Research Article

PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF MALE FLOWER OF Juglans regia L.

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Abstract
Medicinal plants are the richest bio-resources of drugs in the traditional systems of medicine, modern medicine, nutraceuticals, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs. Juglans regia L. is one of the valuable medicinal plants with a potency to cure various diseases as practiced in traditional medicine. The present work is aimed at primary screening of the phytochemical contents of different extracts (methanol, ethanol and aqueous) of male flower of Juglans regia L. (MFJR) and to evaluate the antibacterial activity of these extracts against two species of pathogenic bacteria. The main objective of the present work was to check the presence or absence of various botanicals in different extracts of the plant and also to compare their abundance in each of the solvent extracts. The antibacterial activity of various extracts was evaluated against two bacterial species (Pseudomonas aeruginosa and Klebsiella pneumoniae). The zone of inhibitions and minimum inhibitory concentration (MIC) of the various extracts were determined by running the experiments five times. The study reveals the presence of a variety of phytochemicals including alkaloids, carbohydrates, glycosides, proteins, phytosterols, lipids, phenolic compounds, flavonoids and other minor phytochemicals in the analyzed extracts. However, higher amounts of these phytochemicals were present in the methanolic extract of male flower of Juglans regia (MEJR). All the extracts showed significant levels of antibacterial activity. Methanol extract was the most active one with remarkable antibacterial activity on the various species tested. MICs of the extracts revealed methanol extract as the most potent one with the lowest inhibitory concentration of 4.6 mg/mL on P. aeruginosa. The findings of the present study indicated that the male flower of Juglans regia possesses various secondary metabolites having the potential for developing pharmaceutical drugs, especially antimicrobial ones.

Key words: Juglans regia, Phytochemistry, Botanicals and Antibacterial activity.

1. Introduction
Medicinal plants serve as the best raw materials and rich sources of bioactive molecules that have wide range of applications in food as well as medical field. Globally, many people use botanicals for treatment of different types of ailments since ancient times without any major adverse effects. Juglans regia L. belongs to family Juglandaceae and is a native of Asia minor, India,
South-eastern Europe and China. It is a common and highly valued plant in Jammu and Kashmir, India, where it is generally known as ‘Doon’. People in this area traditionally use various parts of the plant to treat wide range of health complaints.

Shell, green husk (epicarp) and leaves of *Juglans regia* have received great attention in cosmetic, nutraceutical and pharmaceutical industries. Its nuts are very popular, costly and are largely consumed as royal food in the whole world (Oliveira et al., 2008). The stem bark of the plant is reported to have alterative, anthelmintic, astringent, bactericidal, depurative, digestive, diuretic, laxative, detergent, stimulant, tonic and even insecticidal properties (Chopra et al., 1986). Moreover, oil from nuts of *Juglans regia* possesses anti-wrinkle and anti-aging properties due to its moisturizing as well as free radical scavenging capacity (Espin et al., 2000). Leaves of *Juglans regia* have been widely used in folk medicine for treatment of venous insufficiency and haemorrhoidal symptomatology and have also antidiarrhoeal, anthelmintic, depurative, astringent fungicidal and insecticidal properties (Neji and Kanwal, 2009; Cosmulescu and Trandafir, 2011). Other properties viz., antibacterial, anticancer, antiproliferative, antioxidant, hypoglycaemic, hypotensive, anti-scorfulous and sedative have also been attributed to various parts of *Juglans regia* (Pereira et al., 2008; Carvalho et al., 2010).

In general, several studies have demonstrated the antimicrobial activity of phenolic content and phenolic extracts (Pereira et al., 2008) from different plants, making them a good alternative to antibiotics and chemical preservatives. There is an extended interest in using natural antimicrobial compounds, due to the increasing resistance among the microbes to antibiotics (Oliveira et al., 2007). *Juglans regia* is a rich source of secondary metabolites such as flavonoids, sterols, pectic substances, phenolics, fats, proteins, vitamins and minerals. However, the nutritional contents differ from cultivar to cultivar, influenced by genotype, cultivator, ecology and soil (Caglarirmak, 2003; Crews et al., 2005; Martinez et al., 2010; Muradoglu et al., 2010).

