Int J Biol Med Res.2018 ;9(2):6304-6308

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original Article

Phytochemically analyzed *Juglans regia* root extracts and their bactericidal facets against some urinary tract infection causing bacteria

Aashaq Hussain Allaie , Ranu Singh, Aijaz Ahmad Ganaie, Ravi Prakash Mishra, Shoket Ali

[1,3,5] *Research Scholar at Environmental Biotechnology Lab. Department of P.G. Studies & Research in Biological Sciences, R.D. University, Jabalpur-482001.*
[2] *Assistant Professor at Department of Botany and Microbiology Mata Gujri Mahila Mahavidyalaya (Autonomous), Jabalpur. 482001.*
[4] *Professor at Department of P.G. Studies & Research in Biological Sciences, R.D. University, Jabalpur-482001.*

ARTICLE INFO

Keywords:

UTIs
Nosocomial
Phytochemical
Juglans regia
Jabalpur

ABSTRACT

Background: In this study, root extract of medicinal plant *Juglans regia* was analyzed for antibacterial potential against various uropathogenic bacteria, the increasing resistance of pathogenic bacteria to conventional antibiotics and their side effects, the root of the *Juglans regia* was analysed for phytochemical constituents, and their different solvent extracts screened against six clinical isolates of UTI causing bacteria, to ascertain their effectiveness in UTI treatment, and provide basis for future clinical trials of the plant. **Methods:** Antibacterial study was carried out on clinically isolated urinary tract infections (UTI) causing bacteria. Identification of isolated bacteria was done by biochemical tests. The phytochemical analyzing of root extracts of *Juglans regia* was done by method of Raman (2006) and Harbone (2005). Antibacterial activity of crude extract was done by disc diffusion method using ciprofloxacin as a control. **Results**: The inhibitory activities of extracts of *Juglans regia* against isolates *Escherichia coli*, *Staphylococcus aureus*, *S. saprophyticus, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* compared to the control. Among the three different root extracts tested against six pathogenic bacteria causing urinary tract infections (UTIs), ethyl acetate extract was effective against all pathogenic bacteria with the highest inhibition followed by methanolic extract while, hexane extract was also effective against causative agents with moderate inhibition zone. Preliminary phytochemical screening shows the presence of phytoconstituents *viz* alkaloids, glycosides, saponins, carbohydrates, proteins, aminoacids, flavonoids, steroids, tannins. **Conclusion**: The plant showed that extracts of *Juglans regia* contain significant antibacterial activity against uropathogenic bacteria and hence, in future it could be used to obtain novel therapeutic compounds for the treatment of patients suffering from urinary tract infections.

Introduction

Urinary tract infections (UTI) are the most common bacterial infections, affecting people throughout their lifespan. Urinary tract infection is the most common bacterial infection that occurs in urinary tract and accounts for 40% of all nosocomial infections [1]. UTI are the most common infections after upper respiratory tract infections [2]. Each year, over 150 million people are diagnosed with urinary tract infections (UTI) and it is the second most common infectious disease in community practice worldwide [3]. Around 80-85% of UTIs are caused by *Escherichia coli* and 5-10% by *Staphylococcus saprophyticus* [4]. The other bacteria known to cause UTI infections are *Enterobacter, Klebsiella, Proteus*, *Staphylococcus aureus* and *Pseudomonas*.

Recently, drug resistance to human pathogenic bacteria has been reported worldwide. This has lead to search for newer therapeutic modalities including extracts obtained from medicinal plants. Many parts of the world have shown that the choice of drugs for the treatment of UTI is quite narrow today. Currently, researchers are focused on the therapeutic and pharmacological effects of plant-derived phytochemicals, such as plant polyphenols, as antimicrobial agents [5-6]. Medicinal plants represent a rich source of antibacterial agents [7]. The effects of plant extracts on bacteria have been studied by a large number of researchers in different parts of the world [8]. Although *Juglans regia* has been used as folk medicine but not much has been known about its antibacterial potential against bacterial pathogens causing urinary tract infection. *Juglans regia*, known as Akhrot in India, a native of Eastern Europe to North Asia is a member of Juglandaceae family. This valuable tree has a long history of medicinal use to treat a wide range of health complaints. Almost all parts of the plant are medicinally important [9]. It is reported to be astringent, antifungal, diuretic, laxative, tonic, blood purifier, anthelmentic antiulcers, anticancer and detoxifier [10-14]. The species is also utilized in the treatment of tuberculosis and tuberculosis of cervical glands [15]. The antibacterial properties of the plant material may be due to the presence of phenolic compounds, terpenoids, alkaloids, flavonoids and steroids [16]. Hence, the present study was done to assess the phytochemical and antibacterial activity of *Juglans regia* extracts against common uropathogenic bacteria causing UTI

