure of fungal growth, 30 μL of 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma) was added to each well after incubation. The lowest concentration at which TTC addition does not result in color change was found to be the Minimum Inhibitory Concentration (MIC) after a 30 minutes incubation period at 37 °C. Each experiment was carried out in triplicate, and the three data were averaged to get the MIC.

The determination of Minimum Fungicidal Concentration (MFC) and MFC/MIC ratio based on the MIC assay

Cultures that were treated and had concentrations that were both equal to and more than the MIC value were swabbed onto an agar plate for the minimum fungicidal concentration (MFC) assay. After incubation, the concentration that produced no subculture growth on agar was considered to be MFC ([Novita & Sutandhio, 2019](#)).

On Sabouraud's agar plates, 10 μL from each well exhibiting growth inhibition was grown for 48 hours at 37 °C. The MFC of the extract on the strain under test signified the lowest concentration that occurred when no colonies existed. To ascertain if the investigated substance had a fungicidal (MFC/MIC ratio < 4) or fungistatic (MFC/MIC ratio $\geq$ 4) action, the MFC/MIC ratio was calculated ([Peixoto et al., 2017](#)).

2.7 Statistical analysis

To undertake the statistical analysis of the samples, the standard deviation and the average of the three replicates for each event were determined. IBM® SPSS® Statistics, Version 21 (IBM Corp., Armonk, NY, USA) is used to analyze the data. The result variable's mean values were compared using a one-way analysis of variance. Tukey's post-hoc test was utilized to verify the significance of differences between the mean values at $P < 0.05$.

3. Result and discussion

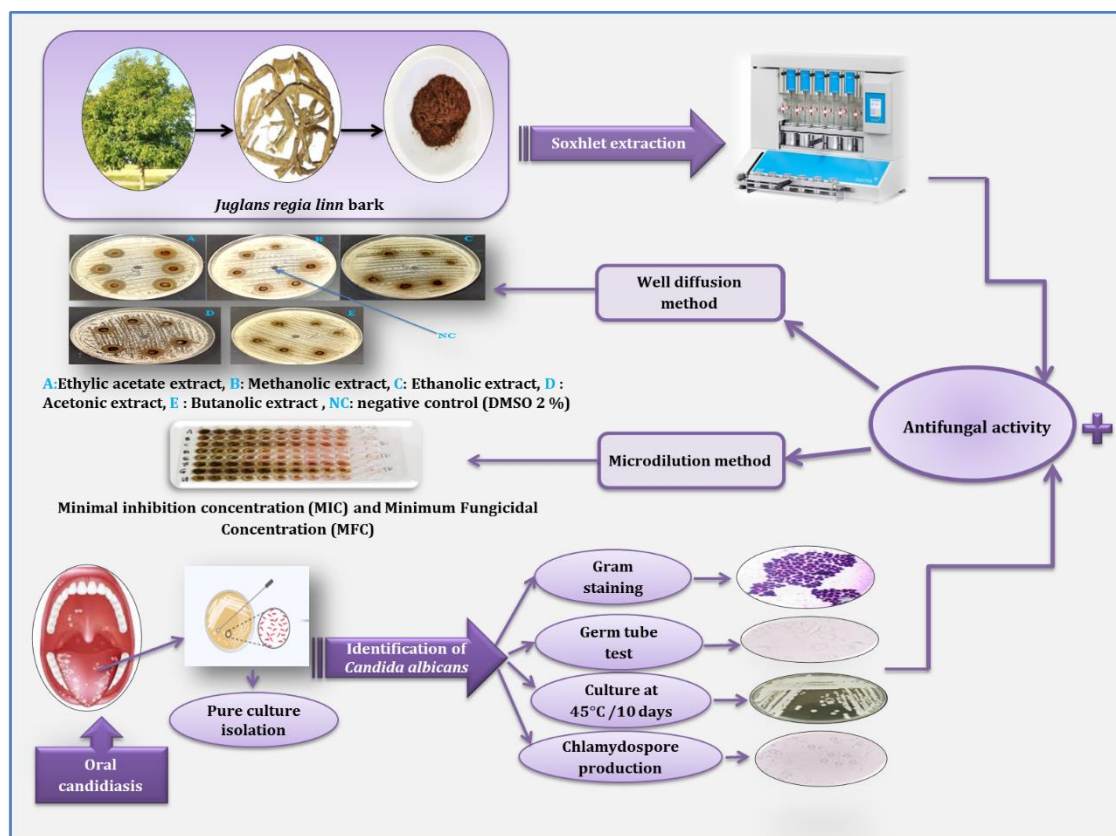
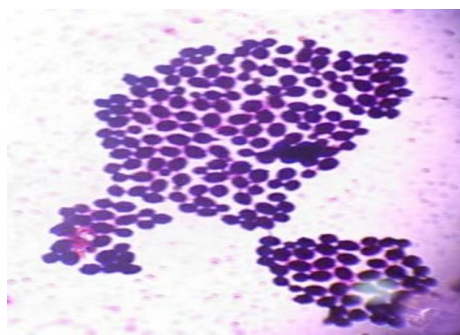


