

COMPARATIVE EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF METHANOLIC AND AQUEOUS *JUGLANS REGIA* L. BARK EXTRACTS AGAINST BACTERIAL AND FUNGAL PATHOGENS

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Abstract

The comparative antibacterial and antifungal activity of methanolic and aqueous bark extracts of *J. regia* from Upper Dir, Khyber Pakhtunkhwa, Pakistan was evaluated. Three different concentrations (30, 60, 90 mg/mL) of *J. regia* extracts were tested on four bacterial and four fungal strains using agar well diffusion and minimum inhibitory concentration (MIC) methods, respectively. The antimicrobial activity of both extracts was concentration-dependent, with different efficiencies against different microorganisms. The methanolic extract showed more antibacterial activity with an inhibition zone of 18-22 mm at 90 mg/mL, whereas the aqueous extract showed an inhibition zone of 8-18 mm at 90 mg/mL. Two-way ANOVA results confirmed that there were significant differences in antibacterial activity between the type of extract and concentrations for all the microorganisms ($p < 0.001$), while only for *Escherichia coli* there was a significant interaction between extract type and concentration ($p = 0.031$). The antifungal activity was more species-dependent, with the aqueous extract being more active against *Aspergillus niger*, *Candida albicans* and *Trichophyton rubrum* at 90 mg/mL, while methanolic extract was more active against *Fusarium oxysporum*. Significant type $\times$ concentration interactions were found for all fungi ($p \leq 0.001$). The methanolic extract consistently showed lower MIC values than the aqueous extract. The antimicrobial activity of *J. regia* bark was generally considered to be significant, and the results were related to the extraction solvent, concentration and type of microorganism. More phytochemical and mechanistic research should be conducted in methanolic and aqueous extracts to determine the compound responsible for this activity.

INTRODUCTION

Antimicrobial resistance (AMR) has become one of the greatest threats in the field of public health, as it has affected the efficacy of existing

antimicrobial treatments and made the treatment of infectious diseases increasingly difficult (Ho et al., 2025). The continued emergence and dissemination of antimicrobial-resistant

microorganisms have significantly increased the demand for new antimicrobial strategies and chemical scaffolds. In this context, plant-derived natural products have attracted increasing scientific interest because plants produce structurally diverse secondary metabolites with antibacterial, antifungal, antibiofilm, and other biological activities (Uttam et al., 2025). A number of studies have been recently conducted, identifying the potential of natural products derived from plants as sources of novel antimicrobial compounds, as well as complementary strategies for the prevention of antimicrobial resistance (AMR) related infections. Many plant secondary metabolites, including phenolic compounds, flavonoids, tannins, terpenoids, alkaloids, quinones, and other compounds, have been reported to show antimicrobial activities via various biological mechanisms, leading to the need for further exploration of medicinal plants as sources of antimicrobial compounds (Daglia, 2012; Ramata-Stunda et al., 2022).

Several factors such as the plant species, component of plant extracts, geographical origin, environmental conditions, extraction solvent, extraction procedure, and the susceptibility of the target microorganism affect the antimicrobial activity of plant-derived preparations (Zouine et al., 2024). Plant extracts constitute chemically complex mixtures in which biological activity may result from individual constituents or interactions among multiple compounds. The polarity and physicochemical properties of the extraction solvent are particularly important because they determine the classes and relative quantities of phytochemicals recovered from plant material. Consequently, extracts prepared using different solvents can exhibit substantially different antimicrobial activities (Wang et al., 2025). It is important to perform comparative assessment of extraction systems not only to establish the relative antimicrobial activity of crude plant preparations but also to define the conditions that could be helpful in the subsequent isolation and characterization of bioactive constituents (Li et al., 2024).

Juglans regia L. (Juglandaceae), Persian or English walnut, is an economically important tree species found throughout Central Asia, southeast Europe and the Himalayas. The plant parts such as bark, leaves, roots, green husk, fruits and oil have been utilized for a long time in traditional medicine and studied for different biological activities (Gupta et al., 2019). A wide variety of compounds such as phenolic acids, flavonoids, tannins, and naphthoquinones are identified in *J. regia*, depending on the part of the plant and the extraction method (Bhat et al., 2023a). Among these constituents, the characteristic naphthoquinone of Juglone (5-hydroxy-1,4-naphthoquinone), has been of special interest due to its antimicrobial and other biological activities reported (Bhat et al., 2023b). Recent studies have also shown antimicrobial properties in various parts of *J. regia* plants, and the variations in phytochemical composition and extraction systems also influence the magnitude and spectrum of the observed activity (Barekat et al., 2023). These findings support the continued investigation of *J. regia*, particularly its bark, as a potential source of plant-derived antimicrobial preparations.

In particular, comparative information on the antibacterial and antifungal activity of aqueous and methanolic *J. regia* bark extracts from northwestern Pakistan remains limited. Therefore, the present study aimed to comparatively evaluate the in vitro antibacterial and antifungal activities of aqueous and methanolic extracts of *J. regia* bark collected from Upper Dir, Khyber Pakhtunkhwa, Pakistan. Antimicrobial activity was assessed against selected bacterial and fungal microorganisms using agar well diffusion and minimum inhibitory concentration (MIC) assays. The study further examined concentration-dependent responses and differences in susceptibility among the tested microorganisms. This comparative approach provides region-specific preliminary evidence regarding the influence of extraction solvent on the antimicrobial activity of *J. regia* bark and establishes a basis for future phytochemical and mechanistic investigations.

