ANTIBACTERIAL ACTIVITY AND PRELIMINARY PHYTOCHEMICAL ANALYSIS OF STEM BARK EXTRACT OF *JUGLANS REGIA* LINN.

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Summary

Juglans regia Linn. also called as Walnut and Akhrot is a deciduous, monoecious tree belonging to family Juglandaceae. In traditional literature, it was found that stem bark was used as folk medicine for centuries as antibacterial for the treatment of various infectious diseases The stem bark of *Juglans regia* Linn. was collected from Upadhyay Orchards in Kullu (Himachal Pradesh) in May, 2010 and authenticated by Dr. K. Madhava Chetty, Asst. Prof., Dept. of Botany, Sri Venkateshwara University, Tirupathi (A.P). Stem bark was shade dried, powdered and extracted using different solvents viz., petroleum ether, benzene, chloroform, acetone, methanol, ethanol and distilled water in ascending order of polarity. Preliminary phyto-chemical screening of the crude extracts revealed the presence of carbohydrates, cardiac glycosides, flavanoids, steroids and tannins. The presence of these bioactive constituents is associated with the antimicrobial activity of the drug. Agar well diffusion method revealed high activity against gram positive microorganisms viz. *Bacillus subtilis*, *Staphylococcus aureus*, a group of gram- positive bacteria that frequently cause enteric infections in humans. Gram negative bacteria were not susceptible as gram- positive bacteria. Results were compared with the standard drug, Ampicillin. Minimum inhibitory concentration (MIC) values ranged from 50µg/ml to 300 µg/ml. The results confirm that *Juglans regia* Linn. can be used as source of drugs to fight infections caused by susceptible bacteria.

Keywords: *Juglans regia* Linn., phytochemical, antibacterial activity.

Introduction

There is an increasing demand for medicinal plant and plant products as alternatives to orthodox medicines especially in developing countries. The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the Near east, but it is doubtless an art as old as man-kind. Random screening as a tool in discovering new biologically active molecules has been most productive in the area of antibiotics[1] . Even now, contrary to common belief, drugs from higher plants continue to occupy an important niche in modern medicine. On a global basis, at least 130 drugs, all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons.

The tree *Juglans regia* Linn. belonging to family Juglandaceae is commonly known as Walnut in English and Akhrot in Hindi[2] . It is a native of Central Asia and found in temperate Himalayas at an altitude of 1000-3000mtrs[2]. *Juglans regia* Linn. stem bark contains chemical constituents viz. β-sitosterol, ascorbic acid[3], juglone, folic acid, gallic acid, regiolone, and quercitin-3-α-L-arabinoside[4]. This tree is reputed to possess varied medicinal properties. Traditional literature revealed that the leaves of *Juglans regia* Linn. were used as folk medicine as anthelmintic, antibacterial[5], aphrodisiac and tonic whereas the fruits were used as carminative and astringent[6]. The antidiabetic[7], antifungal, anticancer, of the leaf had been reported.

Hence the present work is done to investigate the antibacterial activity of different extracts of the stem bark of *Juglans regia* Linn.

Materials and Methods

Collection of plant material

The fresh bark of. *Juglans regia* Linn. was collected in the month of May, 2010 from Upadhyay Orchards in Kullu (H.P.) and authenticated by Dr. Madhava Chetty, Asst. Prof., Dept. of Botany, Sri Venkateshwara University, Tirupathi. (A.P.)

Processing of plant material

The bark was shade dried over a period of two weeks. The dried samples were milled into fine power by pounding manually with a clean and sterile mortar, stored in sterile cellophane bags in a cool dry place till further use.

Preparation of extracts

Extraction of plant material

The dried coarse powder (300gm) was extracted in Soxhlet Apparatus sequentially in 1.5 ltrs of various solvents viz., petroleum ether, benzene, chloroform, acetone, methanol, ethanol and distilled water in the ascending order of polarity. The process was run till the decolourisation of the solvent, after which the sample was concentrated using rotary evaporator and freeze dried to powdered form. The dried extracts were weighed and kept in labeled sterile specimen bottles.

The extracts obtained were suspended in dimethyl sulphoxide (DMSO) to prepare different concentrations ranging from 200 µg/ml to 1200µg/ml and used for screening the antibacterial activity.

Preliminary Phytochemical screening

The major secondary metabolites classes such as alkaloids, carbohydrates, cardiac glycosides, flavanoids, saponins, steroids and tannins were screened according to the standard phytochemical methods[8] .