Figure 1. Graphical abstract of the antimicrobial activity of *Juglans regia* Linn bark extracts against oral *Candida albicans*

Figure 2 illustrates that *Candida* develops as creamy, smooth, pasty convex colonies on SDA at 45 °C and shows gram positive oval budding yeast cells. A germ tube test reveals the formation of germ tubes. On CMA, *Candida* produced abundant chlamydospores and pseudohyphae with clusters of spores.



A



B

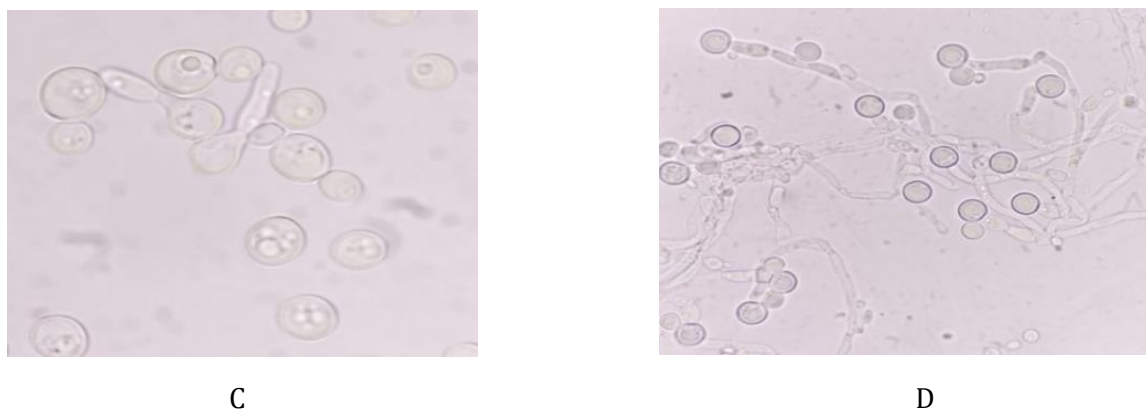


Figure 2. (A) *C. albicans* on Sabouraud dextrose agar after incubation at 45 °C; (B) Gram's staining of *C. albicans* under optical microscope (100×); (C) Germ tube formation in human serum under optical microscope (40×); (D) *C. albicans* with abundant chlamydospores on Corn meal agar incubation for 3 hours at 37 °C

The presence or absence of inhibition zone diameter, MIC, and MFC values were used to qualitatively and quantitatively evaluate the anti-candida properties of *J. regia* bark. Table 1 provides an overview of the outcomes of the well diffusion method recorded in Sabouraud dextrose agar. The present research revealed that while all five of the *J. regia* L. extraction solvents were effective against *C. albicans*, their degree of activity varied. The inhibitory zones varied from 7.33–12.83 mm (ethanolic extract) to 8.33–15.16 mm (methanolic extract) and from 15.16 to 20.83 mm for ethylic acetate extracts to 14.83–19.33 mm (acetonic extract) to 7–12.33 mm (butanolic extract). On the other hand, the standard drugs AMB and FLC showed an inhibitory effect on the *Candida* under test. *Candida* with zones of inhibition of 7.33 and 6.66 mm respectively, using a concentration of 15 mg mL⁻¹.

Bark from *J. regia* L. proved effective and showed antifungal properties against *C. albicans*. The ethylic acetate extract had the strongest antifungal properties (zone of inhibition: 20.83 mm), followed by the acetonic extract (zone of inhibition: 19.33 mm) using a concentration of 200 mg mL⁻¹. However, at a concentration of 15 mg mL⁻¹, among the plant extracts, the butanolic extract showed the lowest level of activity (zone of inhibition: 7 mm). However, oral *Candida* isolate was sensitive to all extract doses, and the ethanol extract of *J. regia* L. bark showed lower effectiveness of inhibition against the examined *Candida* than the methanolic extract. Moreover, ethanolic and methanolic extracts showed greater effectiveness in opposing the examined *Candida* than butanolic extract did. Acetonic and ethylic acetate extracts showed the highest levels of action.

