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Research Article

PHYTOCHEMICAL ANALYSIS AND ANTIMICROBIAL POTENTIAL OF LEAF EXTRACTS OF THUJA ORIENTALIS

JOGINDER S. DUHAN[1*], POOJA SAHARAN[1], SUREKHA[2]

[1] Department of Biotechnology, Ch. Devi Lal University, Sirsa-125 055, India,[2] Department of Botany, Government National PG College, Sirsa -125 055, India, E-mail: duhanjs68@gmail.com; duhanjs@rediffmail.com

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ABSTRACT

Plants are rich source of antibacterial components. Different plant extracts are being used in daily life to combat bacterial and fungal infections. In this study, methanol, acetone and ethyl acetate extracts prepared from leaves of *Thuja orientalis* were explored for their antimicrobial activity against various bacterial (i.e. *S. aureus*, *B. subtilis*, *P. aeruginosa*, *A. faecalis* and *K. pneumoniae*) and fungal (*A. flavus* and *A. niger*) pathogens. Among the tested strains of bacteria, *B. subtilis* (i.e. causal organism of dysentery) was found most sensitive against acetone extract of leaf with maximum zone of inhibition (15.55 mm) followed by *A. faecalis* (15.50 mm). These results were even better than synthetic antibiotics i.e penicillin and ampicillin. Methanol as well as ethyl acetate extracts of leaf were found quite effective against *S. aureus* (14.83 and14.0 mm) which were more potent than the penicillin and streptomycin where as inhibition of *P. aeruginosa* (14.00, 13.83 mm) was comparable to penicillin. None of the extract was able to inhibit *A. flavus* effectively but *A. niger* was inhibited maximum by methanol extract (13.00 mm) which was equally effective to synthetic antifungal drug ketoconozole followed by acetone (12 mm) and ethyl acetate extracts (11.83 mm). These results showed that the extracts prepared from *Thuja orientalis* can be used as natural remedy for treatment of various bacterial and fungal infections. Finally, it was concluded that many complications associated with resistant bacterial infections can be overcome by the use of drugs prepared from natural sources in order to replace synthetic antibiotics.

Keywords: Plant extracts, antibacterial activity, minimal inhibitory concentration, phytochemical screening.

INTRODUCTION

Many of the plant materials used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine[1]. According to recent findings of Mamidala and Gujjeti[2] World Health Organization (WHO) estimates that about 80% of the population living in the developing countries relies almost exclusively on traditional medicine for their primary health care needs. Plant derived substances which are used for drug preparation could be found in various parts like roots, leaves, shoots and bark of plants[3,4]. The modern drugs used today are based on natural compounds[5]. Due to excessive use of synthetic antibiotics, microorganisms are developing resistance towards them. Recently, Margaret Chan, director general of the WHO, warned that bacteria were starting to become so resistant to common antibiotics that it could bring "the end of modern medicine as we know it"[6]. Keeping in view all these above reasons and facts, many researchers are exploring different plants sources for natural drug development which can overcome side effects of synthetic drugs. Like, Soniya[7] investigated the antibacterial activity of methanol, aqueous, ethanol, acetone and petroleum ether extracts of Murraya koenigii, Syzygium aromaticum, Piper nigrum, Ocimum tenuiforum, Laurus nobilis, Cinnamomum zeylanicum, Phyllanthus niruri, Cuminum cyminum, Trilobatum sp. and Hibiscus rosasinensis against Gram positive and Gram negative bacteria. Similarly, Wendakoon[8] tested different parts of different plants i.e. Peumus boldus (boldo leaf), Agathosma betulina (buchu leaf), Echinacea angustifolia (echinacea root), Humulus lupulus (hops strobile), Glycyrrhiza glabra (licorice root), Mahonia aquifolium (Oregon grape root), Usnea barbata (usnea lichen), and Anemopsis californica (yerba mansa root) for their antibacterial activity against human pathogens.

A variety of phytochemicals such as phenolic acids, flavonoids, tannins, lignin, and other small compounds are present in plant-derived products[9] and these compounds possess numerous health-related effects such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities[10].

Based on the above facts, in present study, various extracts of leaf of Thuja orientalis (family Cupresseaceae) were screened for their antimicrobial activity along with phytochemical screening.

MATERIALS AND METHODS

Collection of plant material

Plant material was collected from locality of Sirsa (India). It was thoroughly washed with tap water in order to remove the dust particle and debris from them and rinsed with distilled water. Leaves were separated, dried in shade and then grinded to fine powder and stored for extract preparation.

