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Antimicrobial Activity of *Juglans regia* Root Bark Extracts

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ABSTRACT

Background: *Juglans regia* tree is widely found in several parts of India, Europe and Central Asia. Different parts of J. regia are rich sources of alkaloids, flavonoids, glycosides, saponins, amino acids, and terpenoids. *J. regiais* has antiasthmatic, anti-atherosclerotic, antihypertensive, antioxidant, antidiabetic, antimicrobial, cardioprotective, hepatoprotective, hypolipidemic, osteoblastic, and wound healing activities. The bark and roots are used against tooth decay and ringworm infection.

Purpose: In the present study we evaluated antibacterial and antifungal activities of *J. regia* root bark.

Methods: Extracts of root bark of *J. regia* were prepared using methanol, water, and petroleum ether. Methanolic and aqueous extracts in concentrations 50, 100 and 200 mg/ml were tested against three Gram negative bacterial strains (*S. typhi, P. vulgaris*, and *E. coli*), one Gram positive strain (*S. aureus*) and fungi (*S. cerevisiae, P. chrysogenum*, and *A. fumigatus*).

Results: The extracts effectively inhibited growth of cultured microorganisms. Methanolic extracts showed maximum zone of inhibition (ZOI) for all the microorganisms ranging from 12.8 – 17.0 mm. Aqueous extract disclosed maximum effectiveness against *S. typhi*.

Conclusions: Quantification of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) revealed potent antibacterial activities of methanolic extracts (200 mg/ml) of *J. regia* root bark.

1. Introduction

Juglans regia (Akhrot tree) is a deciduous medium-sized to tall tree belonging to family Juglandaceae. It is native to temperate areas (e.g., Himalayan region, Nepal, Sri Lanka, Spain, Turkey, China, Europe, South America) and is also commercially cultivated in many portions across the globe (Jaiswal and Tailang, 2017). The useful portions of *J. regia* tree are nut-fruits, bark, fleshy portion of green fruits, leaves, and timber. The fruit of *J. regia* "Walnuts" or "Akhrot" is highly popular for its physical and mental health benefits owing to polyunsaturated fatty acids (e.g., linoleic acid, α-linolenic acids, oleic acid, omega 3- and omega 6-fatty acids), stearic acid, palmitic acid, melatonin, vitamin E, amino acids (e.g., lysine, glutamate, aspartate, arginine), and minerals (e.g., calcium, potassium, and magnesium) (Raja et al., 2012; Amaral et al., 2003; Fukuda et al., 2003). Plants belonging to the Juglandaceae family possess diverse phytochemicals of therapeutic significance such as alkaloids, steroids (e.g., lupeol, betulinic acid, daucosterol, and β-sitosterol), tannins (e.g., casuarinin, stenophyllarin, glansrins A, B and C), flavonoids (e.g., catechin, epicatechin, gallo-catechin, procyanidin B2, epigallocatechin, and epicatechingallate) (Zhao et al., 2014), phenolic acids (e.g., syringic, ferulic, caffeic, ellagic, gallic, *p*-coumaric, and sinapic acids) (Ahmed, 2015), saponins, hydroxycinnamic acid derivatives, and terpenoids. The oil of *J. regia* consisted of juglone (A and B), reglolone, 4-hydroxy-a-tetralone, α-pinene, β-pinene, germacrene-D, β-caryophylline, limonene, camphene, sabininene, myrcene, and eugenol (Abdallah et al., 2016; Devi et al., 2011; Colaric et al., 2005).

Earlier *J. regia* has been associated with anti-asthmatic, anti-atherosclerotic, anti-cancer, antihypertensive (Ebrahimiyan et al., 2016), anthelmintic, astringent, keratolytic, antidiarrheal, antioxidant (Pereira et al., 2007), antidiabetic (Ahmad et al., 2012; Fathi Azad et al., 2006), antimicrobial (Pereira et al., 2007), cardioprotective, hepatoprotective (Eidi et al., 2013), hypolipidemic (Hosseini et al., 2014), osteoblastic, and wound healing activities (Delaviz et al., 2017). It is used for skin disorders such as