* Corresponding Author : **: Dr. Ranu Singh**
ranusing4@gmail.com

Material and Methods

Collection and preparation of the plant material

Juglans regia was collected from the Humza Pora Bijbehara area of Kashmir Valley, India in the month of March, 2016. Humza Pora having latitude 33.80 N and 75.10E and altitude of 1, 591 m the plant was identified at Centre of Biodiversity and Taxonomy, Department of Botany, University of Kashmir. The plant was selected based on reports of its widespread use among the local communities in J&K region. The plant was further identified on morphological basis as per description given in the literature [17-19]. After collection, the roots were sun dried for 7 days and pounded using pestle and mortar and stored at 35 - 37°C until required.

Extraction procedure

Fifty grams powdered roots were taken with 250ml of methanol, ethyl acetate and hexane solvents for 12h at 30 °C temp. in soxhlet apparatus and then filtered using Whatman No.1 filter paper. The filtrate evaporated to dryness using rotary evaporator and the resultant extract stored in a reagent bottle at 4 –8°C.

Test bacteria

The uropathogenic bacteria included in our study were isolated from urine samples are four Gram negative bacteria that is *E. coli, P. aeruginosa, K. pneumoniae* and *Proteus mirabilis* and two Gram positive bacteria that is *S. saprophyticus, S. aureus* from patients suffering with urinary tract infections (UTI).

Phytochemical analysis

The phytochemical screening for the crude methanolic, ethyl acetate and hexane extract of *Juglans regia* was carried out by standard protocols of [20-21]. The presence of alkaloids, glycosides, saponins, carbohydrates, proteins, aminoacids, flavonoids, steroids, tannins was detected as shown in Table I.

Isolation and Identification of UTI bacteria

The bacteria used were *Escherichia coli*, *Staphylococcus aureus*, *S. saprophyticus Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* isolated from clinical specimens obtained from patients diagnosed with urinary tract infections at the Bombay Hospital & Research Centre Jabalpur, M.P. Isolation and identification of the organisms were done following standard procedures in handling clinical specimens [22]. The organisms were maintained on nutrient agar slants at 2 - 8°C. All the bacterial strains were grown on Blood agar or MacConkey agar plates at 37°C and maintained on nutrient agar slants and were differentiated using the gram staining procedure into gram positive and gram negative organisms. The organisms were transferred to cystine lactose electrolyte deficient (CLED) agar medium for further differentiation of urinary organisms. Urine samples were shake well in their containers for even distribution of bacteria. A calibrated wire loop with internal diameter 3.26mm that hold 0.004 ml of urine was inoculate into the above media. The inoculums were spread with the wire loop on the media plate. Plates were incubated aerobically at 37°C for 24 hours.

Antibacterial Activity

The antibacterial potential of root extract of *Juglans regia* was tested by disc diffusion method [23]. Mueller-Hinton agar plates were used for determining the antibacterial activity. The plates were lawn cultured with inoculum of bacterial suspension (equivalent to 0.5 McFarland standard) with the help of sterile swabs. Solution of known concentration of the test samples in calculated volume of solvents dried and sterilized filter paper discs (6mm diameter) were then impregnated with known amount of the test substances using micropipette. Standard antibiotic (Ciprofloxacin) disc was used as a positive control. These plates were then kept at low temperature (4 °C) for 24 h to allow maximum diffusion. There was a gradual change in concentration in the media surrounding discs. The plates were then incubated at 37 °C for 12 h to allow maximum growth of the microorganisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter.