Although the antimicrobial activity of *Juglans regia* has been studied before but the plant part, geographical source, extraction solvent, extraction conditions and the different microbial species tested can be significant factors in the antimicrobial activity. Specifically, there is a scarcity of comparative data on the antimicrobial activity of the aqueous and methanolic extracts of *J. regia* bark from the northwest of Pakistan. Thus, the current study was designed to assess the in vitro antibacterial and antifungal activity of the bark extract of *J. regia* both in aqueous and methanolic solvent was carried out and compared from Upper Dir, Khyber Pakhtunkhwa, Pakistan. The antimicrobial activity was tested against selected bacterial and fungal microorganisms by using agar well diffusion and minimum inhibitory concentration (MIC) methods. Additionally, the responses and susceptibility of the microorganisms tested were analyzed for concentration-dependent responses and differences. This comparative method gives the preliminary data on the antimicrobial activity of the bark of *J. regia* and gives a basis for further phytochemical and mechanistic studies in order to obtain the detailed information about the effects of extraction solvent on the antimicrobial activity of *J. regia* bark at regional level.

1 Materials and Methods

1.1 Plant Material Collection and Preparation

The *Juglans regia* L. bark was collected from the local area in Upper Dir District, Khyber Pakhtunkhwa, Pakistan. The collected bark was thoroughly washed with tap water and subsequently rinsed with distilled water to wash away soil and other external contaminants. The material was air dried by keeping it at room temperature (24-27 °C). The dried bark was then crushed to a fine powder and kept in sealed plastic bags until extraction. The plant material was used to prepare aqueous and methanolic extracts. All the bacterial and fungal strains for antimicrobial evaluation were obtained from the Infectious Disease Diagnostic Laboratory, Department of Biotechnology, Abdul Wali Khan University Mardan, Pakistan.

1.2 Preparation of Juglans regia Bark Extracts

Aqueous and methanolic extracts of *J. regia* bark were prepared using a plant material-to-solvent ratio of 1:5 (w/v). Briefly, 100 g of the powdered bark was separately mixed with 500 mL of ethanol and 500 mL of distilled water. The mixtures were maintained on an automatic shaker for 72 h to facilitate extraction. Following extraction, the mixtures were filtered through Whatman filter paper to remove the plant residues. The filtrates were concentrated using a rotary evaporator. The resulting crude extracts were dark brown in appearance and were stored at 20 °C until further antimicrobial analysis. Extract yield was determined as the percentage of dry extract obtained relative to the initial dry mass of plant material.

1.3 Sterility Testing of Plant Extracts

The prepared plant extracts were assessed for microbial contamination before antimicrobial testing. For sterility assessment, 0.2 mL of each extract was inoculated onto sterile Mueller-Hinton agar (MHA) and incubated at 37 °C for 18–24 h. The extracts were also assessed for fungal contamination by inoculating the corresponding samples onto Sabouraud dextrose agar (SDA), followed by incubation at 25 °C for 3 days. Following incubation, the plates were examined for visible microbial growth. Extracts showing no visible bacterial or fungal growth were considered free from detectable microbial contamination and were subsequently used for antimicrobial assays.

1.4 Test Microorganisms

Four bacterial strains were evaluated for antibacterial activity: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Shigella* spp., and *Salmonella enterica*. The bacterial cultures were obtained from the Infectious Disease Diagnostic Laboratory, Department of Biotechnology, Abdul Wali Khan University Mardan, Pakistan. The identity and strain designations of all cultures were verified according to the records provided by the

supplying laboratory. Four fungal strains were evaluated for antifungal activity: *Candida albicans*, *Aspergillus niger*, *Trichophyton rubrum*, and *Fusarium oxysporum*. The fungal cultures had previously been preserved in the laboratory and were revived on Sabouraud dextrose agar (SDA) before antimicrobial testing. The strain or accession numbers, where available, were recorded according to the laboratory's culture collection records.

1.5 Antibacterial Activity

The antibacterial activity of *J. regia* bark extracts was evaluated using the agar well diffusion method. A single colony of each bacterial strain was inoculated into sterile Mueller-Hinton broth (MHB) and incubated overnight with shaking. The bacterial suspensions were adjusted to a 0.5 McFarland standard and uniformly inoculated onto Mueller-Hinton agar (MHA) plates using sterile cotton swabs. Wells of 6 mm diameter were prepared in the agar using a sterile cork borer. Aqueous and methanolic extracts were tested at concentrations of 30, 60, and 90 mg/mL, with the appropriate volume of each extract added to the respective wells. Tetracycline (30 µg) served as the positive control, while dimethyl sulfoxide (DMSO) served as the negative control. The plates were incubated at 37 °C for 24 h, after which the diameter of the inhibition zones was measured in millimeters. All experiments were performed in triplicate.

1.6 Antifungal Activity

The antifungal activity of aqueous and methanolic extracts was tested for four fungal strains by agar well diffusion method. Preserved fungal cultures were revived by growing on Sabouraud dextrose agar (SDA) for 3 days at 28 °C. After growth, the fungus suspensions were prepared and adjusted to 0.5 McFarland turbidity standard. A fungal suspension was standardized and then 100 µL were evenly distributed on the surface of SDA plates with a sterile swab for the assay. Sterile cork borer was used to prepare wells, 6 mm in diameter, with appropriate spacing on the surface of the agar. The aqueous

and methanolic extracts were tested at different concentrations (30, 60 and 90 mg/mL) and 100 µL of each extract concentration was added to the appropriate wells. Nystatin (50µL) was used as the positive control and DMSO was used as the negative control. The plates were incubated for 3 days (72 h). After incubation the diameters of the inhibition zones were measured in millimeters. Each assay was repeated three times.