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dermal inflammation, eczema, sunburns, scrofula, and itching. In the parts of Himalaya, leaves are used against insects such as mosquitoes and lice. Leaves are also beneficial against frost bite and in the treatment of chronic dysentery and itching. Traditionally, fruits are used as aphrodisiac, nervous tonic, and relieve constipative and rheumatism symptoms. Walnut oil mitigates muscular pain when applied topically and can improve eyesight. Oil is also massaged on the scalp, which is traditionally thought to have memory enhancing property (Jaiswal & Tailang, 2017). In traditional system of medicine, roots were used to arrest hair-loss, deterioration of tooth, and applied on the wounds due to its antiseptic activities (Zakavi et al., 2013). In animal studies, *J. regia* exhibited antidepressant, anxiolytic, and memory enhancing activities. The extracts of *J. regia* leaves, fruits, and stem bark showed significant antibacterial, antifungal, antiviral, and anthelminthic activities (Jahanban-Esfahlan et al., 2019).

The use of *J. regia* in traditional medicine in support of experimental studies demonstrate that phytochemicals present can be used as template for production of antioxidant, anti-inflammatory, antimicrobial, lipid-lowering, antidiabetic, and liver protective drugs. Recently, antiplatelet and anticoagulant properties of *J. regia* extract (root bark) was observed (Amirou et al., 2018). However, none of the studies explored the antimicrobial activity of *J. regia* root bark. In purview of the aforementioned background, in the current study we designed to gauge antibacterial and antifungal activities of *Juglans regia* root bark.

2. Collection of Plant Material

The root bark of *Juglans regia* was gathered from the "Shangus" region of district Anantnag, Jammu and Kashmir (India) where the tree occurs widely. The root bark was authenticated in the "Raw Materials Herbarium and Museum" (CSIR-NISCAIR), Delhi (RHMD) voucher specimen No. NISCAIR/RHMD/consult/2019/3508-09 (Dated 21/10/2019) by Dr. Sunita Garg (Emeritus Scientist, CSIR-NISCAIR). A sample specimen of collected root bark was deposited in RMHM for future reference.

3. Preparation of Extracts

After shade drying for 20 days, the root bark fragments were macerated to fine powder using an automated grinder. The powdered material was initially defatted using *n*-hexane (60-80°C). For extraction polar solvents viz. methanol and water were used and non-polar solvent petroleum ether was used. Successive hot extraction in a Soxhlet apparatus was carried out. 500 g of root bark were extracted with petroleum ether (450 ml) for 18-20 h. The PE extract was subjected to rotavapor under reduced pressure in order to obtain concentrated form and subsequently this marc was again extracted with methanol (350 ml) for 20-22 h. The methanolic extract was again subjected to rotavapor to obtain a concentrated form. Afterwards, the left-over marc was subjected to extraction for 6-8 h with 300 ml water. The extracts were concentrated. Storage (4°C) of the extracts was accomplished in air-tight glass (high grade) containers prior to their use. The crude extracts were weighed and % yield was quantified as air-dried pulverized crude matter (Sukhdev et al., 2008). The color and consistency of the extracts were noted. The extracts were then exposed to qualitative phytochemical screening followed by evaluation of antimicrobial potential using *in-vitro* methods. Different concentrations (50, 100, and 200 mg/ml) of aqueous and methanol extracts of *J. regia* root bark were formulated by means of 10% DMSO for assessment of the antimicrobial activity.

4. Qualitative Phytochemical Analysis

A 1% w/v stock solution of every extract was formulated using solvents *viz.* petroleum ether, methanol, and water. Active phytochemicals such as anthraquinone glycosides, amino acids, alkaloids, carbohydrates, flavonoids, cardiac glycosides, tannins, saponins, triterpenoids, phytosterols, and proteins were detected in the sample extracts by adopting standard techniques (Mohamed et al., 2020).

5. Test Microorganisms

As per the pharmacological and clinical relevance Gram +ve (*Staphylococcus aureus*) and Gram -ve (*Salmonella typhi*, *Escherichia coli*, *Proteus vulgaris*) bacteria strains, and fungal strains *Saccharomyces cerevisiae*, *Penicillium chrysogenum*, and *Aspergillus fumigatus* were chosen. The microbes were procured from Government Medical College and Hospital, Chandigarh (India). The bacteria were discretely cultured by streak plate method (temp 37°C) using Muller-Hinton Agar (MHA) (sterilized) for 24 h. Each cultured colony was transferred in 5.0 ml sterilized 0.9% normal saline within test tubes with a flamed wire-loop, then vortexed to make a uniform suspension. This suspension was incubated (temp 37°C) overnight to fine-tune the turbidity to 0.5 McFarland standards of turbidity. The turbidity of inoculum test tubes was adjusted and juxtaposed in white background and distinct black lines against 0.5 McFarland turbidity equivalence typical under adequate light to compare naked eye. The fungus was cultured for 24 h period on sterilized Sabouraud dextrose agar (SDA) (temp 28°C). Inoculum was prepared using sterile normal saline (37°C