Microbial strains

The Microbial strains used are *Bacillus subtilis*, *Escherichia coli* , *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The bacterial isolates were cultured on nutrient agar and incubated at 37˚C for 24 hrs and the microorganisms were repeatedly sub-cultured in order to obtain pure isolation. Morphological and biochemical reactions were carried to ascertain proper identification. They were inoculated into nutrient agar slants and stored at 4˚C. For MIC bacterial inocula were agitated for 15 sec with a Vortex mixer and were diluted 1:100 using sterile saline (0.9%) to get a concenteration of 1.5×108 CFU/ml respectively. MIC was defined as the lowest concentration of extract that inhibited the visible growth on agar.

Preparation of media

The medium was prepared by dissolving nutrient agar (HiMedia Laboratories Pvt. Ltd) in distilled water and autoclaving at 121^0 C for 15 minutes. It was used for preliminary antibacterial study.

Preparation of inoculum

Stock cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* were maintained at 4^{o} C on slopes of nutrient agar. Active cultures for experiment were prepared by transferring a loopful of microorganisms from stock cultures to test tubes of nutrient broth and incubated for 24 hours at 37^{o} C. The cultures were diluted with fresh nutrient broth.

Antibacterial susceptibility test /Agar well diffusion assay

Antibacterial method of Bauer *et al* [9]. was adopted. Nutrient agar medium (100 ml) in sterile Petri plates were used for the test cultures (bacteria 108 CFU/ml). The nutrient agar (NA) (Hi Media Laboratories Pvt. Ltd. Mumbai) plates were prepared by pouring 100 ml of molten media in to sterile petriplates. Nutrient agar plates were swabbed using sterile cotton swabs with the adjusted broth culture of the respective bacterial strains. . The plates were allowed to solidify and inoculum suspension was spreaded uniformly with glass spreader. Sterile discs (6.0mm in diameter) were dipped in solution of the different concentration (50µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml & 300 µg/ml) of various extracts dissolved in dimethyl sulfoxide (DMSO, Merck) till saturation and dried at 40°C for 30 minutes were used for the purpose. The disc was dipped in DMSO and used as a negative control. The standard antibacterial agent ampicillin (20µg/ml, 40µg/ml/disc) was used as positive controls. Extracts were allowed to diffuse at room temperature for 2 hrs and the plates were incubated to 37°C for 24 hrs. The diameter of the Zone of inhibition was measured in mm and the antibacterial experiments were performed in triplicates.

MIC determinations

The extracts were dissolved in DMSO (Stock -1mg/ml) and this was serially diluted to obtain concentration of 50µg/ml to 300 µg/ml and added to the nutrient broth. Sterile discs of each concentration was added to the plates containing standard inoculum. The negative control consists of nutrient broth and of the standard inoculum . The plates were covered with a sterile plate scale and incubated at 37°C for 24hrs. The assay was repeated thrice. The lowest concentrations that yielded no growth after this subculturing was considered as the MIC (Minimum Inhibitory Concentration). MIC values of different extracts are tabulated in table 3.

Chemicals used

- Ampicillin (standard drug)
- Dimethyl sulphoxide (DMSO)
- Nutrient agar medium

Statistical analysis

The values are represented as mean ± standard error of mean (SEM) for triplicate set of experiments.

Results and Discussion

Preliminary Phytochemical screening

Phytochemical screening of the crude stem bark extracts of *Juglans regia* Linn. revealed the presence of carbohydrates, cardiac glycosides, flavanoids, steroids and tannins. Results of preliminary phytochemical screening are tabulated in table 1.

These compounds have potentially significant application against human pathogens, including those that cause enteric infections[10] .The presence of glycosides moieties like saponins, cardiac glycosides and Flavonoids which

are known to inhibit tumor growth and serve also to protect against gastrointestinal infections are of pharmacognostic importance and give evidence to the use of the plant in ethnomedicine. The increasing reliance on the use of medicinal plants by a sizeable proportion of the people in the so called industrial world has been traced to the extraction and development of several drugs from these plants as well as from traditionally used rural herbal remedies[10].

