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Indian Herb *Hemidesmus indicus* - A Potential Source of New Antimicrobial Compounds

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Abstract

The results of present study support the view that *Hemidesmus indicus* and its isolated bioactive compound (2H4MB) showing potential antibacterial and antifungal activities that could be utilized to control infectious diseases caused by the test pathogens and *Candida albicans* in the human system. Analysis of essential oil from root of the plant obtained by using Clevenger apparatus by hydro distillation was undertaken through GC-MS. 2-Hydroxy-4-methoxybenzaldehyde (2H4MB) was the major chemical entity (99.41%) amongst 10 identified compounds. The *in vitro* antibacterial activity of 3 different extracts (Hexane, Methanol and Aqueous) and Bioactive compound 2-Hydroxy-4-methoxybenzaldehyde (2H4MB) as well as MIC values of isolated compound (2H4MB) were performed. The most active extract was found to be the hexane extract showing the maximum zone of inhibition of 22 mm against *Staphylococcus aureus*. The isolated bioactive compound (2H4MB) showed the highest diameter of zone of inhibition of 23 mm against *Staphylococcus aureus*. The Minimum Inhibitory Concentration values of the Bioactive compound 2H4MB on test pathogens varies from 80 µg/ml to 250 µg/ml, Minimum Bactericidal Concentration values varies from 150 µg/ml to 250 µg/ml. The MFC values and MIC values of 2H-4-MB on *Candida albicans* were 200 µg/ml and 150 µg/ml respectively. The active extract was also tested for cytotoxicity using freshly obtained sheep erythrocytes and it was revealed zero cytotoxicity.

Keywords: *Hemidesmus indicus*; Antibacterial activity; Anticandidal activity; Bioactive compound

Introduction

Almost 50,000 people killed every day due Infectious diseases which are the prominent cause of premature deaths. The most common pathogens for infections are *Escherichia coli, Salmonella* spp., *Staphylococcus aureus*. Drug resistance to common pathogens is the world major problem [1-3]. The microorganisms are now resistant due to continuous and indiscriminate use of antibiotics. Adverse reactions hypersensitivity, immunosuppressant and allergic reactions are also reported from antibiotics [4,5] which may associate with many clinical problems to treat infections [6]. Now this is a high time to discover and develop new antimicrobial drugs to control infectious diseases caused by drug resistant microbes. One of the way is to evaluate and study the local herbs for their antimicrobial potentials. Medicinal plants always remain an ultimate source to treat serious diseases. As per WHO [7] estimates 80% of the population in the world is depend on the traditional therapies using plant extracts or their phytochemical constituents. Searching of new chemical entities from plants with antimicrobial properties taking advantage of recent biological and medical technologies is a new and developing field. Since a long time, medicinal herbs and their bioactive constituents like essential oils have been known for their antimicrobial properties [8-11]. In recent times, the search for potent antimicrobial agents has been credited to medicinal plants. A number of plants are useful as a medicine in the treatment of diseases in the body and in most of cases the antimicrobial efficacy value attributed to some plants is beyond belief. Claims of effective therapy for the treatment of dysentery, diarrhea, respiratory disorders, skin diseases, syphilis, fever, leprosy, eye diseases and kidney and urinary disorders by traditional herbalist and revived our curiosity in the scientific exploration of such herbal medications [12-15]. It was estimated that at one time about 10% of all flowering plants on earth have been used by local communities throughout the world but only 1% have scientifically evaluated. Now we have about 120 plant-based drugs from just 95 plant species which are prescribed worldwide. The pharmaceutical potential of only 5000 medicinal plants are scientifically evaluated from approximately 250,000 species. The current medicines and antibiotics showed a steady decline in treating infectious diseases and pose a great problem due to significant increase in the incidence of bacterial resistance to several antibiotics [16]. The work on new antimicrobial compounds due to increased resistance of many microorganisms towards established antibiotics, has become desirable. Traditional herbs are basic health needs in the developing countries and there are many published researches on the efficacy of herbs against Gram-positive and Gram-negative microorganisms.

