

Original article

Antimicrobial Evaluations of *Juglans Regia* Bark Against Oral Pathogenic Microorganisms

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ABSTRACT

Keywords:

Juglans regia, Antimicrobial Activity, Oral Pathogens, Minimum Inhibitory Concentration, Phytochemicals, Natural Remedies.

Oral pathogenic microorganisms significantly contribute to various diseases, impacting both oral and systemic health, including chronic periodontitis and dental caries. Natural plants, including *Juglans regia* (walnut tree), have gained momentum for their therapeutic potential due to their antibacterial properties, offering a promising avenue to combat antibiotic resistance. This study fundamentally aimed to validate the traditional use of *Juglans regia* bark in maintaining oral health; it sought to evaluate the antibacterial and antifungal effects of *Juglans regia* bark. The methanolic extracts of *Juglans regia* bark, imported from China, were prepared using the maceration technique, various microorganisms procured from the American Type Culture Collection (ATCC), including Gram-positive bacteria (*Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA)), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and the local fungal strain *Candida albicans*. Antimicrobial activity was assessed using the agar well diffusion assay at concentrations of 250 mg/ml and 125 mg/ml, with chlorhexidine and phenol serving as reference standards. Minimum inhibitory concentrations (MIC) were determined using the microdilution technique, with serial dilutions ranging from 125 mg/ml down to 3.9 mg/ml. At 250 mg/ml, *Escherichia coli* exhibited the largest zone of inhibition (27mm), followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* (25mm, 23mm, and 25mm, respectively). At 125 mg/ml, *Candida albicans* showed the best results (22mm). The lowest effective inhibitory concentration (MIC) for *C. albicans* was 15.6 mg/ml, while *S. aureus* and *P. aeruginosa* also showed equal MICs.

Introduction

Oral pathogenic microorganisms play a crucial role in the development of various diseases, significantly impacting both oral and systemic health. Although chronic periodontitis, for instance, is characterized by inflammation leading to the loss of tooth attachment, which can result in systemic issues such as cardiovascular diseases and diabetes complications [1]. Nevertheless, the biofilm formed by these microorganisms is essential in the pathogenesis of periodontal disease, where different species such as *Streptococcus*, *Actinomyces*, *Neisseria*, and *Lactobacillus* species cooperate to create a disease-provoking microbiota [2]. Moreover, pathogenic bacteria can enter the bloodstream, linking oral health to systemic diseases and highlighting their significance in overall health. On the other hand, opportunistic pathogens as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*), which thrive in individuals with compromised immune systems and poor oral hygiene, further exacerbate oral microbial diseases [3]. Additionally, the presence of cariogenic bacteria, primarily *Streptococcus mutans*, in dental plaque contributes to dental caries, a prevalent condition caused by acid production from fermentable carbohydrates [4]. Thus, understanding the role of these microorganisms is vital for developing effective preventive and therapeutic strategies to mitigate their impact on oral and systemic health.

Recently, natural plants as Centipede cunninghami, Aloe vera, and Neem have been recognized for their therapeutic potential in treating periodontal diseases due to their antibacterial and anti-inflammatory properties [5]. Their extracts work by inhibiting bacterial growth and reducing gum inflammation, alongside other beneficial ingredients like coenzyme Q10 and folic acid [6-8]. Nowadays, medicinal plants offer a promising avenue for overcoming the persistence of antibiotic resistance in oral pathogenic bacteria [9, 10]. For instance, polyphenols can complex with macromolecules, inhibiting their functions and thereby helping to offset resistance mechanisms; on the other hand, flavonoids and terpenoids disrupt microbial membranes, which not only provides a direct antimicrobial effect but also enhances the permeability of bacterial membranes, making them more susceptible to antibiotics. Additionally, alkaloids serve as inhibitors of efflux pumps, a common bacterial resistance mechanism. The integration of these phytochemicals into treatment regimens could significantly improve outcomes against drug-resistant bacteria, particularly in cases where conventional antibiotics fail.