for 24 h) and turbidity of suspension was adjusted to 1.0 McFarland turbidity standards. The adjusted bacterial and fungal suspensions were used as inoculate within 15 min (Mohamed et al., 2020).

6. Evaluation of Antimicrobial Activity

For determination of "zone of inhibition" (ZOI), the test bacteria were spotted on the plate of MHA and thereafter for 24 h incubated (temp 37°C) (Bhalodia et al., 2011). Then, the inoculated media was bored peripherally 4 wells of 4 mm using a disinfected "cork-borer" of diameter 4 mm. The peripheral wells were poured with 50 μl of aqueous and methanol extracts prepared in 10% DMSO of strength 50, 100, and 200 mg/ml. Streptomycin disc (10 μg/disc) was placed at the center of each petriplate that served as control. The inoculums were allowed to diffuse (for 30 min) at 25°C and then at temp 37°C incubated (18-24 h). Post-incubation, establishment of a clear region round the well was noted in petriplates, which correlates with the antimicrobial effect of test samples. Antifungal action of the extracts was explored by disc diffusion technique using the medium SDA. The discs were positioned in solidified SDA plates prior to addition of 20 μl of different concentration extracts (50, 100, and 200 mg/ml). The petriplates were again subjected to incubation (temp 28°C) for 24 h period. A vernier caliper was used measure the diameter of the inhibitory zones (ZOI) and assess the antifungal activity of the test extracts. Antibiotic fluconazole (25 μg/disc) was used as control. The ZOI was measured in mm and considered as subtle (> 16 mm), transitional (12–15 mm), and resilient (< 12 mm) (Barry et al., 1970).

7. Investigation of Minimum Inhibitory Concentration (MIC)

The MIC for extracts was gauged using 96-well microplates (Manandhar et al., 2019; Wiegand et al., 2008). 50 μl of Mueller Hinton broth (MHB) was poured in each well or bore. Then 150 μl of the extract (final concentration 200 mg/ml) was poured to the 1st column of the microplate (200 μl volume of 1st column of each plate). A two-fold serial dilution was made initiating from first column to tenth column. From the last column, 50 μl of the broth and test extract were removed. MHB (1 ml) was added to 100 μl of bacterial suspension and 50 μl added to the bores up to the last column under aseptic conditions. Afterward, the microplates were (at temp 37°C) incubated for 24 h period. MIC was assessed by addition of 30 μl of 0.02% *p*-iodonitrotetrazolium chloride (INT) (2 mg/ml) and then (at temp 37°C) incubated for a 32 min duration. INT indicated bacterial progress by color change to pink. No change in color post INT addition in the wells indicated nil growth of the microorganisms. The lowermost extracts concentration that entirely repressed the bacterial progress was taken as MIC.

8. Exploration of Minimum Bactericidal Concentration (MBC)

The bottom most extract strength that impedes bacterial development is MBC. The contents of wells were aseptically sub-cultured with reference to the results of MIC for specific bacterium to agar-medium devoid of any antimicrobial. A 3 μl of test material from MIC serial dilution content was added to colony counting agar media aseptically by using a micropipette and then (at temp 37°C) incubated for 24 h. The media was checked for bacterial growth and the lowermost extract concentration that displayed nil post-incubation bacterial development was noted for each of the 4 well and marked as respective MBC (Debalke et al., 2018).

9. Results

Physical characteristic and percentage yield of *J. regia* root bark extracts

The methanol, aqueous, and petroleum ether extracts of *J. regia* root bark had 6.34%, 5.52% and 1.34% (w/w on dry weight basis) yield respectively. The three extracts showed different physical characteristics (Table 1).

Table 1: Characteristic features of extracts obtained from *J. regia* root bark.