1.7 Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of the aqueous and methanolic extracts were determined using two-fold serial dilution in Mueller-Hinton broth to (MHB). After incubation for 24 h at 37 °C, MIC was determined as the lowest concentration of the extract which did not show any visible growth of the microbes. The MIC values of the tested bacterial and fungal strains were noted and presented in the results.

1.8 Statistical Analysis

Each antimicrobial assay was performed in triplicate, and the diameters of the zones of inhibition were reported as mean ± standard deviation (SD). The effects of extract type (aqueous and methanolic), extract concentration (30, 60, 90 mg/mL) and their interaction on inhibition-zone diameter were evaluated by two-way analysis of variance (ANOVA) separately for each bacterial and fungal strain. Statistical significance was set at $p < 0.05$.

2 Results

2.1 Antibacterial Activity of *Juglans Regia* L. Bark Extracts

The antibacterial activity of methanolic and aqueous bark extracts of *Juglans regia* L. was evaluated against four bacterial strains, *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Shigella dysenteriae*, using the agar well diffusion assay. Extract concentrations of 30, 60, and 90 mg/mL were tested. Tetracycline (30 µg) was used as the positive control for the bacterial assays. The inhibition-zone diameters varied according

to extract type, concentration, and bacterial strain.

2.1.1 Antibacterial Activity of the Methanolic Extract

The methanolic *J. regia* bark extract exhibited antibacterial activity against all four tested bacterial strains at all three concentrations (Figure 1; Table 1). Based on the experimental results shown in Figure 1, inhibition zones ranged from 9 to 22 mm across the tested concentrations. At 90 mg/mL, the methanolic extract produced inhibition zones of 19, 21, 22, and 18 mm against *E. coli*, *S. enterica*, *K. pneumoniae*, and *S. dysenteriae*, respectively. The greatest inhibition was observed against *K. pneumoniae* (22 mm), followed by *S. enterica* (21

mm), *E. coli* (19 mm), and *S. dysenteriae* (18 mm). At 60 mg/mL, inhibition zones were 14, 16, 17, and 13 mm against *E. coli*, *S. enterica*, *K. pneumoniae*, and *S. dysenteriae*, respectively. At 30 mg/mL, the corresponding inhibition zones were 9, 11, 10, and 9 mm, respectively. Thus, the methanolic extract generally showed greater antibacterial activity at higher concentrations, with *K. pneumoniae* showing the largest inhibition zone at 90 and 60 mg/mL, whereas *S. enterica* showed the largest zone at 30 mg/mL. Tetracycline produced inhibition zones of 13, 31, 30, and 18 mm against *E. coli*, *S. enterica*, *K. pneumoniae*, and *S. dysenteriae*, respectively (Table 1).

Table 1. Antibacterial activity of methanolic *Juglans regia* L. bark extract against selected bacterial strains.

Bacterial strain	Methanolic extract 90 mg/mL	Methanolic extract 60 mg/mL	Methanolic extract 30 mg/mL	Tetracycline (30 µg)
<i>Escherichia coli</i> ATCC 25922	19 ± 0.31	14 ± 0.30	9 ± 0.80	13 ± 0.20
<i>Salmonella enterica</i> ATCC 13312	21 ± 0.20	16 ± 1.00	11 ± 0.90	31 ± 2.00
<i>Klebsiella pneumoniae</i> ATCC 700603	22 ± 0.40	17 ± 0.40	10 ± 0.80	30 ± 1.30
<i>Shigella dysenteriae</i> ATCC 13313	18 ± 0.40	13 ± 0.60	9 ± 0.70	18 ± 0.90

Values are presented as mean ± SD. Inhibition-zone diameter is expressed in mm.