As research continues to uncover the potential of these natural compounds, they may play a crucial role in addressing the demanding public health challenge of antibiotic resistance [10-14]. However, *Juglans regia*

(Figure 1), commonly known as the walnut tree, is notable for its bark, which possesses various medicinal and ecological properties [15]. Although the bark is rich in phenolics and flavonoids, contributing to its antibacterial, antioxidant, anti-inflammatory, and anticancer activities [15,16]. Additionally, it has been studied for its heavy metal accumulation capabilities (Figure 2), revealing significant concentrations of metals like copper and lead, which can indicate environmental health [17]. Nevertheless, tannins present in the *J. regia* bark give the material a reddish-brown stain and help in the precipitation of proteins [18]. Some volatile compounds like gallic acid and saponins, which are responsible for the soapy texture and bitter taste, are also present. It also contains ash and has a mild abrasive effect as it is tough and fibrous [19]. Yet, one of the most important compounds in the *J. regia* bark is the organic compound called juglone. Juglone is formed from glucose and has a distinct phenyl group. Due to this compound, the bark of this plant holds numerous medicinal benefits. It acts as an antifungal, antibacterial, antiviral, antiparasitic, antioxidant, and antitumor agent [20-22].



Figure 1: Juglans Regia (walnut tree) and Extract Features.

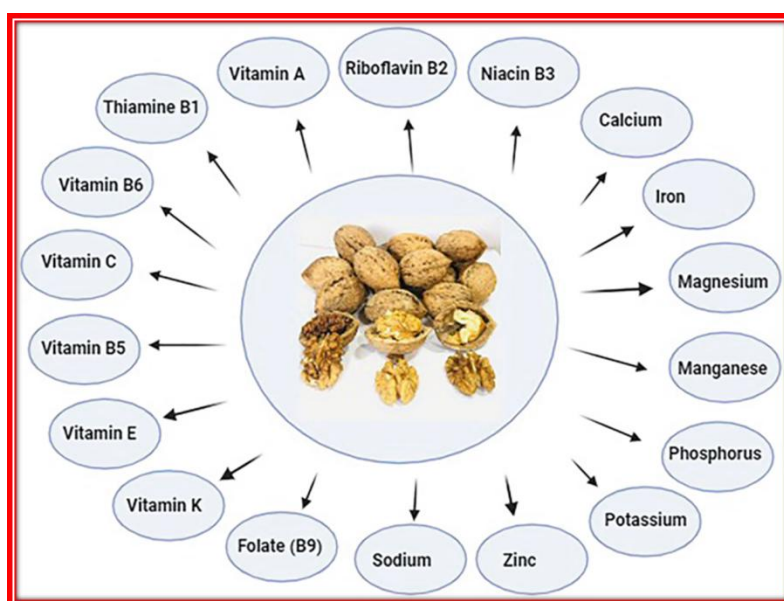


Figure 2: Vitamins and metals that are incorporated in Juglans Regia [23]

The antibacterial effect of Juglone was due to disrupting the cell membrane through various mechanisms, inhibiting DNA synthesis, interfering with energy production, and hindering bacterial proteins' function [24]. Tannins *J. regia* bark, on the other hand, bind to bacterial proteins, causing them to clump and lose their functionality. This multi-pronged approach likely contributes to the bark's efficacy against diverse pathogens [25]. In contrast, while the bark shows promising bioactivity, the potential toxicity of some compounds, such as juglone, necessitates careful evaluation. Further research is essential regarding heavy metals accumulated within the *J. regia* bark, warranting further toxicity investigation to balance its use in traditional medicine with safety [26].

In Libya, *J. regia* bark was used traditionally as a topical application to enhance gum and tooth health, in addition to natural colouring of lips [27]. In other countries *J. Regia* bark had been used contrarily as a topical home remedy for dermal inflammation and excessive perspiration of the palms and soles. It is also a common home remedy for the treatment of eczema [28]. The ethanolic extract found in it gives it excellent antioxidant properties. Some parasitic infections have also been cured with the help of *J. regia* bark extracts [28]. Therefore this study fundamentally aimed to prove and validating the traditional use of *J. regia* bark in maintain oral health by preventing pathogenic infections microorganisms through evaluating the

antibacterial and antifungal effects of *Juglans regia* bark against common oral pathogens, determine the minimum inhibitory concentration (MIC) of *Juglans regia* bark extract, and compare the effectiveness of plant extract with commercially available chlorhexidine as mouth wash.

