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Evaluation of Antifungal, Antioxidant Activities and Determination of Total Phenolic Compounds of Ethanolic Extract from Juglans Regia Bark †

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Abstract: *Juglans regia* (walnut tree) belong to Juglandaceae family and the Juglans genus, it is widely used in traditional medicine for its therapeutic effects. The purpose of the present work was to evaluate the *in-vitro* antifungal and antioxidant activity of ethanolic extracts prepared from *Juglans regia* bark and to determine the total phenolic compounds of the prepared extracts. The results of the antifungal activity study showed that *J.regia* bark ethanolic extracts were active against the three tested *Candida albicans* yeast strains with interesting MICs values (0.039 mg/mL). For both extract under study, the obtained results revealed appreciable antioxidant activity and a richness in total polyphenols with contents of 258 and 285 mg AGE/g of extract. The obtained data confirmed the correlation between the total phenolic compounds and biological activities of medicinal plants extract. In addition, *Juglans regia* bark extracts demonstrate notable antifungal and antioxidant activities, which explains their use in traditional medicine and making them suitable for use in pharmaceutical preparations.

Keywords: *Juglans regia*; bark extract; antifungal activity; antioxidant; phenolic content

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1. Introduction

In response to environmental factors such as abiotic stress, herbivore attack or interspecific interactions, plants produce an inexhaustible source of secondary metabolites. The vital importance of botanical products for humanity is mainly due to these secondary metabolites or phyto-compounds, which are active constituents with therapeutic properties [1].

Invasive fungal infections pose an ongoing and serious threat to human health and are associated with at least 1.5 million deaths worldwide annually [2]. The literature estimates a mortality of 30-40% for invasive candidiasis, 20-30% for disseminated cryptococcosis and a similar percentage for invasive aspergillosis [2,3].

Unfortunately, the treatment of these infections has been hampered by the emergence of antimicrobial resistance, which is considered one of the most serious threats to the world healthcare system this century [4]. On a global scale, phytochemicals have been tested as possible sources of new antimicrobial compounds, food preservation agents, and alternatives for treating infectious disorders because of their antifungal, antibacterial, antioxidant and antiviral properties [5].

The genus Juglans (family Juglandaceae) includes various species and is widespread throughout the world. *J. regia L.*, commonly known as walnut, is an important tree species, producing edible wood and nuts [6]. It is on the FAO's list of priority plants and is considered a strategic species for human nutrition. *J. regia* is an herb that has been used

traditionally in medicine for its antibacterial, antiviral, hepatoprotective, anthelmintic, and antidiarrheal properties. It also has anticancer properties. Additionally, antifungal activity has been reported [7].

The purpose of the present work was to evaluate the *in-vitro* antifungal and antioxidant activity of ethanolic extracts prepared from Juglans regia bark from western Algeria using maceration and soxhlet methods and to determine the total phenolic content of the prepared extracts.

2. Material and Methods

2.1. Plant Material

The barks of *J. regia* were harvested from Nedroma, Tlemcen (Algeria) during the period extending from October to November 2021. The plant material was identified, washed and then dried in the shade at room temperature. After drying, the plant materials were ground well into a fine powder using a mechanical blender.

2.2. Preparation of Bark Extract

In the current study, the ethanolic extract from *J. regia* barks was prepared in ethanol by two distinct methods: maceration at 37 °C for 72 h at 100 RPM and soxhlet extraction for approximately 4 h. After that, the extract was filtered using Whatman filter paper No. 1 and concentrated under vacuum on a rotary evaporator at 40 °C. The crude extracts were weighted in order to calculate the yield and then stored at 4 °C.

2.3. Total Phenolic Contents

Total phenolic content of the studied extracts was assessed using the the Folin-Ciocalteu colorimetric reagent according to the method described by b Slinkard and Singleton (1977) [8] using gallic acid as a standard. The titration was performed by mixing 0.5 mL of the ethanolic extract solution with 2.5 mL of Folin-Ciocalteu reagent (10 times diluted in distilled water) and 2.5 mL of freshly prepared 7.5% aqueous $NaHCO_3$ solution. The mixture was shaken and incubated at 45 °C for 45 min. After incubation, the absorbance was measured at 765 nm. The same method was used with standard solutions of gallic acid to obtain a standard curve.Each experiment was performed in triplicate and obtained results were reported as Means ± standard deviation.