Despite a good number of research works have been carried out on various parts of *Juglans regia* (Fukuda et al., 2003; Zhang et al., 2009), no major reports are available regarding the phytochemistry and antimicrobial activity of the male flower of this plant. Therefore, attempts have been made in the present study to analyze the phytochemistry of the male flower of *Juglans regia* L., as well as on the antibacterial activities of three solvents (aqueous, ethanolic and methanolic) extracts of the same.

### 2. Materials and Methods

#### Plant material and chemicals

Male flower of *Juglans regia* L., (MFJR) were collected from the Himalayan region of Jammu and Kashmir, India and the plant was identified and taxonomically authenticated by the Department of Botany, Annamalai University, India, with herbarium voucher no. ABH-2023. Flowers were dried in shade and powdered in a grinder before the commencement of the experiment. Chemicals, solvents and other reagents were procured from SD Fine (analytical grade), India and Hi-Media, India.

#### Preparation of extracts

Five hundred grams of the powdered material each was individually cold extracted separately with methanol (MEJR), ethanol (EEJR) and water (AEJR) (1:3) for 72 hours and then filtered. The solvents from the extracts were evaporated under vacuum in a rotary evaporator and the dried extracts were stored at 4 ºC.

#### Phytochemical tests

Preliminary phytochemical analysis of all the extracts was performed by following standard methods of Trease and Evans (1989) and Sofowara (1993).

#### Bacterial strains

Pure bacterial strains viz., *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* used for the study were procured from the Department of Microbiology, Faculty of Agriculture, Annamalai University. Separate sterile Nutrient agar slants were prepared and the bacterial strains were individually inoculated into separate slants under...
aseptic conditions and incubated at 37 °C for 24 hrs. Colonies were harvested separately under aseptic condition from the slants and individually inoculated into sterile nutrient broths in separate test tubes and kept in refrigerated condition.

Antimicrobial assay

Antimicrobial activities of the MFJR and the standard (Ciprofloxacin 5 mg/mL) were tested using Disc diffusion method of Peach and Tracey (1956). Petri dishes and the Mueller Hinton agar medium were sterilized for 20 min at 120 °C. Twenty five milliliters of the medium was poured into sterile Petri dishes and allowed to get solidified under laminar airflow. From the respective Nutrient broth sub-culture (bacterial concentration of $5 \times 10^5$ CFU/mL), bacteria were swabbed on the medium in Petri dishes separately using sterilized cotton swabs under sterile conditions. Filter paper (Whatman No. 1) discs of 5 mm diameter were prepared and sterilized. Extracts to be tested were prepared by making three different concentrations of 12.5, 25 and 50 mg/mL in DMSO. The impregnated sterile discs with the extracts and standard were then placed carefully on the surface of the respective inoculated Petri dishes with separate sterilized forceps and gently pressed down to ensure complete contact of the disc with the agar surface. Filter paper discs soaked in DMSO alone were used as controls and the respective solvents as negative controls. After incubation for 24 hrs, the diameters of the inhibition zones were measured with micro scale. Each experiment was repeated at least five times and the mean of the diameter of the inhibition zones was calculated.

Determination of minimum inhibitory concentration (MIC)

The MICs (the lowest concentration that will inhibit the visible growth of the microorganism) of all the above extracts for all the two strains of bacteria were determined using broth dilution method of Talaro and Talaro (2002) and Forbes et al. (2007). To determine the MICs, the extracts were added in serial dilutions to a series of tubes containing Mueller Hinton broth so that the concentrations ranged from 1 to 20 mg/mL. The bacterial concentration in each tube was $5 \times 10^5$ CFU/mL. Growth control and negative growth controls were also prepared along with ciprofloxacin standard. After 24 hrs of incubation at 37 °C, the lowest concentration of the respective extract that led to the inhibition of the growth of bacteria was considered as MIC. Data represent at least five replicated experiments per microorganism.

Statistical analysis

Experiments were replicated five times and the mean and standard deviation ($X \pm SD$) of data were calculated statistically.