Culture media and chemical

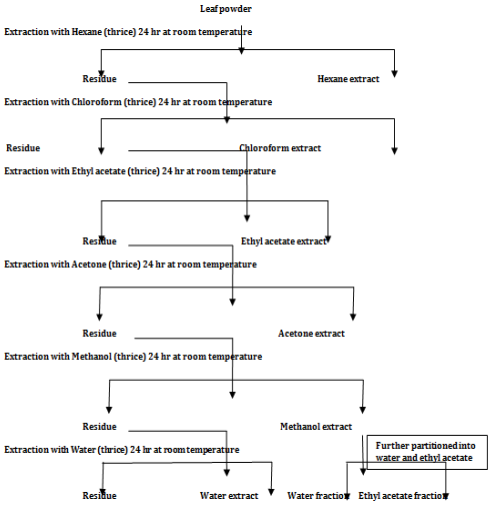
The culture media like nutrient agar, nutrient broth and Czapek yeast extract used for the growth of microorganism were procured from from Hi-media Laboratory Pvt. Ltd. Bombay. All other chemicals including organic solvent used for the extraction of metabolite were of analytical grade.

Test microorganisms

A total of five human pathogenic bacteria (i.e. two Gram positive viz., Bacillus subtilis MTCC 441, Staphylococcus aureus MTCC 87 and three Gram negative viz. Pseudomonas aeruginosa MTCC 424, Klebsiella pneumoniae MTCC 109, Alcaligens faecalis MTCC 126 and two fungal strains viz.,Aspergillus niger MTCC 404, Aspergillus flavus MTCC 873 were used for the evaluation of the antimicrobial activity. Authentic cultures of above microbes were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. All the microbes were preserved as stab slant cultures.

Preparation of plant extracts

The different extracts of leaf of Thuja orientalis were prepared by increasing order of solvent polarity (Flow chart 1). In this method, the extraction was started with a lowest polar solvent, i.e. Hexane, which was followed by chloroform, ethyl acetate, acetone, methanol and water. The filtrate of each solvent was evaporated at 45ºC for solvent elimination and the extracts of each solvent were kept in refrigerator until use.



Flow chart 1: Leaf powder of Thuja orientalis extracted in increasing order of solvent polarity

Antibacterial activity testing assay

Agar well diffusion assay described by Perez[11] was used for testing antibacterial and antifungal activity. Hundred µl suspension of 24 h old culture of test organism was inoculated on the agar plates and spread on to the surface of the agar with the help of a sterilized glass spreader. After five minutes of inoculation of test microorganism, wells (2.5 mm diameter) were prepared with the help of sterilized steel cork borer. Wells of each plate were loaded with 60 µl of crude plant extracts along with control (respective extraction solvent). Same method was repeated for antibiotics viz., tetracyclin, streptomycin, ampicillin, penicillin and fungicides viz., ketoconazole and fluconozole (used as positive control) under similar conditions. The plates were then aerobically incubated at 30±2^{0}C for S. aureus, at 37±2^{0}C for 24 h for other bacterial strains and at 28±2^{0}C for 72 h for fungal test organisms.

Minimum inhibition concentration (MIC) of the extracts

The MIC was defined as the lowest concentration that completely inhibited the growth of microorganisms for 24 h[12]. MIC of the extracts was also carried out using agar well diffusion technique. The bacterial strain was cultured in nutrient broth and then incubated at 37^o C for 18-24 hrs. In this method 60 µl of 24 h old culture of test organism was inoculated on the agar plates and spread on to the surface of the agar with the help of a sterilized glass spreader. After five minutes of inoculation of test microorganism, wells (2.5 mm diameter) were prepared with the help of sterilized steel cork borer. Each well of agar plate was loaded with 60 µl of different concentrations (serial dilution ranging from 250 - 800 µg/ml and 1.0 - 3.5mg / ml of extracts were prepared) of tested plant extract. The plates were then aerobically incubated at 30±2^{0}C for S. aureus, at 37±2^{0}C for 24 h for other bacterial strains and at 28±2^{0}C for 72 h for fungal test organisms. The lowest concentration of the extracts showing a clear zone of inhibition was considered as the MIC.

Preliminary phytochemical analysis of the extracts

To assess the chemical composition of the various extracts qualitatively, a preliminary phytochemical analysis was conducted according to the standard methods[13, 14]. Using these methods, the presence of several phytochemicals like sterols, tannins, proteins, sugars, alkaloids, flavonoids, saponins, anthraquinones, terpenoids, and cardiac glycosides was evaluated.

Test for sterols (Salkowaski reaction)

A few milligrams of the plant extract were dissolved in 2 ml chloroform and then 2 ml of conc. H_2SO_4 was added from the sides of the test tube. The test tube was shaken for a few minutes. Red colour development in the chloroform layer indicated the presence of sterols.

Test for tannins (ferric chloride reagent test)

The test sample of each extract was taken separately in water, warmed and filtered. To a small volume of this filtrate, a few drops of 5 % (w/v) solution of ferric chloride prepared in 90 % alcohol were added. Appearance of a dark green or deep blue colour indicated the presence of tannins.

