ISSN 0976 – 3333

Available Online at www.ijpba.info.

International Journal of Pharmaceutical & Biological Archives 2010; 1(5):442-447

ORIGINAL RESEARCH ARTICLE

Antifungal Activity and Preliminary Phytochemical Analysis of Stem Bark Extracts of *Juglans regia* linn.

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Received 20 Nov 2010; Revised 28 Nov 2010; Accepted 06 Dec 2010

ABSTRACT

Juglans regia, the royal species from *Junglandaceae* family, well-known for its valuable medicinal uses isgrown in the forests of Himalayas in India. It is a woody, deciduous and frost-tender tree. The root, stem bark, leaves, seeds, cotyledons and seed oil are useful to treat various health complaints including cancer in the folk medicines. The stem bark was collected from Upadhyay Orchards in Kullu (Himachal Pradesh) in May, 2010 and authenticated by Dr. K. MadhavaChetty, Asst. Prof., Dept. of Botany, Sri Venkateshwara University, Tirupathi (A.P). It 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.Crude extract of the bark of *Juglans regia* Linn.were tested for antifungal activity by observing the zone of inhibition against four species of fungi. Antifungal activity was done by disc diffusion method at concentrations of 100, 200 and 300 µg/ml/disc of the extracts, using ketoconazole(40µg/ml/disc) as the standard. The extracts showed selective fungistatic action against some species.All extractsshowed varying degrees of inhibitory activity against allfungal species. Methanolic extract showed significant activity against *Aspergillus niger*, Acetone extract significantly inhibited the growth of *Alternalia alternata*, Chloroform extract exhibited promising activity against *Trichoderma virens* and *Fusariumsolani*.

Keywords:*Juglans regia*Linn.,phytochemical, antifungal activity, ketoconazole.

INTRODUCTION

The use of higher plants and their preparations to treatinfectious diseases is an age-old practice and in the pastpossibly the only method available. However, thesystematic study of higher plants for detecting antifungal activity is of comparatively recent origin.Hence, the plant kingdom is being screened for newer and effective chemotherapeutic agents. Higher plants can serve both as potential antimicrobial crude drugs as well as asource of new anti-infective agents[1].

Juglans regia, known as Akhrot in India, a native of Eastern Europe to North Asia i.e. China, Iraq, Mexico, Spain, Turkey, Nepal, India (forests in Himalayas) is a member of *Juglandaceae* family. It is a woody, deciduous and frost-tender tree growing to 20m. height[2]. The wood is heavy, durable and polishes well. The bark is resinous and scented. The tree is in flower in June and a seed ripe in October. 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. The dried green husks contain 2.5-5% ascorbic acid(vitamin C) which can be extracted and used as a vitamin supplement. The rootand stem bark are anthelmentic, astringent[3], antibacterial[4] and detergent. The stem bark is dried and used as a toothcleaner. The decoction of leaves and bark is used with alum for staining wool brown. The cotyledons are used in the treatment of cancer since a long time. Some extracts of the plant have shown anticancer activity. *Juglans regia* Linn.stem bark contains chemical constituent's viz. β-sitosterol, ascorbic acid[5], juglone, folic acid, gallic acid, regiolone, and quercitin-3-α-L-arabinoside[6]. Juglone found in the leaves and its derivatives show a wide spectrum of applications in the field of cosmetics, pharmacology and ecology. This tree is reputed to possess varied medicinal properties.Considering the vast medicinal applications of the royal species, an

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attempt is made to technically analyze the stem bark for *in vitro* antifungal activity as there is no report on antifungal activity of this plant.The present work therefore, attempts to evaluate the antifungal activity of the stem bark of *Juglans regia* Linn.

MATERIALAND 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. MadhavaChetty, 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;

Thedried 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 100 µg/ml to 300µg/ml and used for screening the antifungal activity.

Preliminary Phytochemical screening;

The secondary metabolites classes such as alkaloids, carbohydrates, cardiac glycosides, flavanoids, saponins, steroids and tannins were screened according to the standard phytochemical methods[7].

Fungal strains;

The fungal strains used are *Aspergillus niger, Alternalia alternata,Trichoderma virens and Fusarium solani.* The bacterial isolates were cultured on Sabouraud Dextrose agarand incubated at ˚C37 for 2 4 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 SDA slants and stored at 4˚C. For MIC fungal 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 Sabouraud dextrose agar.