3. Results and Discussion

Phytochemical screening

Preliminary screening of all the extracts showed the presence of a wide variety of phytochemicals including alkaloids, carbohydrates, glycosides, proteins, phytosterols, lipids, phenolic compounds, flavonoids, volatile oil, saponins, aldehydes, gums and mucilages. The respective tests for the various phytochemical moieties and their results are presented in Table - 1. When compared to the aqueous extract, the abundance of alkaloids in ethanolic and methanolic extracts was evidenced by the formation of strong yellow precipitate in the test tube by the Hager’s test. Methanolic extracts showed the presence of higher amounts of phenolic compounds than ethanolic one. Results of the present study also showed increased presence of saponins in the methanolic extract in comparison to other extracts, by the formation of about 1.5 cm thick foam layer in the test tube. While flavonoids were present in all the three extracts, aldehydes as well as gums and mucilage were found in aqueous extract alone. Similarly, while ethanol and aqueous extracts confirmed the presence of sugars, volatile oil was present in methanolic as well as aqueous extracts (Table - 1).

Due to the diverse structure and chemical composition of the plant materials, their behavior in a solvent may be different from another and therefore, cannot be predicted precisely and according to Al-Farsi and Lee (2008) the selection of the most appropriate solvent is a determinant factor on extract properties. In the present study,
different solvents (water, methanol and ethanol) were used for the extraction of MFJR and were assayed for preliminary phytochemicals.

The present study has revealed the presence of carbohydrates, proteins, fixed oils and fat, phytosterols, alkaloids, saponins, phenolic compounds and flavonoids. Among the various solvents used, even though ethanolic and aqueous extracts have a slight edge over methanolic one with regards to the number of phytochemicals getting extracted, methanolic extract is rich in terms of quantity of the phytochemicals. Methanolic extracts especially showed the presence of higher amounts of phytosterols, alkaloids, saponins, phenolic compounds and flavonoids, which are highly significant on a pharmacological point of view (Qadir et al., 2015). The significance of the methanolic extract was also reflected by its enhanced antimicrobial activity as revealed by the present study (Table - 2). The capacity of methanol to extract more quantities of phytochemicals is also evidenced by the lower yield of carbohydrates and proteins in the aqueous extract when compared to methanolic one (Table - 1). This finding is in agreement with the previous report on aqueous and methanolic extract of Faidherbia albida legume by Ismail et al. (2016). Previous studies using different parts of J. regia L., have also reported the presence of rich unsaturated fatty acids (linoleic acid and oleic acid), protein (arginine, leucine), carbohydrates (dietary fibre), vitamins (vitamin A, C, E), pectic substances, minerals (magnesium, potassium, phosphorus, sulphur, copper, and iron), fibres, melatonin, plant sterols, phenolic acids, and flavonoids in the plant (Chopra et al., 1986; Kris-Etherton et al., 1999; Prasad, 2003; Labuckas et al., 2008; Pereira et al., 2008).

Phenolic compounds, alkaloids, and glycosides detected in the extracts (Table 2) are compounds that have been documented to have medicinal properties including antibacterial activity (Okwu, 2004; Afolabi et al., 2007). The toxicity of polyphenols in microorganisms is generally due to iron deprivation or hydrogen bonding with microbial enzymes affecting their activity or enzyme inhibition by the oxidation of phenolic compounds (Qadir et al., 2015). Therefore the conspicuous antimicrobial activity exhibited by different extracts in the present study may be attributed to the presence of phenolic compounds in it. The presence of volatile oil in different extracts of MFJR (Table - 1) might also be imparting the antibacterial activities as essential oils are reported to have excellent antibacterial and anti-inflammatory properties (Doughari, 2012). According to Sahoo et al. (2012), volatile oils can cause disruption of membranes in microbes by the action of the lipophilic compounds in these oils and thereby imparting antibacterial activity. Flavonoids are an important group of polyphenols and are known to have antimicrobial (Miller, 1996) activity. Due to these inherent properties, flavonoids are often referred to as nature’s biological response modifiers. According to Cowan (1999), the antibacterial activity of flavonoids is due to their ability to complex with extra cellular and soluble proteins as well as with the bacterial cell wall. Hence the antibacterial property might also be associated with the amount of flavonoids present in the extract.