Test for proteins (Xanthoproteic test)

The extract (few mg) was dissolved in 2 ml water and then 0.5 ml of conc. HNO_3 was added in it. Yellow colour indicated the presence of proteins.

Test for sugars (Fehling's test for free reducing sugar)

About 0.5 g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

Test for flavonoids (Ferric chloride test)

About 0.5g of each extract was boiled with 5 ml of distilled water and then filtered. To 2 ml of this filtrate, a few drops of 10% ferric chloride solution were added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group.

Test for saponins

One gram of each extract was boiled with 5 ml of distilled water and filtered. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins.

Test for anthraquinones

An aliquot of 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for terpenoids (Salkowski test)

To 0.5 g of each extract, 2 ml of chloroform was added, followed by a further addition of 3ml of concentrated H_2SO_4 to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for cardiac glycosides (Keller-Killiani test)

To 0.5 g of the extract diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Statistical analysis

Antimicrobial activity of various extracts of leaf extracts was statistically analysed along with standard antibiotics by applying t-test.

RESULTS AND DISCUSSION

Different extracts of leaf possess antimicrobial activity almost against all the tested microbes as shown in table 1. Among all the prepared extracts of leaf, acetone extract was found most potent against B. subtilis with maximum zone of inhibition (15.55 mm) and then against A. faecalis (15.50 mm) while S. aureus and P. aeruginosa were found moderately sensitive against acetone extract (12.66 mm and 12.50 mm inhibition zone, respectively). When compared, acetone extract was found even better than synthetic antibiotics (i.e penicillin and ampicillin) (Table 2). Maximum antibacterial activity was shown by ethanol extract of Z. officinale, P.

granatum and acetone extract of T. chebula against E. coli with the inhibition zone of 14 mm in size as studied by Sharma[15].

S. aureus and P. aeruginosa was inhibited effectively by ethyl acetate extract (14 mm and13.83 mm) followed by B. subtilis with 12.86 mm zone of inhibition.

Antifungal activity was found maximum (i.e. 13.00 mm against A. niger) in methanol extract which was also able to inhibit the growth of S. aureus (14.83 mm) and P. aeruginosa (14 mm). Activity of methanol extract was found more than to penicillin and streptomycin (i.e. 13.00 mm and 13.83 mm, respectively) against S. aureus while it was comparable to penicillin against P. aeruginosa (Table 2).As shown in table 2, methanol extract was found equally effective to ketoconozole (synthetic antifungal drug). But on the other hand, all the extract was found unable to inhibit growth of A. flavus effectively.

Minimum inhibitory concentration (MIC) was also measured for determining effectiveness of various extracts used. Lower MIC value of extract proves its better efficiency against the tested pathogens. Only acetone extract was able to inhibit the growth of P. aeruginosa at lowest concentration i.e. 800 µg/ml while chloroform extract inhibited A. faecalis, K. pneumonia, S. aureus and B. subtilis at MIC of 1 mg/ml.Ethyl acetate extract was also able to inhibit A. flavus at same concentration i.e. 1 mg/ml (Table 3).Results of Audipudi[16] on antimicrobial susceptibility reveals that methanol and chloroform extracts have great antibacterial activity against S. typhi, S. aureus, Mycobacterium spp. and has shown no activity on E. coli and P. vulgaries.

The antimicrobial activity of different extracts of leaf of Thuja oreintalis along with standard antibiotics were statistically analysed by applying t-test. The results showed that the antimicrobial activity of methanol and acetone extracts was found significant at P<0.02% and P<0.01% level while ethyl acetate extract data was found non-significant (Table 4).

After preliminary phytochemical screening (Table 5), it was revealed that extract prepared from leaf of Thuja orientalis contains active phytochemicals (i.e. alkaloids, flavanoids, tannins, free reducing sugars etc.) which were responsible for inhibition of pathogenic bacteria and fungi. These phytochemicals are known to have antimicrobial effects[17, 18].

Present study was supported by work done of Umamaheswari[19] on antimicrobial activity of different solvent extracts of leaf of Bougainvillea spectabilis against various bacterial strains i.e. S. aureus, B. subtilis, Streptococcus faecalis, Micrococcus luteus, E. coli, P. aeruginosa, Salmonella typhii, K. pneumoniae, Proteus vulgaris, Serratia marcescens, Shigella flexneri and Vibrio cholerae.