One possible approach is to screen/unexplored Indian medicinal bioactive plants extracts for their potential to be used against multiresistant bacteria. India has one of the world's richest flora with about 120 families of plants comprising 1,30,000 species and about 119 secondary plant metabolites are used globally as drugs. The WHO reported that 80% of world population rely chiefly on traditional medicines/herbs for primary healthcare have steadily increased worldwide in the recent years. Keeping in view this study is designed to evaluate the antimicrobial potentials of *Hemidesmus indicus* and their bioactive phytochemicals.

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Materials and Methods

Collection of plant materials

The roots of *Hemidesmus indicus* were obtained from the Himalaya Drug Company Dehradun India. The collected plant material was identified by the department of Pharmacognosy, The Himalaya Drug Company Dehradun. Roots were washed with potable water 2-3 times and once with sterile distilled water and then dried, a homogenous fine powder was made and stored in air tight container till further use.

Preparation of solvent root extraction

For the preparation of the plant extract, the modified method of Alade and Irobi [17] was used. The powdered root (25 g) were soaked in 100 ml each of the organic solvents (Hexane, methanol) and water in separate flasks and kept on rotating shaker for 72 hours, filtered using Whatman filter paper No.1. The extracts were concentrated to half its volume using rotary evaporator while water extract was concentrated on water bath.

Culture media

Soya bean casein digest agar/broth of Hi Media Pvt. Ltd., Bombay, India were used for this study.

Inoculum

The Soya bean casein digest Agar were inoculated with approximately 10^5 CFU/ml of 4 h growth that was incubated at 37°C in Soya bean casein digest broth.

Isolation of volatile bioactive component of *H. indicus* through clevenger apparatus

The method of Peyson and Richard [18] was used with little modification. The dry root (1 Kg) was taken in a round bottom flask of a Clevenger type apparatus for 4 hr. at 100°C. The distillate obtained was washed with diethyl ether and dried over anhydrous sodium sulphate. After filtration, the yield of essential was 1.2 g (0.12% w/w) and then it was stored in an airtight glass vessel at 4°C until required.

GC analysis of essential oil

The essential oil of *Hemidesmus indicus* was analyzed by GC/MS technique using an GCMS-QP2010 Ultra (Shimadzu Company) GC system, Mass Spectrometer for composition. The identification of volatile oil component was done by comparison of their spectra with NIST 11 lib. /Wiley 8 lib. library data of the GC-MS system and compared their retention indices (IR) with available relevant data. The percentage of peak area relative total peak area represent to relative amount (RA) of each individual component of the essential oil. Determination of RI value of each component relative to the retention time (RT) of series C8-C40 4-alkanes with linear interpolation on the Rtx-5 MS (30 meter × 0.25 i.d. × 0.25 Um film thickness)-column was done.

HPLC analysis of 2hydroxy4-methoxybenzaldehyde (2H4MB)

The fine powder of the root was poured into a glass vessel containing 75% of ethanol and then was filtered and evaporated. The residue of obtained was mixed with n-butanol and water in the ratio of (2:1) and both the layer of n-butanol and water were separated and evaporated under vacuum. The residues were washed with first with petroleum ether then with methanol. The methanolic extract was concentrated and analyzed using HPLC as per standard method of Shimizu et al. [19] with slight modifications. The extract was passed through Sartorius RC-membrane syringe filter (0.20 m) and 20 µl of filtrate was used for analysis in the HPLC. Shimadzu HPLC (Model SPD-10A UV-VIS Detector) and supercoil LC-18 column (25 cm × 4.6 mm, 5 m) with mobile phase prepared with water, acetonitrile, and acetic acid in the ratio of (50:50:0.1) was used into performing the chromatography. The flow rate and back pressure was consistently retained at 1.0 ml/minute and 250 psi respectively. UV detector at 210 nm was used to read the compound. The initial total run time was 20 min but after that it was preferably extended up to 40 min [19]. The results obtained were compared with standard.

Microorganisms

The antimicrobial activity of the plant extract and the essential (bioactive compound) were tested individually on G+ve and G-ve bacterial strains. All bacterial test strains were received from IMTECH, Chandigarh, India. The G+ve strain used was *Staphylococcus aureus MTCC 737* and G-ve bacterial strains were *E. coli MTCC 1687*; *Pseudomonas aeruginosa MTCC 1688* and *Salmonella enteric MTCC 3858* and *Candida albicans MTCC 3017*.