Material and methods

Chemicals, reagents, and cultures

All chemicals' reagents used throughout this study for either identification and/or antibiotic susceptibility test were obtained from Sigma-Aldrich company, U.K., unless otherwise mentioned. All cultures were of the purest available grade BDH U.K. unless otherwise indicated; all bacterial growth media were obtained from Oxoid Company.

Plant Collection

Juglans regia bark used in this study was imported from China, commercially obtained from Tripoli markets. The best time to collect *Juglans regia* bark is in the spring or early summer. from China, commercially obtained from Tripoli markets. The best time to collect *Juglans regia* bark is in the spring or early summer.

Plant extract

In this study, the Methanol 96% was used to extract active constituents from *Juglans regia* bark and measured its weight, which was 9.708g. Using the maceration technique, the yielded extract was 2.036g.

Anti-microbial activity

Microorganisms

The microorganisms employed in the current study were procured from the American Type Culture Collection (ATCC). Deep freeze-stored cultures were employed for all bacterial strains and reactivated using brain heart infusion broth. Strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213), *Methicillin-resistant Staphylococcus aureus* (ATCC. 35126), Gram-negative bacteria *Escherichia coli* (ATCC.25922), *Pseudomonas aeruginosa* (ATCC. 24846), and a fungal local strain *Candida albicans* were obtained from Tripoli University Hospital.

Agar Well Diffusion Assay

In this study, Mueller-Hinton agar plates were inoculated with 0.1 ml of 10^6 CFU/ml bacterial suspension, while Sabouraud dextrose agar plates were inoculated with corresponding fungal strains. Following the creation of wells in the agar, 50 microliters of extract at concentrations of 250mg/ml and 125 mg/ml were added, and the zones of inhibition were measured after incubation at 37 °C for 18 hours, with chlorhexidine and phenol serving as reference standards.

Table 1: Muller-Hinton agar (MHA) medium

Ingredients	Gram/Litre
Beef Extract	gm 2.00
Acid Hydrolysate of Casein	gm 17.50
Starch	1.50 gm
Agar	17.00 gm
Distilled Water	ml 1000

Table 2: Sabouraud's dextrose agar (SDA) medium

Ingredients	Amount
Dextrose	gm. 40.0
Peptone	gm. 10.0
Agar	20.0 gm.
Distilled Water	1000 ml
pH	5.6

MIC assay

The minimum inhibitory concentration (MIC) of any given compound is characterized as the lowest concentration that entirely prevents observable growth (turbidity in liquid media). MIC values were ascertained utilizing the microdilution technique. Solutions of the extract of *Juglans regia* bark were formulated in phosphate buffer at a concentration of 125 mg/ml. From this stock solution, serial dilutions of the extracts (125, 62.5, 31.25, 15.6, 7.8 and 3.9mg/ml) were prepared to determine the MIC. All assessments were conducted in triplicate.

Results

The obtained results indicated that *Escherichia coli* showed the best results at a concentration of 250mg/L with a zone of inhibition of 27mm compared to *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*, 25, 23, and 25mm, respectively. At a concentration of 125mg/ml, the zone of inhibition slightly decreased, but *Candida albicans* demonstrated the best results with a zone of inhibition of 22mm, followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, 21, 19, and 20, respectively. The bark extract of *J. regia* did not affect MRSA (Table 1). *J. regia* did not affect MRSA. Table 1

Table 3. Zone of inhibition of *J. regia* bark in the cup-cut method.

