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Antifungal Activity Screening and HPLC Analysis of Crude Extract from *Tectona grandis*, *Shilajit*, *Valeriana wallachi*

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Abstract

The antifungal activity of methanolic crude extract of *Tectona grandis*, *Shilajit*, *Valeriana wallachi* was investigated against *Alternaria cajani*, *Curvularia lunata*, *Fusarium sp.*, *Bipolaris sp.* and *Helminthosporium sp.* at different concentrations (1000, 2000, 3000, 4000 and 5000 µg/ml). Better antifungal activity was observed with the extracts of *Valeriana wallachi*, that showed excellent inhibitory activity against *Helminthosporium sp.* (96.15%) followed by *Shilajit* extract against *Alternaria cajani* (95.12 %) and *Helminthosporium sp.* (95.00 %) at concentration of 5000 µg/ml. Among different fungi tested *Bipolaris sp.* and *Fusarium* were found to be more sensitive to crude extract when compared to others. The increase in

the production of phenolics in the extract can be correlated with the induction of resistance in treated plants against phytopathogenic fungi. HPLC analysis of the crude extract of medicinal plants showed four different Phenolic acids (Tannic acid, Gallic acid, Ferulic acid and Caffeic acid). The results of the study provide scientific basis for the use of the plant extract in the future development as antioxidant, antibacterial, antifungal and anti-inflammatory agent.

Introduction

Medicinal plants as a group comprise approximately 8000 species and account for around 50% of all the higher flowering plant species of India. Over one and a half million practitioners of the Indian System of Medicine use medicinal plants in preventive, promotive and curative applications. In recent years, secondary plant metabolites (Phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents ¹. The World Health Organization (WHO) has given guidelines to the member states to ensure about genuine use of plants and their parts before their use for human health. ²

Tectona grandis Linn. is commonly known as sagon, sagwan and belongs to family verbenaceae and is one of the most important heart wood of the world over. According ayurveda, wood is acrid, colling laxative sedative to gravid uterus and useful in treatment of piles, leucoderma and dysentery. It allays thirst and possess anthelmintic and expectorant properties ³. *Tectona grandis* leaf extract are widely used in the folklore for the treatment of various kinds of wound, especially burn wound ⁴. Shilajit, described as India's wonder drug, is used in Ayurveda, the traditional Indian system of medicine. The botanical name name of shilajeet is asphaltum (mineral pitch). Shilajit contains atleast 85 minerals in ionic form as well as humic acid and fulvic acid. Shilajit works effectively as a vitality increasing tonic. It helps in metabolism, stimulates our energy levels, fight against diabetes and regulates blood sugar balance. It boots the immune system and acts an anti-oxidant. It can be used in its raw form for animistic ritual and dream enhancement ceremonies or in its super purified form for enhancing both mental and psychospiritual activity of the brain ⁵.

Valerian is a well known and frequently used medicinal herb that has a long and proven history of efficacy. It is note specially for its effect as a tranquilliser and nervine, particularly for those people suffering from nervous overstrain ⁶. Valeriana has been shown to encourage sleep, improve sleep. It is also used internally in the treatment of painful menstruation, cramps, hypertension, irritable bowel syndrome etc. ⁷. It should not be prescribed for patients with liver problem. Externally it is used to treat eczema, ulcers and minor injuries ⁸. The root is antispasmodic, carminative, diuretic, hypnotic, powerfully nervine sedative and stimulant ⁹.

Various extract of medicinal plants have shown inhibitory effects against phytopathogenic fungi in vitro ¹⁰. Diverse pharmacological activities have been accredited to Phenolic acids by HPLC for instance gallic acid has inflammatory ¹¹, antibacterial ¹²; caffeic acid with anti-inflammatory ¹³; ferulic acid with anti-inflammatory ¹³ and antifungal ¹⁴; tannic acid with antioxidant and astringent property ¹⁵¹⁶.

The objective of this research was to aunthenticate the antifungal sensitivity and HPLC analysis of methanolic extracts of phenolic acid present in *Tectona*

grandis, Shilajeet and Valeriana wallachi to lengthen the queue of antimicrobial herbs.

Materials and methods

Collection and extraction of medicinal plant material

The raw material of medicinal plants such as, *Tectona grandis*, Shilajit and *Valeriana wallachi* were collected from different regions of India. Voucher specimens deposited at Institute of Bioengineering and Biological Sciences, Varanasi, India for future reference.