Evaluation of antimicrobial activity

The method of Perez et al. [20] was modified. Soya bean casein digest agar plates were inoculated with test cultures in SCD broth. Wells of 8 mm diameter were made on the inoculated plate through cork borer and filled with test samples and blank of distilled water, hexane and methanol and positive control of standard antibiotic was simultaneously used. The plates were kept for incubation at 37°C for 18 h. The antibacterial/anticandidal activity was determined by measuring the diameter of zone of inhibition that was observed. Wells were filled with 0.1 ml of 20 mg/ml concentration of each sample (2 mg/well). Bioactivity was determined by measuring Diameter of Inhibition Zones (DIZ) in mm.

MIC/MBC Determination of Bioactive compound (2H-4-MB)

MTT staining method of Scudiero et al. [21]; Marshall et al. [22] and Stevens and Olsen [23] was used to determine the minimum Inhibitory Concentration (MIC) value which is defined as the lowest concentration of the sample that inhibited growth of microorganisms. MTT could convert to Formazan only by living organisms and a blue color appeared in the well. To determine the Minimum Bactericidal Concentration (MBC) approximately 10 µl of the sample from the minimum Inhibitory Concentration assay was spread onto freshly prepared and sterile LB plates and incubated at 28°C over night. The MBC were taken as the lowest concentration that did not allow bacterial growth on the surface of agar plates.

Determination of cellular toxicity using sheep erythrocytes

Cellular toxicity was determined by using the method described by Xian-guo and Ursula [24] with minor modifications. The extract was 10-fold serially diluted using phosphate buffered saline, 0.8 ml volume of each dilution was taken in Eppendorf tube. The negative control tube (containing saline only) and a positive control tube (containing tap water) were taken for this study. Freshly prepared sheep erythrocytes were used in each tube with a final volume of 1 ml. The tubes were kept for incubation at 37°C for 30 min and were centrifuged for 5 minutes. All tubes after incubation were check for hemolysis.

Results and Discussion

The yield of the essential oil obtained by hydro distillation of the

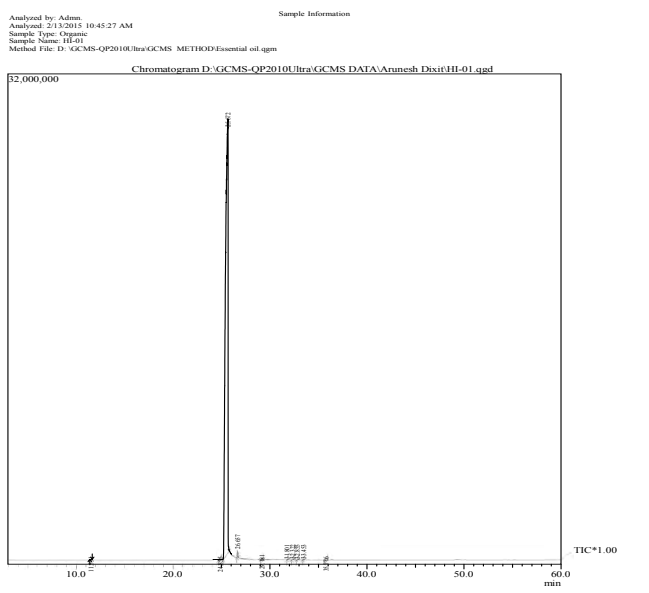

Figure 1: The essential oil evaluation by GC-MS.

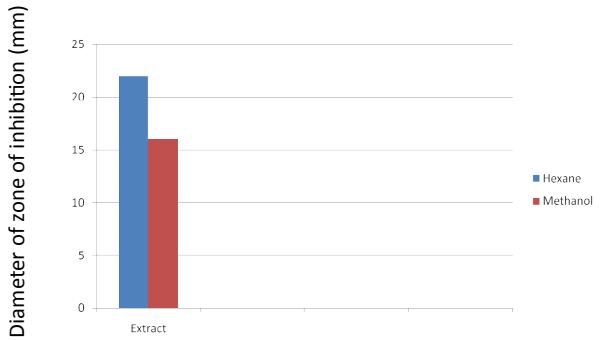

Figure 2: Antimicrobial efficacy of Hexane and Methanol extract of *Hemidesmus indicus* in the form of Diameter of zone of inhibition.