Microorganisms	Zone in mm of 250mg /ml	Zone in mm of 125 mg /ml	Phenol 5%	0.12% chlorohexidine
<i>Pseudomonas aeruginosa</i>	25 mm	19mm	24mm	23mm
<i>Escherichia coli</i>	27mm	20mm	30mm	25mm
<i>Candida albicans</i>	25mm	22mm	35mm	20mm
<i>Staphylococcus aureus</i>	23mm	21mm	32mm	20mm
MRSA	0mm	0mm	29mm	12mm

The result of the MIC of *J. regia* bark against the test microorganisms has shown the lowest effective inhibitory concentration. Of *J. regia* bark extract against *C. albicans* 15.6mg/ml, and such extract reveals equal MIC against both *S. aureus* and *P. aeruginosa*. However, 62.5mg/ml is accordingly considered the MIC of *E. Coli*.

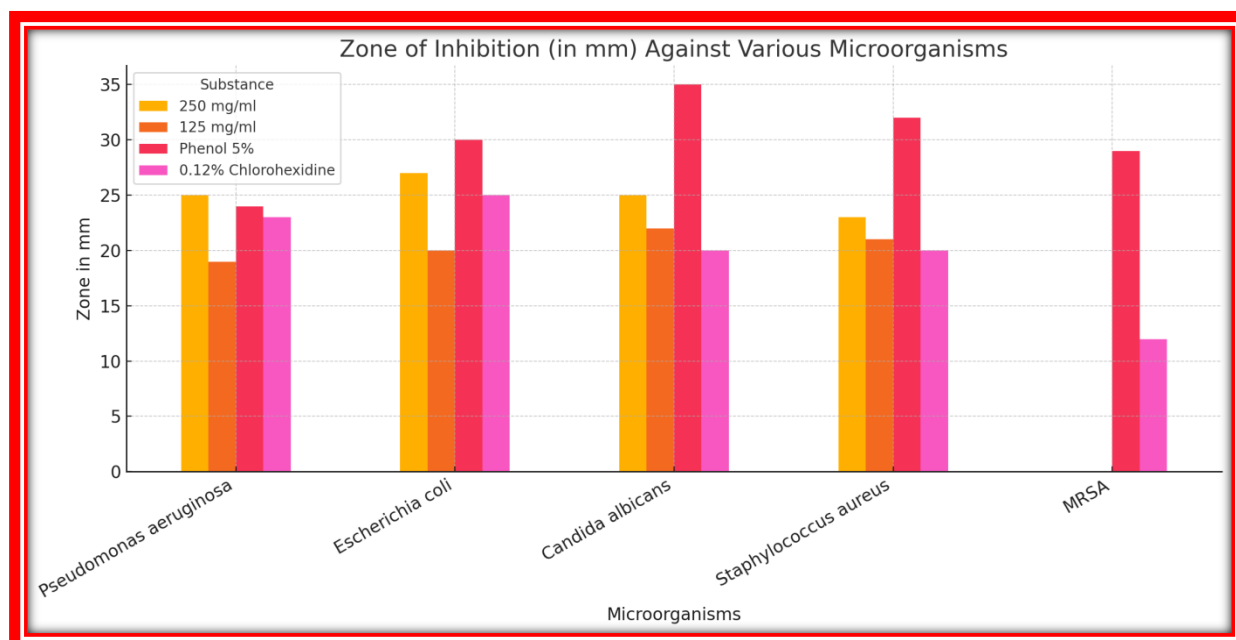


Figure 3. Zone of inhibition of *J. regia* bark in the cup-cut method.

Table 4: MIC results of *J. regia* bark extract

Microorganisms	<i>E. Coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
MIC	62.5mg/ml	31.25mg/ml	31.25mg/ml	15.6 mg/ml