Extraction solvent	Color	Consistency
Methanol	Deep brown	Greasy
Aqueous	Light brown	Sticky
Petroleum ether	Dark brown	Greasy

Primary phytochemical screening of extracts of *J. regia* root bark

The outcomes of initial phytochemical screening conducted on the methanol, aqueous, and petroleum ether extracts of *Juglans regia* root bark showed presence of different phytoconstituents (Table 2). Methanol extract showed a greater diversity and extent of phytochemicals followed by aqueous and petroleum ether extracts.

Table 2: Preliminary phytochemical screening of *J. regia* root bark extracts.

Phytoconstituent	Phytochemical Tests	Extract of root bark of *J. regia*		
		Methanol	Aqueous	Petroleum ether
Alkaloids	Drangendroff	++	+	-
	Hager's	++	+	-
	Wagner's	++	+	-
Triterpenoids	Salkowski	++	+	+
Phytosterols	Liebermann Burchard's	++	++	+
	Salkowski	++	+	+
Flavonoids	Shinoda	++	++	+
	Alkaline reagent	++	+	+
	Lead acetate	+	+	+
Saponins	Foam	+	+	+
	Olive oil stain	+	+	+
Glycosides	Keller Killani	+	-	-
	Anthraquinone glycosides	-	+	-
	Phenolic compounds	++	+	+
Proteins	Biuret	+	-	-
Amino acids	Millon's	+	-	-
	Ninhydrin	-	+	-
	Xanthoproteic	+	+	-
	Pauly's	+	+	-
Carbohydrates	Molisch's	+	+	+
	Barfoed's	+	+	+
	Seliwanoff's	+	+	-

(+ indicates present and–indicates absent)

Antimicrobial activity of *J. regia* root bark extracts

Methanol and aqueous extracts were taken to evaluate the antibacterial/antifungal actions as these extracts were rich in different phytoconstituents identified during phytochemical screening and also showed a higher *in-vitro* antioxidant and anti-inflammatory activities in comparison to the petroleum ether extract.

Methanol and aqueous extracts showed concentration dependent increase in ZOI against each microorganism (Table 3 and 4). When used in same concentrations the methanolic extract of root bark of *J. regia* showed greater ZOI in relation to the aqueous extracts for bacteria and fungi growth both. All the bacterial strains tested showed intermediate sensitivity (ZOI 12 - 15 mm) against aqueous extract (50, 100, and 200 mg/ml) except *S. typhi* that showed high sensitivity (ZOI > 16 mm) against *J. regia* root bark aqueous extract at concentration of 200 mg/ml. Aqueous extract (50, 100, and 200 mg/ml) moderately inhibited the fungal growth (ZOI 12 – 15 mm). *S. cerevisiae* showed no sensitivity (ZOI < 12 mm) towards aqueous extract concentration 50 mg/ml. The methanol extract (200 mg/ml) showed most effectiveness (ZOI 16 mm) against bacteria (*S. aureus*, *S. typhi*, and *E. coli*) and fungi (*S. cerevisiae*, *P. chrysogenum*, and *A. fumigatus*). However, *P. vulgaris* showed intermediate sensitivity (ZOI < 16 mm) against methanol extracts (200 mg/ml) of *J. regia* root bark.

The MIC of methanol and aqueous extracts (200 mg/ml) were in harmony with the ZOI for all the bacterial strains. Methanol extract was more active in impeding the bacteria relative to the aqueous extract (Table 5).

Table 3: ZOI (mm) measured against bacterial strains for methanolic and aqueous extracts of root bark of *J. regia* and standard streptomycin.

Bacteria	Methanol extract (mg/ml)			Aqueous extract (mg/ml)			Streptomycin
	50	100	200	50	100	200	10 μg
S. aureus	13.35±0.332	14.55±0.24	16.140.27	12.93±0.32	14.30±0.31	15.85±0.33	23.41±0.81
S. typhi	14.94±0.21	15.83±0.33	17.01±0.24	13.95±0.56	15.05±0.12	16.95±0.23	24.01±0.83
E. coli	13.93±0.226	14.82±0.26	16.71±0.19	13.45±0.17	14.64±0.17	15.76±0.33	24.53±0.67
P. vulgaris	13.82±0.15	14.56±0.17	15.75±0.27	12.86±0.40	13.98±0.19	14.86±0.15	23.35±0.88

Table 4: ZOI (mm) measured against three fungal strains for methanolic and aqueous extracts of root bark of *J. regia* and standard fluconazole.