Peak	R. Time	Area	Area%	Name
1	11.531	63636	0.01	(S)-3-Ethyl-4-methylpentanol
2	24.855	128396	0.02	2,4-Undecadienal
3	25.672	525430106	99.41	2-Hydroxy-p-anisaldehyde/2-Hydroxy-4-methoxybenzalde
4	26.657	1947873	0.37	Phenol, 2-methoxy-3-(2-propenyl)-
5	29.181	515809	0.10	Caryophyllene <(E)->
6	31.801	61357	0.01	Benzene, 1-(1,5-Dimethyl-4-Hexenyl)-4-Methyl
7	32.322	112830	0.02	Funebrene <alpha->
8	32.855	118869	0.02	Bisabolene <beta->
9	33.453	100649	0.02	6.Alpha-Cadina-4,9-Diene, (-)-
10	35.776	61565	0.01	(-)-5-Oxatricyclo [8.2.0.0(4,6)] Dodecane,12-Tri
		528541090	100.00	

Table 1: Peak Report TIC.

Sample	Retention Time (min)
2H4MB Compound standard	11.03
Hemidesmus indicus	11.14

Table 2: HPLC analysis *Hemidesmus indicus* extract and the standard bioactive compound.

root of *Hemidesmus indicus* was 0.12%. The essential oil was evaluated by GC-MS. 10 compounds were identified. The major compound in the oil was 2-hydroxy-4-methoxybezaldehyde (99.41%) (Figure 1 and Table 1).

The isolated compound 2-hydroxy 4-methoxy benzaldehyde (2H4MB) from *Hemidesmus indicus* was also analyzed by HPLC based on their standard retention time 6.598 min. The *Hemidesmus indicus* root extract was also analyzed through HPLC the results showed almost the same Retention time (Rt) in both root extract (6.881 min) and 2H4MB compound *Standard* (6.598 min). This revealed the presence of 2H4MB compound in root extract of *Hemidesmus indicus* (Table 2).

The antibacterial and antifungal activities of the root extracts of *Hemidesmus indicus* and 2-hydroxy-4-methoxybenzaldehyde compound were evaluated against 5 test microorganisms including one G+ve bacteria, three G-ve bacteria ads one fungi. Their potency were assessed by diameter of zone of inhibition and MIC/MBC values. Among all the tested extracts hexane extract was found to have maximum zone of 22 mm against *Staphylococcus aureus* Plate 1 (Table 3, Figure 2) followed by *E. coli* (18 mm), *Candida albicans* (18 mm), *Pseudomonas aeruginosa* (16 mm) and *Salmonella enteric* (15 mm). The isolated bioactive compound 2H4MB showed the highest diameter of zone of inhibition of 23 mm against *Staphylococcus aureus* followed by *E. coli* 16 mm (Figure 3 and Table 3). The Pure compound 2H4MB was also compared with the isolated compound Plate 2-4 as depicted in Table 3 [25].

The MIC values of the Bioactive compound 2H4MB on test microorganisms ranged from 80 µg/ml to 250 µg/ml, MBC values from 150 µg/ml to 250 µg/ml. The MFC values and MIC values of 2H4MB on *Candida albicans* were 200 µg/ml and 150 µg/ml respectively (Table 4, Figures 4 and 5).

The significant antimicrobial effect of *Hemidesmus indicus* against all the pathogen confirmed that the compound present in the crude extract are responsible for the effective antimicrobial activity.

The traditional uses of *Hemidesmus indicus* appeared to have a fairly good degree of correlation with their antimicrobial activity. The cellular toxicity of the alcoholic extract was examined against sheep erythrocytes. Hemolysis of the erythrocytes was not observed at any dilutions of extract ranging from 1:1 to 1:1000. Only the positive

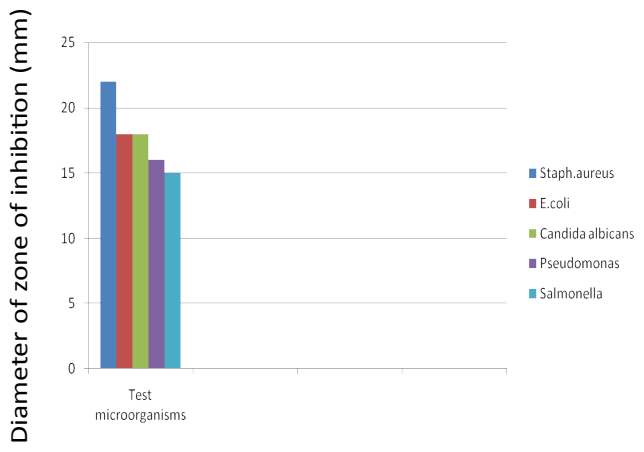

Figure 3: Susceptibility of test microorganisms against Hexane extract of *Hemidesmus indicus* in the form of diameter of zone of inhibition (mm).