Table 1. In vitro anticandidal activity of five extracts derived from *J. regia* bark (expressed as the diameter of inhibition zone in mm)

Extracts	Extract's concentration (mg mL ⁻¹)					
	200	150	100	50	25	15
	Inhibition zone as diameter in mm (mean ± SD) around the well impregnated with 20 µl of plant extract					
Ethanolic extract	12.83±0.76*	12.50±0.50**	11.33±0.57**	9.50±0.50**	8.33±0.28**	7.33±0.57*
Methanolic extract	15.16±0.28**	14.50±0.50**	13.66±0.57**	12.16±0.28**	11.33±0.57**	8.33±0.57**
Butanolic extract	12.33±0.57**	11.16±0.28**	10 ±0	9.33±0.57*	8.16±0.28**	7±0

Continued Table 1

Extracts	Extract's concentration (mg mL ⁻¹)					
	200	150	100	50	25	15
	Inhibition zone as diameter in mm (mean ± SD) around the well impregnated with 20 µl of plant extract					
Ethyllic acetate extract	20.83±0.76**	19.83±0.28***	18.66±0.57**	17.83±0.76**	17±0	15.16±0.76**
Acetonic extract	19.33±0.28***	18.50±0.50**	17.76±0.57**	17.33±0.57**	16.50±0.50**	14.83±0.76**
AMB	ND	ND	ND	ND	ND	7.33±0.57*
FLC	ND	ND	ND	ND	ND	6.66±0.57*
Negative control	0	0	0	0	0	0

All values are expressed as the means± standard deviation of three experiments. The data were analyzed through an unpaired t test. P values <0.05 were considered to be significant (*p<0.05, **p<0.001, ***p<0.0001), ND: not determined.

Table 2 presents the MIC and MFC values of the five extracts of *J. regia* L. bark and the standard drugs amphotericin B and fluconazole using the microdilution method. The lowest MIC was seen for ethyllic acetate, acetonic extracts and amphotericin B (MIC: 0.46875 mg mL⁻¹), followed by ethanolic and methanolic extracts with MICs of 0.9375 mg mL⁻¹. Furthermore, the butanolic extract demonstrated weaker activity (MIC: 1.5625 mg mL⁻¹) than did fluconazole (3.75 mg mL⁻¹). The MFC values varied from 0.9375 to 7.5 mg mL⁻¹ and the strongest one was observed for ethyllic acetate extract (MFC: 0.9375 mg mL⁻¹). However, butanolic extract, ethyllic acetate extracts and fluconazole showed fungicidal activity according to the ratio MFC/MIC < 4, while methanolic extract, ethanolic extract, acetonic extract and amphotericin B were able to prevent the *Candida* from growing with a fungistatic effect. The results reported in Tables 1 and 2 showed that the bark had substantial antifungal activity against yeast tested to different degrees. The walnut was particularly active against *Candida* as compared to the antifungal agents commonly used to prevent diseases caused by this strain (amphotericin B and fluconazole).

Table 2. Values of MFC/MIC ratios for the tested products

Extracts	CMI (mg mL ⁻¹)	MFC (mg mL ⁻¹)	MFC/MIC ratio	Effect
Ethanolic extract	0.9375	7.5	8	fungistatic
Methanolic extract	0.9375	7.5	8	fungistatic
Butanolic extract	1.5625	3.125	2	fungicidal
Ethyllic acetate extract	0.46875	0.9375	2	fungicidal
Acetonic extract	0.46875	7.5	16	fungistatic
FLC	3.75	7.5	2	fungicidal
AMB	0.46875	7.5	16	fungistatic

Different degrees of antifungal capacity against *Candida* specie collected from the mouth were demonstrated using extracts and they were highly significant (P < 0.05) against the microbial fungi (Table 3). Therefore, this plant showed growth-inhibiting properties against *C. albicans*.

Table 3. Difference between groups using one way ANOVA

Extract	F	P-value	Specification
Ethanolic extract	50.336	0,000000	Highly significant
Methanolic extract	79.541	0,000000	Highly significant
butanolic extract	81.840	0,000000	Highly significant
Ethylic acetate extract	39.662	0,000000	Highly significant
Acetonic extract	24.373	0,000007	Highly significant

Effective treatment of this pathogenic yeast depends on an accurate and timely diagnosis (Nadeem et al., 2010). Hereby, the isolated *Candida* sp. can be identified as *C. albicans* based on the findings of the studies conducted by Marinho et al. (2010). However, further biochemical and molecular identification are needed for confirmation.