Results and Discussion

The activity showed by root extract of selected plant was promising against bacteria responsible for urinary tract infection.

A total of 500 patients of all age group clinically diagnosed as urinary tract infection (UTI) were studied to isolate bacteria from urine. Out of 500 UTI 220 (44%) were culture positive.

Table I: Distribution of culture by UTI

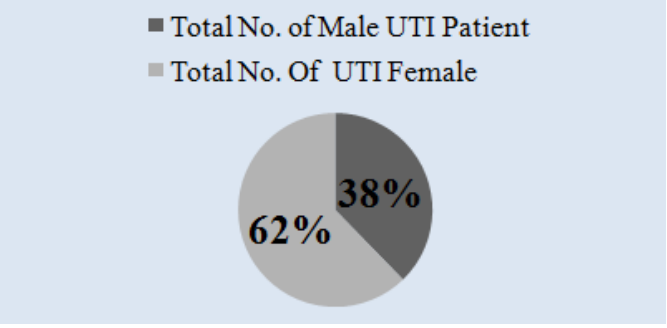
Type of UTI	Urine cultured	Culture positive cases	Percentage
UTI	500	220	44%

Among the samples analyzed and data obtained by the patients of UTI it could be assessed that female is more prone (62.27%) than male (37.72 %) for the above infection

Table II. Distribution of infected patient male and female.

Total No. of Male UTI Patient	Total No. Of UTI Female
83 (37.72 %)	137 (62.27 %)

Fig. 1. Distribution of infected patient male and female.

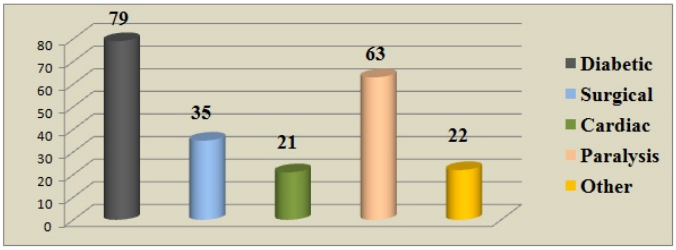


From table iii it is found that Paralysis (64.28%) was identified as risk factor for maximum patients. Diabetic (57.66%) was the next to it.

Table 2 Effect of long term treatment of CMSE on US, UP and BW of severely diabetic rats.

Risk Factor UTI Patient	Total No. of Patients	No. of Infected Patients	Percentage
Diabetic	137	79	57.66%
Surgical	87	35	40.22%
Cardiac	68	21	30.88%
Paralysis	98	63	64.28%
Other	110	22	20%

Fig. 2. Prevalence of risk factor of bacteria among suspected UTI patients



The most common organisms isolated were Escherichia coli (85), Proteus mirabilis (48), Pseudomonas aeruginosa (29), Klebsiella pneumonia (33) Staphylococcus aureus (18) and Staphylococcus saprophyticus (07) form diseased patients.

Table IV: Distribution of isolated bacteria responsible for urinary tract infection

Isolated bacterial species	
E. coli	85
P. aeruginosa	29
Proteus mirabilis	48
Klebsiella pneumonia	33
Staphylococcus aureus	18
Staphylococcus saprophyticus	07
Total	**220**

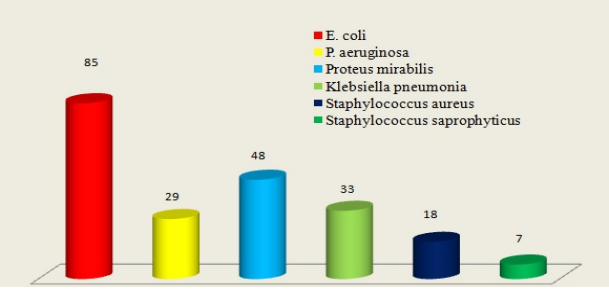


Fig. 3. Distribution of bacteria responsible for urinary tract infection

According to the results of this study, the highest isolate bacteria was E. coli (38.63%) followed by Proteus mirabilis (21.88%), Klebsiella pneumonia (15%) P. aeruginosa (13.18%), Staphylococcus aureus (8.18%) and Staphylococcus saprophyticus (3.18%)

The presence of alkaloids, glycosides, saponins, carbohydrates, proteins, aminoacids, flavonoids, steroids, tannins was detected as shown in Table V.