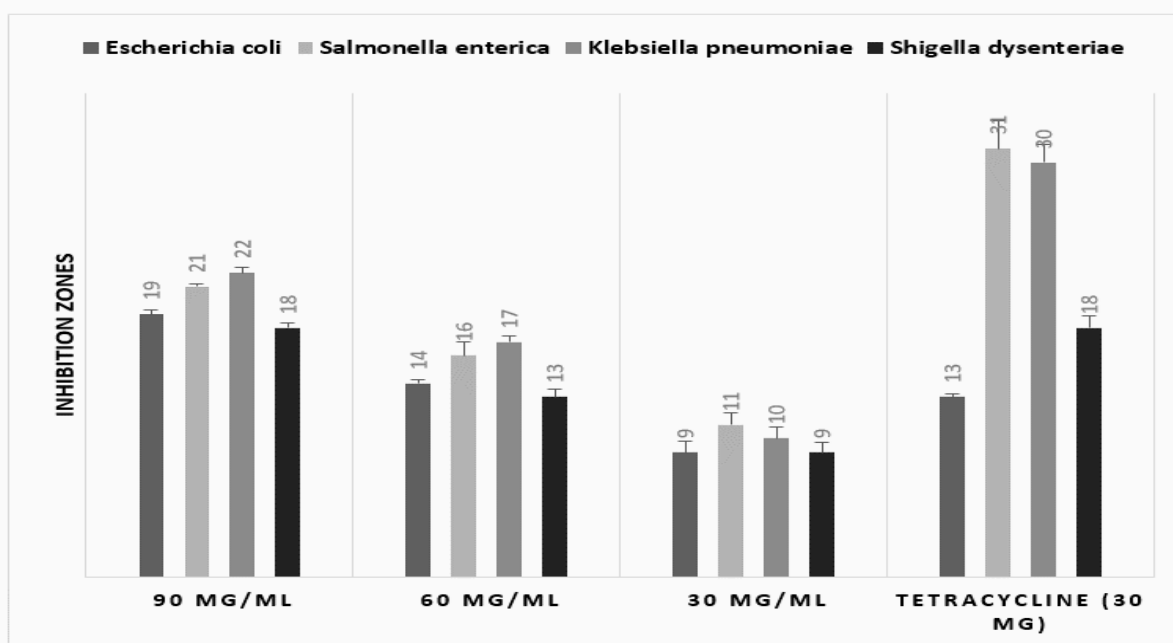


Figure 1: Antibacterial activity of methanolic *Juglans regia* L. bark extract against four bacterial strains.

2.1.2 Antibacterial Activity of the Aqueous Extract

The aqueous *J. regia* bark extract also exhibited antibacterial activity against all four tested bacterial strains at the three evaluated concentrations (Table 2). Inhibition-zone diameters ranged from 3.3 to 18 mm. At 90 mg/mL, the aqueous extract produced inhibition zones of 8, 18, 16, and 13 mm against *E. coli*, *S.*

enterica, *K. pneumoniae*, and *S. dysenteriae*, respectively. The greatest inhibition was observed against *S. enterica* (18 mm), followed by *K. pneumoniae* (16 mm), *S. dysenteriae* (13 mm), and *E. coli* (8 mm). At 60 mg/mL, inhibition zones were 4.7, 11, 10, and 7.3 mm, respectively. At 30 mg/mL, the corresponding inhibition zones decreased to 3.3, 6.3, 5.3, and 4.3 mm.

Table 2. Antibacterial activity of aqueous *Juglans regia* L. bark extract against selected bacterial strains.

Bacterial strain	Aqueous extract 90 mg/mL	Aqueous extract 60 mg/mL	Aqueous extract 30 mg/mL	Tetracycline (30 µg)
<i>Escherichia coli</i>	8 ± 0.31	4.7s ± 0.30	3.3 ± 0.80	13 ± 0.20
<i>Salmonella enterica</i>	18 ± 0.20	11 ± 1.00	6.3 ± 0.90	31 ± 2.00
<i>Klebsiella pneumoniae</i>	16 ± 0.40	10 ± 0.40	5.3 ± 0.80	30 ± 1.30
<i>Shigella dysenteriae</i>	13 ± 0.40	7.3 ± 0.60	4.3 ± 0.70	18 ± 0.90

Values are presented as mean ± SD. Inhibition-zone diameter is expressed in mm.

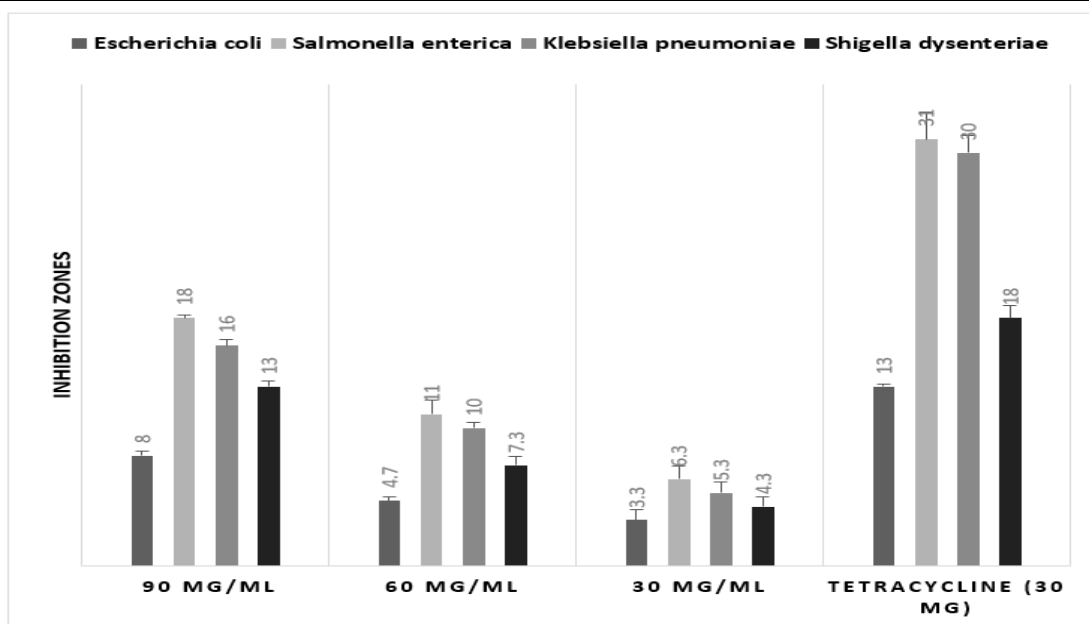


Figure 2. Antibacterial activity of Aqueous *Juglans regia* L. bark extract against four bacterial strains.