The objective of the research was to examine the anti-candida effects of dried *J. regia* L. extracts cultivated in Algeria and used in traditional cosmology in Bechar. The test plant part was its bark. According to our research, the five *J. regia* L. extraction solvents (1-butanol, ethanol, ethyl acetate, methanol and diluted acetone) were active against the *C. albicans* strain; however, their efficacy differed. The more efficient extraction solvent was ethylic acetate extract, which had diameters of inhibition that varied from 20.83 ± 0.76 to 15.16 ± 0.76 mm and MIC: 0.46875; MFC: $0.9375 \text{ mg mL}^{-1}$. The MIC and MFC results were highly effective and competitive with the referenced antibiotics. Regarding the MIC values of fluconazole and amphotericin B, it is important to note that the tested fungal strain was susceptible to the antifungals tested, according to Arendrup et al. (2012).

Moreover, MFC/MIC ratios revealed that *J. regia* has a fungicidal and fungistatic mechanism. This selectivity is the result of the broad spectrum antimicrobial properties of walnut bark extracts that prevented the growth of multiple pathogenic microorganism species, including a pathogenic yeast (*C. albicans*) and both Gram+ (*S. aureus* and *S. mutans*) and Gram- (*E. coli* and *P. aeruginosa*) bacteria (Han et al., 2017). Our findings are in accordance with those found by Noumi et al. (2014). Using an extract concentration of 300 mg mL^{-1} , they demonstrated that the ethylic acetate extract of *J. regia* bark exhibited the highest antifungal activity against the *C. albicans* isolate (zone of inhibition: 18.66 mm), while the acetonic extract demonstrated weaker activity against *Candida* than the methanolic and ethylic acetate extracts. Asma et Yasmin (2021) and Muzafer and Paul (2018) demonstrated the anticandidal action of *J. regia* methanolic and ethylic acetate extracts, which supports our findings. The most successful extract as an antimicrobial against oral microbiota was the acetonic extract of *J. regia* L. bark, according to Deshpande et al. (2011).

Santos et al. (2013) confirm that the primary reason for *J. regia*'s health benefits is its chemical composition. *J. regia* bark remnants are abundant in phenolic chemicals, including flavonoids, phenolic acids, hydrolyzable tannins, and gallic tannins (Ben Ticha et al., 2022). The results of several other studies indicated that polar solvents such as methanol, ethanol, water, acetone, and ethyl acetate are considered to be the best solvents for polyphenol extraction when compared to the extracts of other non-polar solvents, including petroleum ether, chloroform, and n-butanol (Jahanban-Esfahlan et al., 2019), that could explain why butanolic extract has the least amount of action. *J. regia* L. mostly comprises phenolic chemicals called naphthaquinones (Sharma et al., 2009). Due to its chemical reactivity and bioactivity, juglone (5-hydroxy-1,4-naphthoquinone) is one of the several naphthoquinones that is of particular interest (Ghosh & Sinha, 2008). Previous research has demonstrated that the antifungal action may also be connected to the presence of "juglone." (Arasoglu et al., 2016; Wianowska et al., 2016).

Ethylic acetate is a superior extracting solvent in terms of juglone yield and stability, according to Sharma et al.'s findings (2009), which validates the strong anticandidal activity of this extract in our study. Noumi et al. (2014), Sytykiewicz et al. (2015), Naseri et al. (2016), Raja et al. (2017),

Kocaçalışkan et al. (2018), Al-Maliki & Ahmed (2020), Jafer and Naser (2020), Huo et al. (2020), D'Angeli et al. (2021), Ashraf et al. (2022), Ara et al. (2023) have also demonstrated the effect of *J. regia* L. against different *Candida* species.

4. Conclusion

In this study, the results indicate that oral *C. albicans* have different sensitivities towards different extracts and prove that the bark of *J. regia* is a promising source of new antimicrobial agents that can aid underdeveloped nations in reducing mouth infection, particularly oral candidiasis, considering its inexpensive cost and high effectiveness compared with the referenced antibiotics that are usually used to treat this infection. It might also be a good source of beneficial chemicals, such as juglone, one of the most important antifungal compounds in the bark. However, additional research is required to identify the molecules responsible for these activities by using chromatography methods like TLC, HPLC and GC-MS and elucidating their mechanisms of action, making this walnut by-product a promising source of bioactive compounds for pharmacological purposes that can replace synthetic products or at least reduce their use. Additionally, the exploration of synergistic effects through the combination of plant extracts presents a fresh approach to enhancing antimicrobial activity, offering novel insights that could pave the way for innovative treatment modalities.

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Author's declaration and contribution

The authors show no conflict of interest. All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by Hamida. All authors commented on previous versions of the manuscript, read and approved the final manuscript.

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