Preparation of media;

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

Preparation of inoculum;

Stock cultures of *Aspergillus niger,Alternalia alternata,Trichoderma virens* and *Fusarium solani* were maintained at 4^o C on slopes of SDA. Active cultures for experiment were prepared by transferring a loopful of fungal organisms from stock cultures to test tubes of SDA slants and incubated for 24 hours at 37^o C.

Antifungal susceptibility test /Agar disc diffusion assay;

The method of Bauer*et al*[8] was adopted for the study.Sabouraud dextrose agar mediamedium (100 ml) in sterile Petri plates was used for the test cultures. TheSDA (Hi Media Laboratories Pvt. Ltd. Mumbai) plates were prepared by pouring 100 ml of molten media in to sterile petriplates.SDA plates were swabbed using sterile cotton swabs with the adjusted broth culture of the respective fungal 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 (100 µg/ml, 200 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 antifungal agent, ketoconazole (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 antifungal experiments were performed in triplicates. The results of antifungal activity are tabulated in table 2.

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 were 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 subculturingwas 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)
- Sabouraud Dextrose 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 (T**able 1**).

The increasing reliance on the use of medicinal plants worldwide has been traced to the extraction and development of several drugs from these plants as well as from traditionally used rural herbal remedies. Further, detailed investigation needs to be underway to determine the exact phytoconstituents and isolate the active principles which are responsible for the antifungal activity of the stem bark of *Juglans regia* Linn.

Agar disc diffusion assay;

In vitro antifungal activity of pet.ether, benzene, chloroform, acetone and methanol extracts of stem bark of *Juglans regia* Linn. were evaluated by measuring the diameters of zones of growth inhibition of the fungal colonies and the results are tabulated in table 2. The highest zone of growth inhibition was shown by methanol and chloroform extracts (300µg/ml) against *Aspergillu sniger* and *Trichoderma viren s*respectively (9.33mm).At same concentration (300µg/ml) benzene, acetoneandpet.ether extracts also inhibited the growth of *Fusarium solani, Alternalia alternata and Aspergillus niger* (9mm,8.33mm,7.33mm) respectively,butthe pet. ether, chloroform and methanolicextracts did not show any activity against *Fusarium solani, Alternalia alternata and Aspergillus niger* at all. These results show that these fungal species are known to be resistant to the action of tested extracts. The methanolic extract was found to be more effective than other extracts which indicates the potency of the bioactive components of the plant against all the test species. The lowest zone of growth inhibition was found to be of acetone extract against *Fusarium solani*. The antifungal 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	-	-	-	+	+	+	+

Table 2**: Antifungal Activity of the Different Extracts of the stem bark of J*uglans regia* linn.**