The dried powdered of plant materials (roots and aerial parts) were extracted separately with methanol: sterile water (1:1) using soxhlet apparatus for 48 hrs. The solvent was distilled off at lower temperature under reduced pressure in rotary flash evaporator and concentrated on water bath to get the crude extract which is stored in dessicator for future use.

Antifungal activity

Three different medicinal crude extract which showed in vitro antifungal activity against some plant pathogens such as *Alternaria cajani*, *Helminthosporium* sp., *Bipolaris* sp., *Curvularia lunata* and *Fusarium* sp., were used in the present experiment. Test fungi were isolated on potato dextrose agar (PDA) (peeled potato 250 g, dextrose 20 g, agar 15 g, distilled water 1 L) medium from their respective hosts collected from experimental farm of Banaras Hindu University, Varanasi, India. The cultures were further purified by single spore isolation technique and maintained at 25 ± 2 °C on PDA slants. 7-10 days old culture were used in the experiment.

Stock solution (5000 µg/mL) of the crude extract were prepared by dissolving 5 mg of the culture in 1 ml of distilled water. Required concentrations (1000, 2000, 3000, 4000 and 5000 µg/mL) were prepared from each stock solution by diluting with distilled water. One drop (40 µL) from each concentration was placed on grease-free glass slides. Fungal spores (200-300) were picked up from 7-10 days old culture with sterilized inoculation needle and mixed in solution of the fraction of different concentrations separately. The slides were placed in moist chambers made by placing two sterile filter papers each on the lid and base of the petriplates. The slides with spores were then incubated at 25 ± 2 °C for 24 hr. Germination was observed after staining with cotton blue prepared in lactophenol under binocular microscope (Nikon, Japan Type 102). Spores mixed in sterile distilled water only served as control. All the experiments were conducted in triplicate.

Sample Preparation of phenolic compounds

The phenolic acids were extracted as per the method of Singh et al. 17 . Three crude extracts of *Tectona grandis*, Shilajeet and *Valeriana wallachi* were collected from different places of India. One gram of each extract was macerated and suspended in 5 ml methanol-water (80:20; v/v). The collected samples were subjected to ultrasonication (Branson Sonifier, Danbury, CT, USA) for 15 min at 4°C followed by centrifugation at $12\ 500 \times g$ for 15 min. The clear supernatant was subjected to charcoal treatment. The residue was re-extracted twice with the same extracting solution and the supernatant was pooled prior to evaporation under vacuum (Buchi Rotavapor Re Type, Labco, India; Ambala Cantt. India). Dried extract were resuspended in 1.0 ml high-performance liquid chromatography (HPLC)-grade methanol by vortexing and filtered through ultra membrane filter (pore size 0.45 µm: Millipore) before HPLC analysis.

HPLC analysis

Quantitative analysis of the sample was performed according to the method of Singh et al. 17 . The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 AVP UV-VIS detector and a Rheodyne Model 7725 injector with a loop size of 20 μ l. The peak area was calculated with a Winchrom integrator. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 x 4.6 mm i.d., particle size 5 μ m, Luna 5 μ C-18(2); phenomenex, Torrance, CA, USA) at 25°C. Running conditions included: injection volume, 5 μ l; mobile phase, methanol: 0.4% acetic acid (80: 20 v/v); flow rate, 1 ml/min; and detection at 290 nm. Samples were filtered through an ultra membrane filter (pore size 0.45 μ m; E-Merck, Darmstadt, Germany) prior to injection in the sample loop. Tannic, gallic, caffeic, ferulic, benzoic, cinnamic, capachin and salicylic acids were used as internal and external standards. Phenolic acids present in each sample were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards. The amount of each phenolic acid is expressed as micrograms per gram of fresh weight unless otherwise stated.