2.1.3 Comparison of Methanolic and Aqueous Extracts

The methanolic extract generally exhibited greater antibacterial activity than the aqueous extract, particularly at 90 and 60 mg/mL. At 90 mg/mL, the methanolic extract produced inhibition zones of 19, 21, 22, and 18 mm against *E. coli*, *S. enterica*, *K. pneumoniae*, and *S. dysenteriae*, respectively, compared with 8, 18, 16, and 13 mm for the aqueous extract. At 60 mg/mL, the corresponding inhibition zones were 14, 16, 17, and 13 mm for the methanolic extract and 4.7, 11, 10, and 7.3 mm for the aqueous extract. At 30 mg/mL, the difference between extracts was smaller for some strains, although the methanolic extract remained equal to or more active than the aqueous extract against most strains. Among the tested bacteria, *K. pneumoniae* showed the highest inhibition zone with the methanolic extract at 90 mg/mL (22 mm), whereas *S. enterica* showed the highest response to the aqueous extract at the same concentration (18 mm). Tetracycline produced larger inhibition zones than either plant extract against *S. enterica* and *K. pneumoniae*, while the plant extracts produced inhibition zones

comparable to or greater than tetracycline against some of the other tested strains.

"The inhibition-zone diameters of the plant extracts were comparable to those of tetracycline for some bacterial strains, although tetracycline produced substantially larger zones against *S. enterica* and *K. pneumoniae*."

Statistical Analysis of Antibacterial Activity

2.1.4 Two-way ANOVA of Antibacterial Activity

A two-way analysis of variance (ANOVA) was performed separately for each bacterial strain to assess the effects of extract type (methanolic and aqueous) and extract concentration (30, 60, and 90 mg/mL) on inhibition-zone diameter. The model also included the extract type × concentration interaction to determine whether the effect of concentration differed between the two extracts. Statistical significance was considered at $p < 0.05$.

The analysis revealed that extract type had a significant effect on antibacterial activity in all four bacterial strains ($p < 0.001$). Similarly, extract concentration significantly affected inhibition-zone diameter in all strains ($p < 0.001$). A significant extract type × concentration

interaction was observed for *E. coli* ($F = 4.72$, $p = 0.031$), indicating that the difference between methanolic and aqueous extracts varied across concentrations. In contrast, the interaction was

not significant for *S. enterica* ($F = 1.12$, $p = 0.358$), *K. pneumoniae* ($F = 1.70$, $p = 0.223$), or *S. dysenteriae* ($F = 0.62$, $p = 0.552$) (Table 3).

Table 3. Two-way ANOVA of the antibacterial activity of *Juglans regia* L. bark extracts

Bacterial strain	Source of variation	df	F	p
<i>E. Coli</i>	Extract type	1	184.07	<0.001
	Concentration	2	41.42	<0.001
	Extract type × concentration	2	4.72	0.031
<i>S. enterica</i>	Extract type	1	42.22	<0.001
	Concentration	2	112.17	<0.001
	Extract type × concentration	2	1.12	0.358
<i>K. pneumoniae</i>	Extract type	1	80.64	<0.001
	Concentration	2	89.14	<0.001
	Extract type × concentration	2	1.70	0.223
<i>S. dysenteriae</i>	Extract type	1	103.26	<0.001
	Concentration	2	91.51	<0.001
	Extract type × concentration	2	0.62	0.552

Antifungal activity of *Juglans regia* L. bark extracts

The antifungal activity of methanolic and aqueous *J. regia* bark extracts was evaluated against four fungal strains, *Aspergillus niger*, *Candida albicans*, *Trichophyton rubrum*, and *Fusarium oxysporum*, using the agar well diffusion assay. Extract concentrations of 30, 60, and 90 mg/mL were tested. Nystatin was used as the positive control, whereas DMSO was used as the negative solvent control. Both extracts produced measurable inhibition zones against all four fungal strains, although the magnitude of inhibition varied among extract types, concentrations, and fungal strains.

2.1.5 Antifungal activity of the methanolic extract

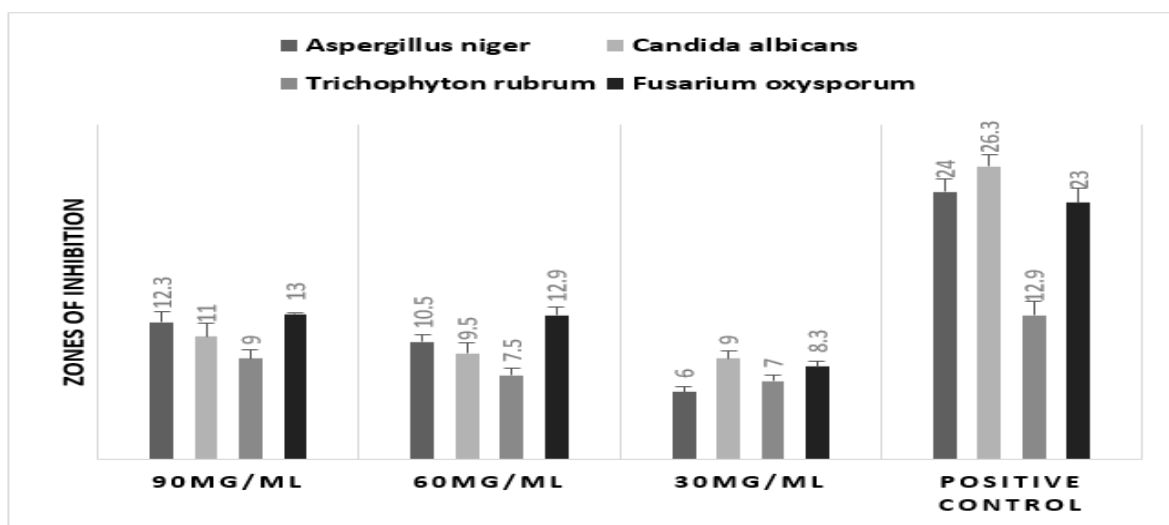
The methanolic extract of *J. regia* bark had antifungal activity against all four fungal strains