Table V. Preliminary phytochemical screening of leaves of *Juglans regia*

Name of the test	Methanol Extract	Ethyl acetate Extract	Hexane Extract
Carbohydrates	+	+	+
Proteins	+	+	-
Aminoacids	+	+	+
Steroids	+	+	+
Cardiac glycosides	+	+	+
Flavonoids	+	+	+
Alkaloids	-	+	+
Tannins	+	+	-

Seven biochemical tests were performed for each organism catalase activity, indole production test, citrate utilization test, oxidase, urease test, methyl red and voges proskauer‟s test were done as given in Table VI

Table VI. Biochemical tests of recovered clinical isolates

Catalase	Indole	Oxidase	Citrate	MR	VP	Urease	Organism confirmed
+	+	-	-	+	-	-	*Escherichia coli*
+	-	+	+	+	-	-	*Proteus mirabilis*
+	-	-	+	-	+	±	*Klebsiella pneumoniae*
+	-	+	+	-	-	-	*Pseudomonas aeruginosa*
+	-	-	+	+	+	+	*Staphylococcus aureus*
+	-	-	+	-	-	+	*Staphylococcus saprophyticus*

+= positive, - = negative, ± = Some species are positive some negative

The results of inhibitory effect of root extract of *Juglans regia are* shown in Table VII

Table VII. Antibacterial activity of root extract of *Juglans regia* against selected UTI pathogens

Table VII. Antibacterial activity of root extract of *Juglans regia* against selected UTI pathogens

Bacterial species	Solvents used			Standard
	Methanol	Ethyl acetate	Hexane	Ciprofloxacin
Escherichia coli	27	29	22	36
Proteus mirabilis	16	21	17	32
Klebsiella pneumoniae	18	24	16	39
Pseudomonas aeruginosa	23	26	12	35
Staphylococcus aureus	15	18	09	34
Staphylococcus saprophyticus	16	20	07	38

The results indicate that five bacterial species exhibit different sensitivities towards the extract. The extract was found to be inhibitory to both bacterial isolates but with variable extent. The order of activity against selected bacteria was *E. coli> Klebsiella pneumoniae>P. aeruginosa> Proteus mirabilis>Staphylococcus saprophyticus >Staphylococcus aureus.* The obtained crude extract of root was tested against different bacterial strains and compared to that of antibacterial antibiotic, ciprofloxacin. The results of the sensitivity test are shown graphically in (**Fig. 4)**.

The maximum antibacterial activity was observed of crude extract of *Juglans regia* against four gram negative bacteria and two gram positive strains of bacteria. Ethyl acetate extracts shows maximum activity against all pathogenic bacteria, *E. coli* shows (29 mm) followed by *Pseudomonas aeruginosa* (26 mm), *Klebsiella pneumonia* (24 mm), and moderate activity was observed against *Proteus mirabilis* (21 mm), *Staphylococcus aureus* (18 mm) *Staphylococcus saprophyticus* (20 mm). While the methanolic extracts of *Juglans regia* shows highest against *E. coli* (27 mm), *Pseudomonas aeruginosa*, (23 mm) *Klebsiella pneumonia,* (18 mm) *Proteus mirabilis,* (16 mm) *Staphylococcus aureus* (15 mm) and *Staphylococcus saprophyticus* (16 mm). Inhibition zones noticeable for hexane extract derived from *Juglans regia* against *E. coli* (22 mm), *Pseudomonas aeruginosa*, (12 mm) *Klebsiella pneumonia,* (16 mm) *Proteus mirabilis,* (17mm) and minimum activity was observed against *Staphylococcus aureus* (09 mm) and *Staphylococcus saprophyticus* (07 mm). The diameter of the zones of inhibition is shown in (**Table VII**). The diameters of the zones of inhibition with the standard drug used were 36 mm, 32 mm, 39mm, 35 mm, 34 mm and 38 mm for the six strains respectively. The studied root extract showed a remarkable antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis, Klebsiella pneumonia, Staphylococcus aureus* and *Staphylococcus saprophyticus* Although, the mechanism of action of the extract has not yet been elucidated in detail, the presented data confirm *in vitro* antibacterial activity of root extract against UTI causing bacteria.