Results and Discussion

Comparative analysis of antifungal activity

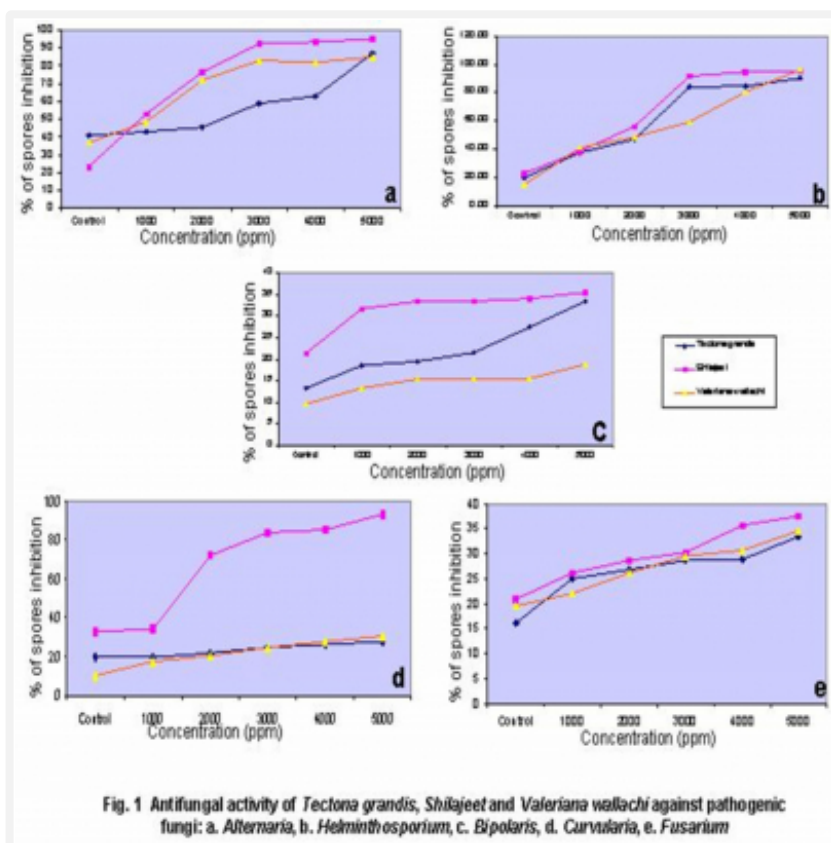
Crude extract of *Tectona grandis*, *Shilajit* and *Valeriana wallachi* were tested against phytopathogenic fungi such as *Alternaria cajani*, *Helminthosporium sp.*, *Bipolaris sp.*, *Curvularia lunata* and *Fusarium sp.* *Alternaria cajani*, *Curvularia lunata*, *Fusarium sp.*, *Bipolaris sp.* and *Helminthosporium sp.* at concentrations of 1000, 2000, 3000, 4000 and 5000 μ g/mL. The effects of the different concentrations of crude extracts on five different phytopathogenic fungi are presented in Table 1 and Fig. 1a, b,c, d, e.

Figure 1

Table 1: Effect of on spore germination of some pathogenic fungi

S. No.	Fungus	Host	Medicinal Extract	Concentration (μ g / mL)					
				Control	1000	2000	3000	4000	5000
1	<i>Alternaria cajani</i>	<i>Cajanus cajan</i>	<i>Tectona grandis</i>	41.18	43.18	45.28	58.82	63.16	86.84
			<i>Shilajit</i>	23.53	53.12	76.47	92.59	93.48	95.12
			<i>Valeriana wallachi</i>	37.14	48.48	72.22	82.93	82.14	85.00
2	<i>Helminthosporium sp.</i>	<i>Saccharum officinarum</i>	<i>Tectona grandis</i>	20.00	37.50	47.06	84.00	84.61	90.00
			<i>Shilajit</i>	22.58	38.71	56.25	91.67	94.59	95.00
			<i>Valeriana wallachi</i>	14.63	41.10	48.61	59.10	80.64	96.15
3	<i>Bipolaris sp.</i>	<i>Oryza sativa</i>	<i>Tectona grandis</i>	13.33	18.57	19.48	21.43	27.40	33.33
			<i>Shilajit</i>	21.28	31.67	33.33	33.33	34.00	35.48
			<i>Valeriana wallachi</i>	9.68	13.21	15.38	15.38	15.49	18.57
4	<i>Curvularia lunata</i>	<i>Sorghum vulgare</i>	<i>Tectona grandis</i>	19.35	20.00	22.22	25.00	26.10	27.78
			<i>Shilajit</i>	32.56	34.48	72.50	84.21	85.71	93.33
			<i>Valeriana wallachi</i>	10.00	17.65	20.41	25.00	27.78	30.91
5	<i>Fusarium sp.</i>	<i>Cajanus cajan</i>	<i>Tectona grandis</i>	16.13	25.00	26.92	28.57	28.79	33.33
			<i>Shilajit</i>	20.93	26.10	28.57	30.23	35.71	37.50
			<i>Valeriana wallachi</i>	19.44	21.95	26.10	29.50	30.77	34.48