Agar disc diffusion assay

In vitro antibacterial activity of benzene, acetone, methanol and ethanol extracts of stem bark of *Juglans regia* Linn. were evaluated by measuring the diameters of zones of growth inhibition of the bacterial colonies and the results are tabulated in table 2. The highest zone of growth inhibition was shown by acetone extract (300µg) against gram - positive bacteria, *B. subtilis* (22mm). At same concentration (300µg) benzene and methanol extracts also inhibited gram-positive bacteria *B. subtilis* (20 mm), whereas ethanol extract showed moderate activity against gram-positive bacteria (16mm), but all these extracts did not show any considerable activity against gram-negative bacteria. These results show that gram-negative bacteria are known to be resistant to the action of tested extracts. The reason for the differential sensitivity between gram-positive pattern and gram – negative bacterial strains could not be ascribed to their morphological differences or adduced to their chemical compositions. Gram-negative bacterial strains have an outer phospholipids membrane with the structural lipopolysaccharide components, which make their cell well impermeable to antimicrobial agents while the gram – positive bacteria should be more susceptible having only an outer peptidoglycan, which is not effective permeability barrier. The acetone extracts are more effective than benzene, methanol and ethanol extracts. The large zone sizes produced by the plant extract against the test bacteria, especially the acetone extracts is an indication of the potency of the bioactive components of the plant against all the test bacteria. The lowest zone of growth inhibition was found to be of ethanol extract. There was no activity in Petroleum ether, Chloroform and Aqueous extracts. The antibacterial potency of the stem bark of *Juglans regia* Linn. maybe attributed to single or the combined effect of the phytoconstituents present in the bark.

Table 1: **Preliminary phytochemical evaluation of the stem bark of *Juglans regia* Linn.**

Phytoconstituents	Petroleum ether Extract	Benzene extract	Chloroform extract	Acetone extract	Methanol extract	Ethanol extract	Aqueous extract
Alkaloids	-	-	-	-	-	+	+
Carbohydrates	-	-	-	-	-	-	+
Cardiac glycosides	-	-	+	+	+	+	-
Flavanoids	-	-	-	+	+	+	+
Steroids	+	+	+	+	+	-	-
Tannins	-	-	-	+	+	+	+

'+' : Present; '-' : Absent

Table 2: **Antibacterial activity of different extracts of the stem bark of *Juglans regia* Linn. against gram positive bacteria.**

	Zone of inhibition (mm)	
	Gram positive bacteria	
	Staphylococcus aureus	***Bacillus subtilis***

Concentration of extract (µg/ml)	**50**	**100**	**150**	**200**	**250**	**300**	**50**	**100**	**150**	**200**	**250**	**300**
Benzene extract	10± 1.154	12± 0.577	14± 0.577	14± 1.154	16± 0.577	16± 1.154	8± 0.577	14± 0.577	16± 1.154	16± 0.577	18± 0.577	20± 0.577
Acetone extract	0±0	0±0	12± 0.577	12± 1.154	14± 0.577	14± 0.577	16± 0.577	18± 0.577	18± 1.154	18± 0.577	20± 0.577	22± 0.577
Methanol extract	0±0	0±0	10± 0.577	12± 0.577	12± 1.154	14± 1.154	10± 0.577	14± 0.577	16± 0.577	18± 0.577	18± 1.154	20± 1.154
Ethanol extract	0±0	0±0	0±0	0±0	0±0	10± 0.577	8± 1.154	8± 0.577	10± 0.577	10± 1.154	12± 0.577	16± 0.577

Antibacterial activity of the standard drug, Ampicillin:

		Zone of inhibition (mm)			
		Gram positive bacteria		**Gram negative bacteria**	
		Staphylococcus aureus	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Escherichia coli***
Ampicillin	**20µg/ml**	28±1.154	10±0.577	28±0.577	38±1.154
	40µg/ml	36±1.154	12±0.577	32±1.154	42±0.577
DMSO (control)		0	0	0	0

Table 3: Minimum inhibitory concentration (MIC) values of different extracts of the stem bark of *Juglans regia* Linn.

	MIC value (in µg/disc) against			
	Gram positive bacteria		**Gram negative bacteria**	
	Staphylococcus aureus	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Escherichia coli***
Benzene extract	50	50	-	-
Acetone extract	150	50	-	-

Methanol extract	150	50	-	-
Ethanol extract	300	50	-	-

Conclusion

The phytochemical assay of the stem bark extracts of *Juglans regia* Linn. revealed the presence of carbohydrates, cardiac glycosides, flavanoids, steroids and tannins. Most of the secondary metabolites were identified in the methanol and acetone extracts.

Thus, antimicrobial activity of *Juglans regia* Linn. is evident due to the active compounds present in the crude extracts. The findings in the present study offer a scientific support to the use of stem bark of *Juglans regia* Linn. as an antibacterial in new drugs for therapy as it showed significant antibacterial activity. Further pursuit on the isolation of bioactive compounds would enable more potential and natural antibiotics against several pathogens..

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