tested, as shown in Table e. Inhibition zones were observed in the range of 9.0 -13.0 mm at 90 mg/mL concentration *F. oxysporum* showed the largest inhibition zone (13.0 ± 0.1 mm), followed by *A. niger* (12.3 ± 0.9 mm) and *C. albicans* (11.0 ± 1.2 mm), whereas *T. rubrum* showed the smallest zone (9.0 ± 0.8 mm). At 60 mg/mL, inhibition zones ranged from 7.5 to 12.9 mm, with *F. oxysporum* showing the largest zone (12.9 ± 0.7 mm). At 30 mg/mL, inhibition zones ranged from 6.0 to 9.0 mm, with *C. albicans* showing the largest zone (9.0 ± 0.7 mm).

Overall, the methanolic extract generally showed greater antifungal activity at higher concentrations, although the magnitude and concentration response varied among fungal strains.

Table 3: Antifungal activity of *Juglans Regia* Methanolic extract against four tested Fungi

Microorganisms	Concentration mg/ml			Positive Control	Negative Control
	90	60	30		
<i>Aspergillus niger</i>	12.3 ± 0.9	10.5 ± 0.7	6 ± 0.5	24 ± 1.2	—
<i>Candida albicans</i>	11 ± 1.2	9.5 ± 0.9	9 ± 0.7	26.3 ± 1	—
<i>Trichophyton rubrum</i>	9 ± 0.8	7.5 ± 0.7	7 ± 0.5	12.9 ± 1.3	—
<i>Fusarium oxysporum</i>	13 ± 0.1	12.9 ± 0.7	8.3 ± 0.5	23 ± 1.3	—

Figure 3. Antifungal activity of methanolic *Juglans regia* L. bark extract against four fungal strains.

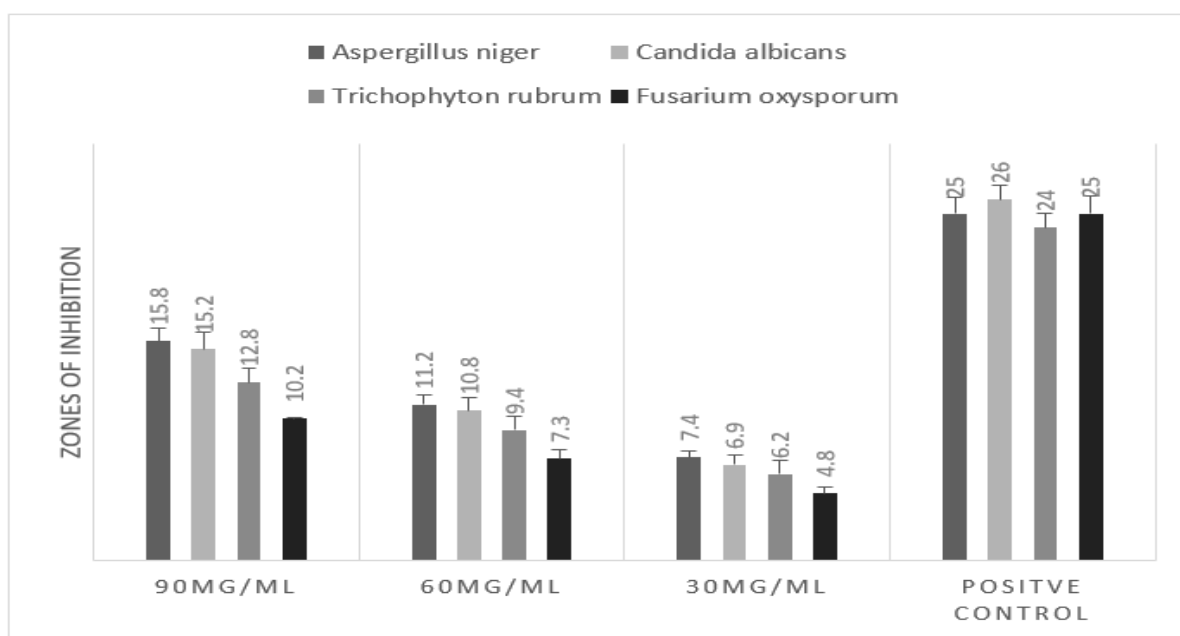
2.1.6 Antifungal activity of the aqueous extract

The aqueous *J. regia* bark extract inhibited all four fungal strains at the tested concentrations (Table 4). At 90 mg/mL, inhibition zones ranged from 10.2 to 15.8 mm. The largest inhibition zones were observed against *A. niger* (15.8 ± 0.9 mm) and *C. albicans* (15.2 ± 1.2 mm), followed by *T. rubrum* (12.8 ± 0.8 mm), whereas *F. oxysporum* showed the smallest zone (10.2 ± 0.1 mm). At 60 mg/mL, inhibition zones ranged from 7.3 to 11.2 mm, with *A. niger* showing the largest zone (11.2 ± 0.7 mm). At 30 mg/mL, inhibition zones ranged from 4.8 to 7.4 mm, with *A. niger* again showing the largest inhibition zone (7.4 ± 0.5 mm).