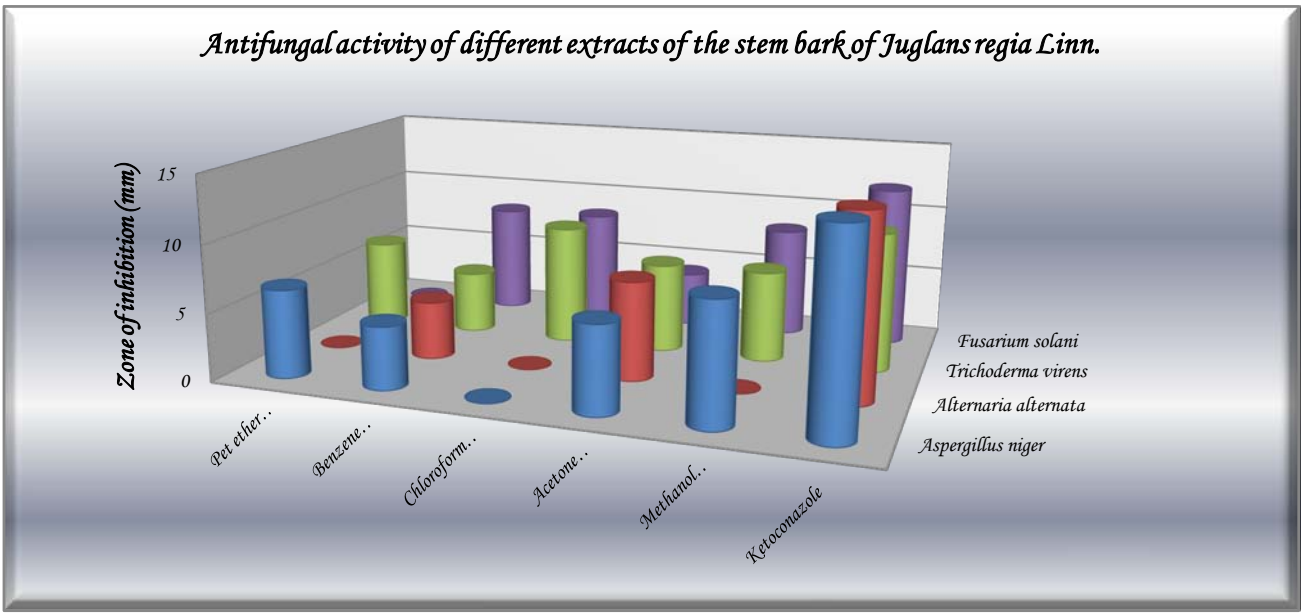
Treatment	Zone of inhibition (mm)											
	Aspergillus niger			*Alternaria alternata*			*Trichoderma virens*			*Fusarium solani*		
	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml
Pet ether ext.	6±0	6.33± 0.999	7.33± 0.999	0±0	0±0	0±0	6±0	6.66± 0.666	6.66± 0.999	0±0	0±0	0±0
Benzene ext.	0±0	6.66± 0.666	7.33± 0.881	0±0	6±0	7± 0.577	0±0	6.33± 0.999	7.66± 0.881	7±0	8.66± 0.999	9± 0.577
Chloroform ext.	0±0	0±0	0±0	0±0	0±0	0±0	8.33± 0.881	9± 0.577	9.33± 0.999	7.67± 0.999	8.33± 0.666	9±0
Acetone ext.	6±0	6.33± 0.999	7±0	6.66± 0.999	7± 0.577	8.33± 0.999	6.33± 0.999	6.66± 0.666	7± 0.577	0±0	6±0	6.33± 0.999
Methanol ext.	8.33± 0.999	9± 0.577	9.33± 0.999	0±0	0±0	0±0	6±0	7±0.5 77	7.33± 0.666	7.33± 0.999	8.33± 0.666	9± 0.577

Treatment	Zone of inhibition (mm) *Aspergillus niger*	*Alternaria alternata*	*Trichoderma virens*	*Fusarium solani*
	40 µg/ml	40 µg/ml	40 µg/ml	40 µg/ml
ketoconazole	14.33±1.201	13.6±0.832	10.3±0.299	12±1.154

TABLE3: MIC VALUES OF DIFFERENT EXTRACTS

	MIC value (in µg/ml/disc) against tested fungal organisms			
Treatment	*Aspergillus niger*	*Alternalia alternata*	*Trichoderma virens*	*Fusarium solani*
Pet.ether extract	80	-	100	-
Benzene extract	150	150	150	75
Chloroform extract	-	-	50	75
Acetone extract	100	100	75	100
Methanol extract	50	-	100	50

GRAPH 1: **Graphical Representation Of The Antifungal Activity Of Stem Bark Of *Juglans regia* Linn.**



PICTURES SHOWING ZONE OF INHIBITON OF DIFFERENT EXTRACTS OF THE STEM BARK OF *Juglansregia*Linn. AGAINST THE ANTIFUNGAL SPECIES

Aspergillus niger *Alternalia alternate* *Trichoderma virens* *Fusarium solani*

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 thesecondary metabolites were identified in the methanol and acetone extracts.

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

ACKNOWLEDGEMENTS:

The authors are highly thankful to The Chairman, Executive Director and the Principal The Oxford College of Pharmacy, Bangalore for providing necessary facilities and support to conduct this work. The authors are also thankful toDr. Bharathi HOD Department of Microbiology, The Oxford college of Science Bangalore for providing the fungal strains and Dr. Madhava Chetty, Asst. Prof., Dept. of Botany, Sri Venkateshwara

University, Tirupathi (A.P.) for authenticating the sample.

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