2.4. DPPH Free Radical Scavenging Assay

The free-radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to assess the antioxidant activity studied extracts, as described in the literature [9]. In this method, one milliliter of different concentrations of the tested extract prepared in ethanol was added to 1 mL of DPPH ethanolic solution at a concentration of 0.2 mmol/L. The obtained mixture was vigorously agitated and left standing for 30 min at in the dark at room temperature. The absorbance was then measured at 517 nm for the resulting solution[9,10]. Ascorbic acid and BHT were used as standard and DPPH mixture without any sample served as blank. The following Equation (1) was used to calculate the inhibition of the free radical DPPH in percent (I %).

$$I\% = (A\ blank - A\ sample/A\ blank) \times 100\% \quad (1)$$

The IC50s were calculated graphically by the linear regression formula of the inhibition percentages as a function of different concentrations of the sample tested.

2.5. Ferric-Reducing Antioxidant Power Assay (FRAP)

In this study the FRAP assay was carried out to evaluate the total antioxidant capacity of tested samples as described by Oyaizu (1986) [11,12]. Different concentrations ethalonic extract (mg/mL) in ethanol were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50 °C for

20 min. After that, trichloroacetic acid (2.5 mL, 10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). Absorbance was read at 700 nm against a blank. Analyses were conducted in triplicates. Ascorbic acid was used as the standard compound.

2.6. Antifungal Activity

The antifungal activity was determined using the disc diffusion and the micro-well dilution methods. The yeast strains used in this study are *Candida albicans (ATCC 26790*), *Candida albicans (ATCC 10 231)*, *Candida albicans (IP444)*. The microorganisms used in this study are belonging to the "American Typed Culture Collections" (ATCC).

2.6.1. Disc Diffusion Assay

Disc diffusion method was carried out according to the recommendations of the Clinical and Laboratory Standards Institute CLSI [13]. Sabouraud supplemented with glucose (2%) was inoculated on the surface with the appropriate microorganisms using a cotton swab. The inoculums were then allowed to dry. Then sterile filter paper discs (6 mm diameter) soaked with the tested extract were placed on the plates. Amphotericin B (0.2 mg/disc) were used as positive controls. Plates were incubated at 28 °C for yeast for 24 h. Each test was performed in triplicate. Afterwards, the zones of inhibition were measured.

2.6.2. Micro-Well Dilution Assay

Minimum inhibitory concentrations (MICs) of the plant extracts studied were performed using the method of microplate (96 wells) [14]. The tested yeasts were inoculated onto Sabouraud broth supplemented with glucose (2%). After 24 h, 100 µL of each inoculum (1 to 5 ×104 CFU/mL were prepared. Then, 100 µL of each sterile broth were placed in each line of the microplate. Afterwards, 100 µL of the extract were introduced into the first well and serially diluted. In the end, 100 µL of each inoculum were added. The microplates were sealed and incubated at 28 °C during 20 h. Each assay was carried out in triplicate incubation. The MIC was defined as the lowest concentration of plant extract that inhibits the visible growth.

3. Results and Discussion

3.1. Extraction Yield

In the present study the extraction from *J. regia* barks was performed by maceration at 37 °C for 72h and soxhlet extraction in ethanol. the yields and organoleptic characteristics are shown in the Table 1.

Table 1. Yield and organoleptic characteristics.

Extraction Method	Yied	Color and Consistence
maceration	0.9 [1]	Dark brown, solid
Soxhlet	4.2	Dark brown, viscous

[1] yield is expressed as a percentage.

From these results, we observe that the yield is variable and significantly depends on the extraction method used, even when employing the same organic solvent. The highest yield was achieved with the Soxhlet extraction method (4.20%), whereas maceration produced a lower average yield (0.9%)

3.2. Total Phenolic Content

In the current stud total polyphenol contents were determined from the linear regression equation of the calibration curve, and results are expressed as mg gallic acid equivalent per gram extract (mg GAE/g extract).the obtained results are shown in Table 2.

Table 2. Total phenolic content.

Extraction Method	Total Phenolic Content [a]
maceration	258 ± 0.018
Soxhlet	285 ± 0.022

[a] mg gallic acid equivalent per g of extract (mg GA/g). Values expressed are means ± SD of three measurements.

These results showed that the ethanolic extract of Juglans regia bark prepared by the soxhlet method has a higher total polyphenol content(285 ± 0.022 mg AGE/g extract) than the ethanolic extract of the same plant prepared by maceration (258 ± 0.018 mg AGE/g extract). Other research on ethyl acetate extracts of J. regia bark from the Mahdia region (Tunisia) revealed a phenolic content of 34.833 mg AGE/g [15]. In a separate study, the phenolic content of J. regia bark extracts prepared using four different solvents ranged from 118.5 to 311.5 mg AGE/g [16]. These differences in phenolic compound content can probably be explained by differences in origin, variety, harvesting season, geographical location, maturity, solvent and extraction method.