Figure 2



The methanolic extract, on the other hand, inhibited growth of the test fungi to varying degrees. Concentrations at 1000 and 2000 $\mu\text{g}/\text{mL}$ brought minimal inhibition (*Valeriana wallachi* showed 13.21% and 15.38 % against *Bipolaris*) against test fungi (Table 1). Based on the inhibition profiles, *Bipolaris* sp. and *Fusarium* sp. were least sensitive to all the three extracts, while *Curvularia lunata* was less inhibited by *Tectona grandis* and *Valeriana wallachi*. A considerable reduction in the sporulation was also recorded. The methanolic extract tested at 5000 $\mu\text{g}/\text{mL}$ against a number of pathogenic fungi were found effective at higher concentrations. Among the five fungi tested, the extract of *Valeriana wallachi* was found to be most effective and evinced excellent inhibitory activity against *Helminthosporium* sp. (96.15%) followed by *Shilajit* extract against *Alternaria cajani* (95.12 %), *Helminthosporium* (95.00 %) and *Curvularia lunata* (93.33%) at the concentration of 5000 $\mu\text{g}/\text{mL}$. Also at concentrations of 3000 and 4000 $\mu\text{g}/\text{mL}$, *Shilajit* exhibited better antifungal activity against *Alternaria cajani* and *Helminthosporium* sp. The extract of *Tectona grandis* showed 90.00% and 86.84% inhibition growth against *Alternaria cajani* and *Helminthosporium*. It is revealed from the above statement, that higher concentration of the methanolic extract impart maximal antifungal activity (Table 1 and Fig. 1)

Various extract of medicinal plants have shown inhibitory effects against phytopathogenic fungi in vitro 17 . The antimicrobial activities of various plants have been reported by many researchers 18 . *Tectona grandis* sawdust extract exhibited the growth of *Aspergillus niger* 4 . Endophtic isolated from *Tectona grandis* could produce inhibitory substances effective against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* and *Candida albicans* in vitro 19 . A lipophilic crude extract of *Valeriana capense* exhibited antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum* 20 .

HPLC analysis

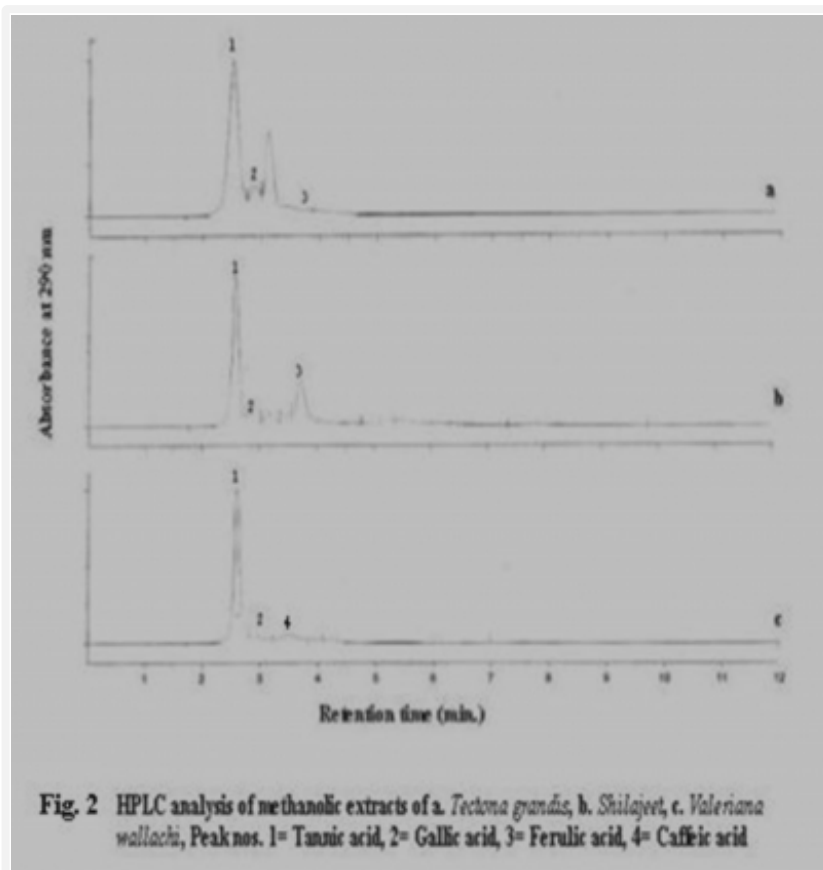
Recent researches indicate that the polyphenols, being secondary metabolites, are present in rich amount in several plants. Many of them possess antioxidant, anti-inflammatory and several others therapeutic properties. The HPLC fingerprints of the crude extracts of *Tectona grandis*, *Shilajeet* and *Valeriana wallachi* showed four types of the Phenolic acids i.e. Tannic acid, gallic acid, Ferulic acid and caffeic acid that are present in varying amount (Table 2 and Fig. 2). Although a primary objective of carrying out HPLC may be to standardize dosage, more information may be obtained during the course of a run, if appropriate detection hardware and software are used.