The aqueous extract therefore showed a generally concentration-dependent reduction in inhibition-zone diameter as the extract concentration decreased.

Table 4: Antifungal activity of *Juglans Regia* Aqueous extract against four tested Fungi

Microorganisms	Concentration mg/ml			Positive Control	Negative Control
	90	60	30		
<i>Aspergillus niger</i>	15.8 ± 0.9	11.2 ± 0.7	7.4 ± 0.5	25 ± 1.2	–
<i>Candida albicans</i>	15.2 ± 1.2	10.8 ± 0.9	6.9 ± 0.7	26 ± 1	–
<i>Trichophyton rubrum</i>	12.8 ± 0.8	9.4 ± 0.7	6.2 ± 0.5	24 ± 1.3	–
<i>Fusarium oxysporum</i>	10.2 ± 0.1	7.3 ± 0.7	4.8 ± 0.5	25 ± 1.3	–

Figure 4. Antifungal activity of aqueous *Juglans regia* L. bark extract against four fungal strains.

2.1.7 Comparative antifungal activity of methanolic and aqueous extracts

The relative activity of the two extracts varied according to fungal strain and concentration. At 90 mg/mL, the aqueous extract produced larger inhibition zones than the methanolic extract against *A. niger* (15.8 vs. 12.3 mm), *C. albicans* (15.2 vs. 11.0 mm), and *T. rubrum* (12.8 vs. 9.0 mm). In contrast, the methanolic extract produced greater inhibition against *F. oxysporum* (13.0 vs. 10.2 mm). At 60 mg/mL, the aqueous extract produced larger inhibition zones against *A. niger* (11.2 vs. 10.5 mm), *C. albicans* (10.8 vs.

9.5 mm), and *T. rubrum* (9.4 vs. 7.5 mm), whereas the methanolic extract showed greater activity against *F. oxysporum* (12.9 vs. 7.3 mm). At 30 mg/mL, the aqueous extract showed greater activity against *A. niger* (7.4 vs. 6.0 mm), whereas the methanolic extract produced larger inhibition zones against *C. albicans* (9.0 vs. 6.9 mm), *T. rubrum* (7.0 vs. 6.2 mm), and *F. oxysporum* (8.3 vs. 4.8 mm).

2.2 Two-way ANOVA of antifungal activity

A two-way analysis of variance (ANOVA) was performed separately for each fungal strain to

evaluate the effects of extract type (methanolic and aqueous) and extract concentration (30, 60, and 90 mg/mL) on inhibition-zone diameter. The extract type $\times$ concentration interaction was also included in the model. Statistical significance was considered at $p < 0.05$.

The analysis showed that extract type significantly affected antifungal activity in all four fungal strains. Extract concentration also had a significant effect on inhibition-zone diameter for

all strains ($p < 0.001$). A significant extract type $\times$ concentration interaction was observed for *A. niger* ($F = 13.09$, $p < 0.001$), *C. albicans* ($F = 39.87$, $p < 0.001$), *T. rubrum* ($F = 12.69$, $p = 0.001$), and *F. oxysporum* ($F = 19.55$, $p < 0.001$) (Table X). These findings indicate that the effect of extract concentration differed between methanolic and aqueous extracts across the tested fungal strains.

Table 4. Two-way ANOVA of antifungal activity of *J. regia* bark extracts

Fungal strain	Source of variation	df	F	p-value
<i>A. niger</i>	Extract type	1	64.44	<0.001
	Concentration	2	334.87	<0.001
	Extract type $\times$ concentration	2	13.09	<0.001
<i>C. albicans</i>	Extract type	1	36.53	<0.001
	Concentration	2	180.81	<0.001
	Extract type $\times$ concentration	2	39.87	<0.001
<i>T. rubrum</i>	Extract type	1	18.33	0.001
	Concentration	2	84.73	<0.001
	Extract type $\times$ concentration	2	12.69	0.001
<i>F. oxysporum</i>	Extract type	1	217.78	<0.001
	Concentration	2	124.47	<0.001
	Extract type $\times$ concentration	2	19.55	<0.001

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) of the methanolic and aqueous *Juglans regia* L. bark extracts were determined using a two-fold serial broth dilution method. For the bacterial isolates, the extracts were tested in Mueller-Hinton broth (MHB) and incubated at 37 °C for 24 h. The MIC was defined as the lowest extract concentration at which no visible microbial growth was observed.

Table 5. MIC values of methanolic and aqueous *J. regia* bark extracts against bacterial strains.

Bacterial strain	Methanolic extract MIC (mg/mL)	Aqueous extract MIC (mg/mL)
<i>Escherichia coli</i> ATCC 25922	12.5	50
<i>Salmonella enterica</i> ATCC 13312	6.25	25
<i>Klebsiella pneumoniae</i> ATCC 700603	6.25	25
<i>Shigella dysenteriae</i> ATCC 13313	12.5	50

The methanolic extract showed lower MIC values against all four bacterial strains than the aqueous extract. *S. enterica* and *K. pneumoniae* exhibited the greatest susceptibility to the methanolic extract, with MICs of 6.25 mg/mL, whereas *E. coli* and *S. dysenteriae* showed higher MICs of 12.5

mg/mL. A similar pattern was observed with the aqueous extract, for which MICs ranged from 25 to 50 mg/mL. These findings were generally consistent with the greater antibacterial activity of the methanolic extract observed in the agar well diffusion assay.