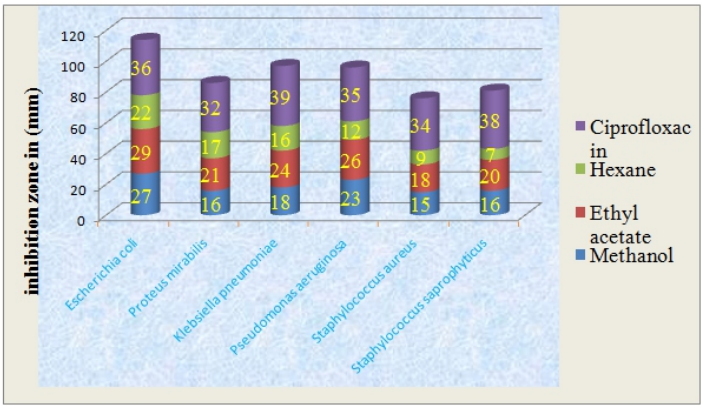
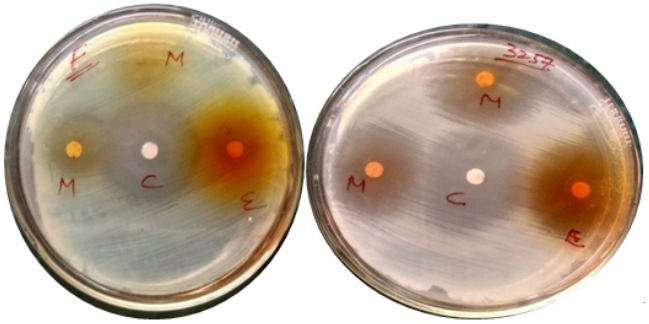


Fig. 4. Antibacterail activity of Juglans regia against six pathogenic bacteria causing UTI



Urinary Tract Infections remains the most common bacterial infection in human population and is one of the most frequently occurring nosocomial infections [24]. Different plant metabolites have shown effective antibacterial activity against uropathogens including drug resistant strains. For example the leaf and bark extracts of *Pimenta dioca* (Linn) Merill (Myrtaceae) and *Anacardium occidentale* L. (Anacardiaceae) exhibited antibacterial efficacy against drug resistant clinical isolates of urinary tract infection [25]. Plants such as *Coccinia grandis* [26], *Caesalpinia pulcherrima, Delonix regia* and *Peltaphorum ferruginum* [27] were reported for antibacterial efficacy against uropathogens. The results in the present study indicate that the antibacterial activity varies according to type of bacteria used for the study. The samples obtained indicated the presence of seven different bacterial strains which were subjected to primary investigation by culturing on nutrient agar and studying the morphological nature of the colonies so obtained, after which was subjected to disc diffusion method using plant extracts as to identify the most suitable antibacterial agent. The use of plant extracts with known antibacterial properties can be of great significance in therapeutic treatments. Intensive care physicians consider antibiotic resistant bacteria a significant problem in the treatment of diseases. A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds. Medicinal plant extracts, therefore offer considerable potential for the development of new agents effective against infections currently difficult to treat.

Acknowledgements

This study was supported by Department of Pathology & Microbiology, Bombay Hospital & Research Centre, Jabalpur, M.P.

Conclusion

The results from the current study indicate that *Juglans regia* roots contained various types of compounds with potential pharmacological activity against bacterial pathogens associated with UTIs. Further research work involving more detailed *in vitro* and *in vivo* investigations is required to establish which components of the extract are biologically active in terms of antibacterial activity versus UTI causing bacteria. The isolation of bioactive components from this readily available natural resource and their utilization as potential natural antibacterial agents could be of high economic value.

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