Table 1: In vitro antimicrobial activity of various extracts of leaf of Thuja orientalis by agar well diffusion assay

S.No.	Extract	Plant part	P. aeruginosa	A. faecalis	K. pneumonia	S. aureus	B. subtilis	A. flavus	A. niger
1		Control	6.00 ± 0.00	-	4.00 ± 0.00	-	-	-	-
	Methanol	Leaf	14.00 ± 0.00(133)	10.66 ± 0.58	12.00 ± 0.00(200)	14.83 ± 0.29	05.66 ± 0.58	8.00 ± 0.00	13.00 ± 0.00
2									
	Acetone	Control	5.50 ± 0.00	-	6.00 ± 0.00	-	-	5.80 ± 0.00	-
3		Leaf	12.50 ± 0.50(127)	15.50 ± 0.58	7.06 ± 0.11(17)	12.66 ± 0.58	15.55 ± 0.50	9.00 ± 0.00(35)	12.00 ± 0.00
	Ethyl Acetate	Control	6.00 ±0.00	8.00 ± 0.58	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	-	-
		Leaf	13.83 ±0.29(130)	11.00 ± 0.00(37)	11.06 ± 0.11(45)	14.00 ± 0.11(133)	12.86 ± 0.32(114)	-	11.83 ± 0.29

Table 2: I*n vitro* antimicrobial activity of different antibiotics (500 mg/ml) by agar well diffusion assay

S.No.	Antibiotics	Zone of inhibition (mm) *P. Aeruginosa*	*A. faecalis*	*K. pneumoniae*	*B. subtilis*	*S. aureus*	*A. flavus*	*A. niger*
1.	Penicillin	14.00±1.00	10.66±0.58	7.00±1.00	20.16±0.76	13.00±0.00	-	-
2.	Ampicillin	16.00±0.00	10.00±0.54	9.33±0.58	26.83±1.04	17.06±0.11	-	-
3.	Tetracyclin	20.66±0.58	17.50±0.00	17.00±0.50	22.00±1.00	21.00±0.00	-	-
4.	Streptomycin	23.00±1.00	14.50±1.00	11.83±0.76	19.00±0.00	13.83±0.28	-	-
5.	Fluconozole	-	-	-	-	-	14.00+00	15.00+0.2
6.	Ketoconozole	-	-	-	-	-	14.00+0.2	13.00+0.2

All values are mean of three replicates; ± = standard deviation; - =No activity

Table 3: The MIC values of different plant extracts of T*huja orientalis* by agar well diffusion assay

S. No.	Microorganisms tested	800 µg/ml	1 mg/ml	1.5 mg/ml	2 mg/ml	2.5 mg/ml	3 mg/ml	3.5 mg/ml
1.	P. aeruginosa	β_4	--	--	α_4	--	--	--
2.	A. faecalis	--	δ_4	γ_4	--	--	--	--
3.	K. pneumonia	--	δ_4	α_4, β_4	--	γ_4,	--	--
4.	S. aureus	--	α_4, δ_4	--	β_4	--	--	--
5.	B. subtilis	--	δ_4	β_4	--	--	--	--
6.	A. flavus	--	γ_4	--	β	γ_4, δ_4	--	--
7.	A. niger	--	--	--	--	--	β_4	α_4

α = Methanol extracts β = Acetone extracts -- = Not tested

γ = Ethyl acetate extracts δ = Chloroform extracts

Table 4: t-test statistics of antimicrobial activity of various extracts of leaves of *Thuja orientalis* along with the standard antibiotics

	Leaf			Standard antibiotics			
	Methanol	Acetone	Ethyl acetate	Penicillin	Ampicillin	Tetracyclin	Streptomycin
t-value	7.063**	8.185*	19.297#	5.026***	19.628#	8.144*	5.988***

*** Significant at 1 % level ** Significant at 2 % level *** Significant at 5 % level # Non-Significant**

Table 5: Phytochemical constituents of leaf extracts of *Thuja orientalis*

S.No.	Phytochemicals	Test	Inference			
			ME	AE	Et. Ac.	ChE
1.	Flavanoids	Ferric chloride	+	+	+	-
2.	Alkaloids	Wagner's test	+	+	+	-
3.	Free reducing sugar	Fehling's test	+	+	-	+

4.	Saponins	Frothing test	+	+	-	-
5.	Tanins	Ferric chloride reagent test	+	+	+	+
6.	Sterols	Salkowski reaction	+	+	-	-
7.	Proteins	Xanthoproteic test	+	+	+	-
8.	Terpenoids	Salkowski test	-	-	-	-
9.	Anthraquinones	Borntrager's test	+	-	-	+
10.	Cardiac glycosides	Keller-Killiani test	+	+	-	-

Key: + = present; - = absent; ME = methanol extract; AE= acetone extract; Et. Ac. = ethyl acetate extract; ChE = chloroform extract

CONCLUSION

Results showed that leaf extracts exhibited notable antimicrobial activity against all the species tested. Some of the extracts of leaf of *T. orientalis* were acceptable with respect to the standard antibiotics and so can be used as natural remedy for treatment of various bacterial and fungal infections without developing resistance among any bacterial/fungal species.

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