Table 6. MIC values of methanolic and aqueous *J. regia* bark extracts against fungal strains.

Fungal strain	Methanolic extract MIC (mg/mL)	Aqueous extract MIC (mg/mL)
<i>Candida albicans</i>	12.5	25
<i>Aspergillus niger</i>	25	50
<i>Trichophyton rubrum</i>	12.5	50
<i>Fusarium oxysporum</i>	12	24

Overall, the MIC results demonstrated greater inhibitory activity of the methanolic extract against the fungal strains, as indicated by its consistently lower MIC values. The MIC findings were broadly consistent with the concentration-

dependent activity observed in the agar well diffusion assay. The differences between the two extracts may reflect variation in the extraction and recovery of antimicrobial constituents.

Discussion

This study showed that *Juglans regia* L. bark extracts have significant in vitro antimicrobial activity against both bacteria and fungi. The Observed results were dependent on extraction solvent, concentration and microorganism. The methanolic extract generally showed higher antibacterial activity while antifungal activity was various among the species. These findings are in line with previous studies reported antimicrobial

activity of the extracts of *J. regia*, which could differ depending on the extraction method and solvent used (Bakht et al., 2017; Zakavi et al., 2013). The methanolic extract showed a significant concentration-dependent antibacterial inhibition with the highest against *Klebsiella pneumoniae* at 90 mg/mL. The antibacterial activity of the extracts was further confirmed by their significant effects on two-way ANOVA ($p < 0.001$). The methanolic extract gave better

result which could be attributed to better extraction efficiency of antimicrobial components from *J. regia* bark contain multiple classes of phytochemicals such as phenolics and naphthoquinones like juglone, which have been linked with antimicrobial activity (Zakavi et al., 2013).

However in the study, extracts were not chemically characterized and thus the activity observed cannot be assigned to any individual compound. The aqueous extract also exhibited concentration-dependent antibacterial activity, with the inhibition zone generally smaller as compared to the methanolic extract. One of the exceptions was *S. enterica*, where the aqueous extract demonstrated relatively high activity when compared to other extracts at 90 mg/mL. Variability among bacterial species is predicted due to the varying cell-envelope structure, permeability and other mechanisms that influence a microorganism's susceptibility to antimicrobial compounds. Results similar to those obtained here have been reported for *J. regia* bark extracts (Bakht et al., 2017). The higher inhibition rate observed in *S. enterica* and *K. pneumoniae* with tetracycline further suggests the plant extracts are good sources of antimicrobial compounds, which should not be considered as a replacement for existing antibiotics. The antifungal results were different from the results of antibacterial findings. Although the inhibition generally increased with concentration, the aqueous extract was more inhibitory against *A. niger*, *C. albicans* and *T. rubrum* at 60 and 90 mg/mL, while the methanolic extract was more active against *F. oxysporum*. The average extract type × concentration interaction that occurred for each fungus suggests that the impact of increasing extract concentration was different for each extract. Therefore, it can be concluded that the antifungal activity of *J. regia* bark was not of solvent-dependent alone, but rather is dependent on the type of extract, extract concentration, and fungal species. This is in agreement with previous reports indicating that extraction conditions affect the antimicrobial activity of *J. regia* extracts

(Bakht et al., 2017). The antimicrobial activity was further confirmed with the MIC results. The methanolic extract was more effective than aqueous extract against all the tested bacteria and fungi with lower MIC values. The bacteria which had the lowest MICs using methanolic extract were *S. enterica* and *K. pneumoniae* at 6.25 mg/mL while the aqueous extract needed 25-50 mg/mL. The methanolic MICs were found to be 12-25 mg/mL whereas the aqueous extract has a 24-50 mg/mL range for fungi. The results are broadly in agreement with the methanolic extract which showed more potent antibacterial activity. But the inhibition zone diameter and MIC should not be equated since the diffusion method is influenced by the solubility of the compound, its molecular characteristics and diffusion through the matrix of the agar (Eloff, 2019).

Hence, the MIC results also give good complementary evidence of inhibitory activity of the extracts. In general, the results showed *J. regia* bark to be a potential source of antimicrobial compounds. Methanol was found to be more effective in recovering antimicrobial activity while antifungal activity was more dependent on the extraction conditions and the organism used. However, it is difficult to draw conclusions about the compounds responsible for these effects when using crude extracts. To identify the active constituents and elucidate their mechanisms of action, further studies with chromatographic and spectrometric methods and subsequent bioassay-guided fractionation should be carried out.

Conclusion

Overall, the findings showed that *Juglans regia* L. bark has antimicrobial potential and highlight the importance of the extraction process in determining its activity against different microorganisms. The methanolic extract exhibited stronger antibacterial activity with lower MIC values compared with the aqueous extract in all the tested bacterial and fungal strains. Antifungal activity, however, was different among the species with the aqueous extract demonstrating better activity against several fungi. Based on these results, it can be

concluded that *J. regia* bark is a good source of antimicrobial components and extraction solvent is a significant factor influencing antimicrobial activity. Comparative study of methanolic and aqueous extracts should be performed in future to find the relationship between the phytochemical profile and the obtained antibacterial and antifungal activity. Identification and isolation of most active constituents and mechanistic and safety evaluations would help to clarify their potential for further development as plant-based antimicrobial agents.

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