Figure 3

Table 2: Phenolic acid in the crude extract of , and

Crude Extract	Phenolic acid ($\mu\text{g/g}$ dry wt)			
	Tannic acid	Gallic acid	Ferulic acid	Caffeic acid
<i>Tectona grandis</i>	285.90	17.80	-	16.10
<i>Shilajit</i>	319.33	20.76	37.55	-
<i>Valeriana wallachi</i>	176.71	17.48	7.27	-

Figure 4



The HPLC 'fingerprint' (Fig. 2) of the methanolic extract of *Tectona grandis*, *Shilajit* and *Valeriana wallachi* show major peaks at the retention times (min.) of 2.61, 2.92, 3.72 and 3.48 at a wavelength of 290 nm. Out of the three extracts, *Shilajit* showed maximum amount of tannic acid (319.33 $\mu\text{g/g}$) followed by gallic acid (20.76 $\mu\text{g/g}$) and ferulic acid (37.55 $\mu\text{g/g}$) (Fig. 2b). *Valeriana wallachi* also revealed three types of Phenolic acids in which tannic

acid (285.90 µg/g) was present in maximal amount whereas gallic acid and caffeic acid were in trace (Fig. 2c) Tannic acid (176.71 µg/g) was also maximum in *Tectona grandis* followed by gallic acid (17.48 µg/gm) and ferulic acid (7.27 µg/g) which were present in minimal amount. HPLC analysis of the samples revealed wide-variability in their Phenolic acid content (Fig. 2a). The results of the antifungal activity of the various crude extracts were in agreement with the uses of the extract of *Tectona grandis*, *Shilajit* and *Valeriana wallachi* in traditional medicine. The rhizome and aerial parts of the plants appeared to be a potential source of broad spectrum antibiotics.

According to Bauer and Tittel 21 and Springfield et al., 22 , HPLC fingerprinting is the best way for chemical characterization, and therefore this study also established HPLC fingerprint for the active phenolic acids that can act as antioxidant, antifungal, antibacterial and anti-inflammatory. diverse pharmacological activities have been accredited to phenolic acids for instance, gallic acid has anti-inflammatory 23 , antibacterial 24 , caffeic acid with anti-inflammatory 13 , antibacterial, antifungal 24 ; ferulic acid with anti-inflammatory 13 , antifungal 25 ; cinnamic acid with antifungal 25 , anthelmintic 15 , natural protection against infections by pathogenic microorganisms 26 ; salicylic acid with antipyretic and anti-inflammatory 27 , externally used as antiseptic, antifungal and for various skin conditions 15 .

Validated HPLC method was developed for *Valeriana officinalis* and this method was validated with respect to system suitability, specificity, accuracy, precision, linearity and ruggedness 28 . For the quantitative analysis of valerianic acid, HPLC method of Hansel and Schulz was adopted 29 . Validated HPLC method was developed for *Valeriana officinalis* and this method was validated with respect to system suitability, specificity, accuracy, precision, linearity, and ruggedness 28 . Valepotriates are considered as one of the main groups of compounds responsible for the sedative activity of *Valeriana*. For the quantitative determination of valepotriates a direct spectrophotometric scanning on TLC plates was compared with HPLC 30 . In *Tectona grandis*, centrifugal partition chromatography was used to isolate the active compound such as deoxylapachol and tectoquinine that indicated fungal cell wall stress 4 . The active ingredients are called valepotriates, research has confirmed that these have a calming effect on agitated people, but are also a stimulant in cause of fatigue 31 . The leaves of the *swietenia macrophylla* plant possess antidiabetic, anti-inflammatory, antipyretic, analgesic, antispasmodic, antimicrobial, cathartia and expectorant activities 32 .

Conclusion

In conclusion, this study provides new scientific information about *Tectona grandis*, *Shilajit* and *Valeriana wallachi*, based on its antimicrobial potential and chemical profiling that has never been reported. The antifungal activity of *Tectona grandis*, *Shilajit* and *Valeriana wallachi* may be attributed to the various phytochemical constituents present in the crude extract. The purified components may have even more potency with respect to inhibition of microbes. Further work on the types of phytoconstituents and purification of individual groups of bioactive components can reveal the exact potential of the plant to inhibit several pathogenic microbes and encourage in developing a novel broad spectrum antimicrobial herbal formulation in future.

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