Bacteria	Methanol extract (mg/ml)			Aqueous extract (mg/ml)			Fluconazole
	50	100	200	50	100	200	25 μg
S. cerevisiae	13.83±0.12	14.95±0.32	16.75±0.25	11.27±0.25	14.56±0.27	15.36±0.24	20.07±0.52
P. chrysogenum	14.21±0.11	15.33±0.11	16.02±0.21	12.64±0.22	14.64±0.33	15.05±0.25	20.03±0.26
A. fumigatus	14.52±0.36	15.23±0.33	16.77±0.25	13.64±0.25	14.73±0.26	15.24±0.45	20.06±0.26

Table 5: MIC and MBC values (mg/ml) of *J. regia* root bark extracts against bacteria.

Test bacteria	Methanol extract (200 mg/ml)		Aqueous extract (200 mg/ml)	
	MIC	MBC	MIC	MBC
S. aureus	25.82±4.22	32.43 ±3.82	27.92±2.52	34.62 ±3.18
S. typhi	20.37±2.28	21.62 ±3.83	22.52±3.13	22.52 ±2.17
E. coli	21.65±2.19	28.27 ±2.92	24.5 ±4.42	30.72 ±3.11
P. vulgaris	48.23±4.22	55.23±4.22	56.57±4.24	67.72 ±3.88

10. Discussion

The outcomes of primary phytochemical screening of extracts of root bark of *J. regia* showed diversity in phytochemicals. The methanolic, aqueous, and petroleum ether extracts showed positive results for multiple phytoconstituents. Methanolic and aqueous extracts exhibited the existence of maximum constituent's *viz.*, phytosterols, alkaloids, phenolics, triterpenoids, flavonoids, and saponins. Petroleum ether showed negative result for alkaloids, proteins, anthraquinone glycosides, and amino acids. These findings prompted to test three different concentrations of methanol and aqueous extracts (50, 100, and 200 mg/ml) against microorganisms.

The methanolic and aqueous extracts (concentration 50, 100, and 200 mg/ml) of root bark of *J. regia* were effective countering Gram negative and one Gram positive bacterial strains. The extracts subdued the progress of *S. aureus*, *S. typhi*, *P. vulgaris*, and *E. coli*. Methanolic extract showed maximum ZOI against *S. aureus*, *E. coli*, and *S. typhi* ranging from 12.8 – 17.0 mm. However, *P. vulgaris* showed some resistance towards methanolic extract with ZOI < 16 mm at 200 mg/ml extract concentration. Aqueous extract showed maximum activity against *S. typhi* with ZOI 16.9 mm. These two extracts were also tested for antifungal activity against three strains (*S. cerevisiae*, *P. chrysogenum*, and *A. fumigatus*). The extracts inhibited the growth of *S. cerevisiae*, *P. chrysogenum*, and *A. fumigatus*. Previous indicated that the observed antibacterial and antifungal activity of the extracts can be attributed toterpenoids (Thoppil et al., 2011) and saponins (Pistelli et al., 2002). The selected extracts displayed ample potential with regard to antimicrobial efficacy against microorganisms and may be utilized in mitigation of infectious pathologies of these microorganisms.

Methanolic extract portrayed a good antibacterial activity and exhibited potent MIC and MBC against various bacterial strains including *S. aureus*, *E. coli*, and *S. typhi*. The MIC and MBC for *P. vulgaris* were high for both the extracts indicated some resistance shown by the bacteria. Aqueous extract also showed antibacterial activity (MIC and MBC) against these strains and in particular showed wider zone of inhibition against *S. typhi*.

Conclusion

The findings showed prominent antimicrobial activity of *J. regia* root bark extracts. *J. regia* should be explored to unearth a few bioactive natural compounds that might assist as lead compound and template for new antimicrobial pharmaceuticals. Thus, further work can be pursued and very much required for the isolation of pure phytochemicals and evaluation of their biological activities.

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Authorship Contribution

Aadil Wani: Experiments, data collection, analysis, manuscript drafting.
Dr. Manish Kumar: Project supervision, research design, methods validation, resources, manuscript drafting and revision.

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Declaration

It is an original data and has neither been sent elsewhere nor published anywhere.

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