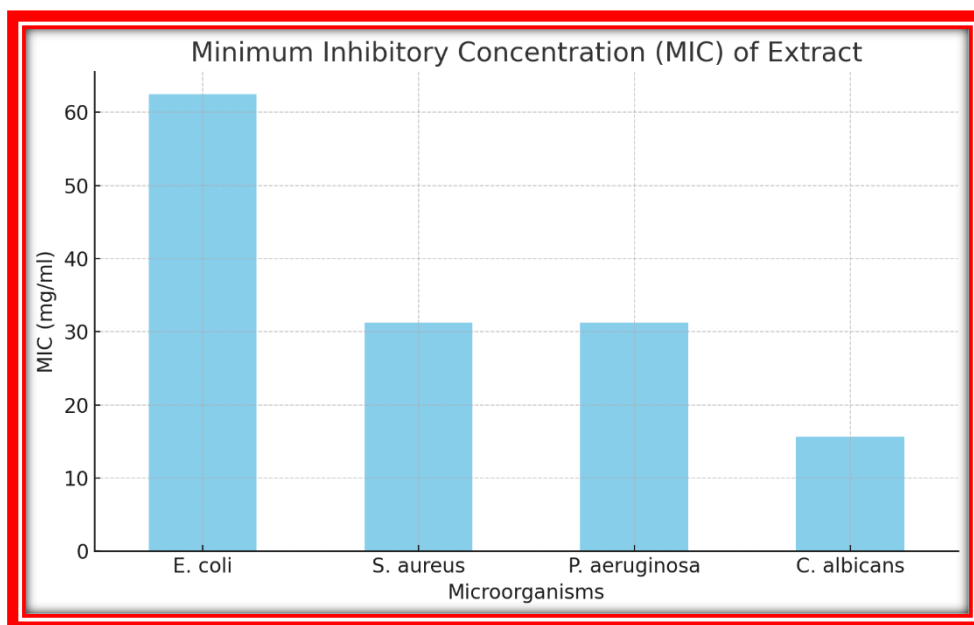


Figure 4: Zone of inhibition of *J. regia* bark in cup-cut method.

Discussion

In this study, the effect of methanolic extracts of *J. regia* bark on selected microorganisms was investigated using a cup-cut assay method, at concentrations of 250 mg/ml and 125 mg/ml, which exhibited significant antimicrobial activity, compared to phenol as a control and chlorohexidine 0.12%. This study reveals that the antimicrobial effect of *J. regia* bark increases with the increase in concentration. However, Zakavi, Faramarz, et al., a study published in 2013 in Iran, advocate the results obtained by this study as they find the high concentrations of ethanol of *J. regia* bark exhibit significant antimicrobial effects against *Streptococcus mutans* and *Staphylococcus aureus*. Moreover, the effect was concentration-dependent, with ethanolic extracts showing larger zones of inhibition compared to aqueous extracts [29]. Nevertheless, a study conducted in 2020 in Kurdistan found that the minimum inhibitory concentrations (MIC) for alcoholic and aqueous extracts of *J. regia* were 14mg/ml and 18mg/ml, respectively, equivalent to the MIC obtained by this study, 15mg/ml of the methanolic extracts [30].

Other research highlighted the capacity of *J. regia* extracts to act against both gram-positive (e.g., *Bacillus cereus*, *Bacillus subtilis*, *S. aureus*) and gram-negative bacteria (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, *Klasiella pneumoniae*) [21]. Further study emphasized the potential of *J. regia* as an alternative to antibiotics to prevent bacterial resistance [29]. This study augments the significant antimicrobial activity of methanolic extracts of *J. regia* against both bacterial and fungal species, supporting the potential use of *J. regia* in improving oral hygiene and as an alternative to traditional antibiotics.

Conclusion

The current study confirms the antimicrobial potential of *J. regia* bark extracts, validating its traditional use as a preventive remedy for various microbial diseases, including dental caries and periodontal disease. These findings indicate that *J. regia* bark could serve as a valuable source of natural antifungal and antibacterial lead compounds for pharmaceutical applications. The study supports the growing interest in utilizing natural remedies over synthetic medications, highlighting the potential of *J. regia* as an effective alternative to chemotherapy. Further research is required to isolate and characterize the specific bioactive compounds responsible for the detected antimicrobial effects. In vivo studies and clinical trials are necessary to confirm the efficacy and safety of these extracts for practical use. Such investigations could pave the way for developing new natural antimicrobial agents, thereby advancing the field of phytotherapy and promoting sustainable healthcare solutions.

Acknowledgment

The writers passionately thank all the technicians and academic staff at the microbiology and pharmacognosy departments at the faculty of pharmacy, University of Tripoli. Without their support, this research work would never have been accomplished.

Conflict of interest. Nil

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