**Antimicrobial assay**

The antimicrobial potential of MFJR against two bacterial strains was evaluated by using disc diffusion method and determination of minimum inhibitory concentration Table - 2. The results revealed that all the extracts of MFJR showed significant antimicrobial activity against both the bacterial strains tested. The results revealed that *Pseudomonas aeruginosa* is more susceptible than *Klebsiella pneumoniae* bacteria. The mean zone of inhibition produced by all the extracts ranged from 19.26 ± 1.7 to 58.33 ±2.21 mm and the MIC value were between 4.6 to 9.7 mg/mL. The MEJR showed highest antimicrobial activity with the highest mean zone of inhibition (58.33 ±2.21 mm) and lowest MIC (4.6 mg/mL) values against *Pseudomonas aeruginosa* followed by EEJR (46.23 ± 1.19 mm; MIC = 6.5) and AEJR (41.16 ± 2.18 mm; MIC = 7.5) (Table - 2). No significant antibacterial activities were observed in the case of DMSO and solvent controls.
### Table 1: Preliminary phytochemical screening of MFJR

<table>
<thead>
<tr>
<th>Phytochemical tests</th>
<th>Methanolic extract</th>
<th>Ethanollic extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molisch’s test (carbohydrates)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Fehling’s test (sugar)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benedict’s test (sugar)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Borntrager’s test (glycosides)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Biuret test (proteins)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ninhydrin test (amino acids)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spot test (fixed oils)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Saponification test (fixed oils and fat)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Liberman Burchard’s test (phytostersols)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mayer’s test (alkaloids)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wagner’s test (alkaloids)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hager’s test (alkaloids)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Foam test (saponins)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ferric chloride test (phenolic compounds)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin test (phenolic compounds)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lead acetate test (phenolic compounds)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline reagent test (flavonoids)</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Volatile oil test (volatile oil)</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Schiff’s reagent test (aldehydes)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Whistler and Miller’s test (gums and mucilages)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = present; ++ = abundantly present; – = absent

### Table 2: Antimicrobial activity of different extracts of male flower of *Juglans regia* L.

<table>
<thead>
<tr>
<th>Name of the organism</th>
<th>Mean zone of inhibition (mm)*b</th>
<th>MIC of the MEJR (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEJR</td>
<td>EEJR</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>27.42 ±1.12</td>
<td>41.15± 1.48</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>25.18 ±1.15</td>
<td>36.3 ± 1.15</td>
</tr>
</tbody>
</table>

Note: MIC, minimum inhibitory concentration; NT, not tested, *Mean zone of five assays, b Inhibition zones including the diameter of the disc (6 mm).*
Recently, Farooqui et al. (2015) reported the antibacterial activity of the bark of *Juglans regia* against 15 bacteria with MIC ranging from 0.31 to >5 (mg/mL). Zakavi et al. (2013), also reported the efficiency of the ethanol and aqueous extracts of *Juglans regia* bark against some oral bacteria (*Staphylococcus aureus, Staphylococcus sanguis, Staphylococcus salivarius* and *Staphylococcus mutans*), with MIC up to 5 mg/mL. According to Cruz-Vega et al. (2008) the leaves and bark as the active aerial part of *Juglans regia* are effective against microbes with the MICs ranging from 100 to 125 μg/mL. Similarly, Noumi et al. (2010) reported the antibacterial effect of bark of *Juglans regia* L. with the MIC values ranging from 0.006 to 3.125 mg/mL. The differences registered between our results and previously reported data could be attributed to the extraction procedure, the plant origin, the tested microorganisms and the size of the inoculums. Hence, the male flower of *Juglans regia* L. could be explored for its highest therapeutic efficacy.

4. Conclusion

The overall results of the preliminary phytochemical screening of the various extracts (methanol, ethanol and aqueous) of male flower of *Juglans regia* reveal that maximum quantity of major phytoconstituents are present in the methanolic one. The comparative antimicrobial efficiencies of the three extracts also clearly indicate the enhanced pharmacological importance of the methanolic extract of male flower of *Juglans regia* L. However, present study suggests that further phytochemical analyses of male flower of *Juglans regia* are essential to determine and refine the different types of compounds present in it.

5. Reference


24) Peach, K. and M.V. Tracey. (1956). Modern Methods of Plant Analysis. Springer Verlag, Berlin,