3.3. DPPH Free Radical Scavenging Assay

The electron or hydrogen atom donating ability of the tested extacts was determined by measuring the bleaching of the purple-colored DPPH ethanolic solution. The obtained results are shown in Table 3. The antioxidant activity was proportional to the concentrations and the lower IC50 value reflects better protective action. The studied extracts prepared by maceration and soxhlet extraction were able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H, reaching 50% of reduction with an IC_{50} of 0.011 and 0.0131 mg /mL respectively. The free radical scavenging capacity of both extracts was higher than that of Ascorbic acid and BHT used as positive control or standards antioxidant.

Table 3. Antioxidant activity as determined by DPPH assay.

Source	DPPH IC50 (mg/mL) [a]
Extract 1 (maceration)	0.011 ± 0.03
Extract 1 (Soxhlet)	0.0131 ± 0.02
Ascorbic acid	0.048 ± 0.1
BHT	1.16 ± 0.05

[a] Values expressed are means ± SD of three measurements.

3.4. Ferric-Reducing Antioxidant Power Assay (FRAP)

In the present study, the reducing power of both tested extracts was positively correlated with their concentrations, as shown in Table 4, with absorbance increasing as the concentration increased. Compared to ascorbic acid, the positive control, both extracts demonstrated higher reducing power. The ethanolic extract of *J. regia*, prepared using the Soxhlet method, exhibited the highest reducing power.

Table 4. Reducing power activity as determined by FRAP assay.

Source	FRAP (A° = 700 nm) [a]				
	0.2	**0.4**	**0.6**	**0.8**	**1** [1]
Extract 1 (maceration)	0.56 ± 0.03	0,93 ± 0.05	1.34 ± 0.08	1.78 ± 0.07	2.18 ± 0.09
Extract 1 (Soxhlet)	1.02 ± 0.07	1.60 ± 0.04	2.36 ± 0.1	2.58 ± 0.00	2.88 ± 0.04
Ascorbic acid	0.18 ± 0.05	0.28 ± 0.1	0.41 ± 0.06	0.48 ± 0.1	0.52 ± 0.02

[a] Values expressed are means ± SD of three measurements; [1] Concentrations are expressed as mg/mL.

3.5. Antifungal Activity

The antifungal activity of the J. regia ethanolic extracts were evaluated against three yeast strains: *C. albicans (ATCC 26790*), *C. albicans (ATCC 10 231)*, and *C. albicans (IP444).* The results are summarized in Table 5. Based on the results, we can conclude that the *J. regia* bark extract prepared using the Soxhlet method exhibited the highest antifungal activity against all three C. albicans strains, with a minimum inhibitory concentration (MIC) ranging from 39 to 78 μg/mL. The observed difference in antifungal activity between the two extracts, prepared using different methods, may be attributed to the loss of thermolabile compounds during the extraction process, grinding, or the method used to preserve the plant material.

Table 5. Antifungal activity of *J.regia* ethanolic extracts.

Yeast	Extraction Method			
	Maceration		**Soxhlet**	
	Zone of Inhibition [1]	**MIC** [2]	**Zone of Inhibition**	**MIC**
C. albicans (ATCC 26790)	12 ± 0.57	0.156 ± 0.00	14 ± 1	0.078 ± 0.00
C. albicans (ATCC 10 231)	10.33 ± 0.88	0.156 ± 0.00	13.66 ± 0.57	0.078 ± 0.00
C. albicans (IP444)	14 ± 0.33	0.078 ± 0.00	16 ± 0.66	0.039 ± 0.00

[a] Values expressed are means ± SD of three measurements; [1] Zones of inhibition are expressed as mm; [2] MIC are expressed as mg/mL.

4. Conclusions

Our results reveal that both ethanolic extracts of *Juglans regia* bark, prepared through maceration and Soxhlet extraction, demonstrate a richnes in phenolic compounds and possess a range of biological properties associated with their chemical composition. These extracts represent promising free radical scavenging activity and interesting reducing power which them promising sources of natural antioxidants with potential applications in the food, pharmaceutical, and cosmetic industries. Furthermore, *Juglans regia* bark extracts exhibit noteworthy antifungal activity, suggesting their utility in pharmaceutical formulations as novel natural antifungal agents to aid in the battle against multidrug-resistant fungal infections. Further research is essential to fully explore these benefits and their potential application.

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