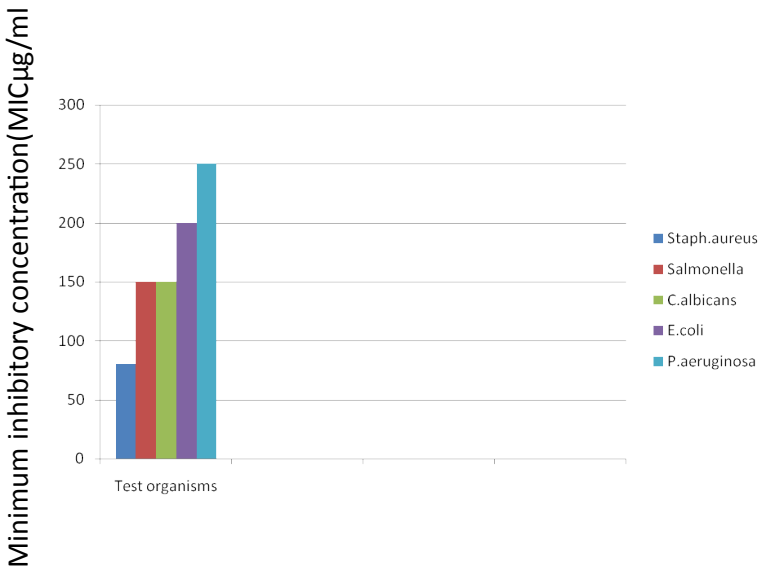

Figure 4: Minimum inhibitory concentration (MIC µg/ml) of isolated compound (2-H-4-MB) against test organisms.

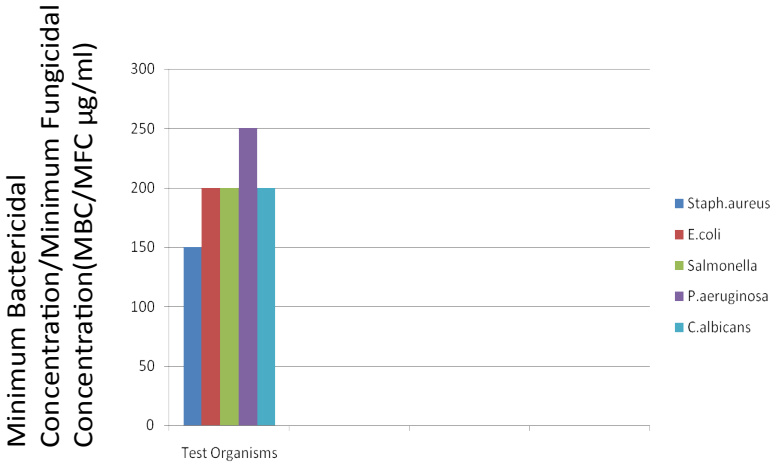

Figure 5: Minimum Bactericidal Concentration/Minimum Fungicidal Concentration (MBC/MFC µg/ml) against test organisms.

S No	Bacterial/Fungal strains	Inhibition zone diameter (mm)					
		Hexane extract	Methanol extract	Aqueous extract	Isolated Bioactive Compound (IBC) 50 mg/ml	Reference Compound (RC) 50 mg/ml	+ve Control Ciprofloxacin 30 µg/ml
1.0.	*Staphylococcus aureus MTCC 737*	22	16	NAD	23	27	25
2.0.	*E. coli MTCC 1687*	18	14	NAD	16	17	21
3.0.	*Pseudomonas aeruginosa MTCC 1688*	16	12	NAD	14	15	22
4.0.	*Salmonella enteric MTCC 3858*	15	13	NAD	16	17	21
5.0.	*Candida albicans MTCC 3017*	18	16	NAD	20	20	----

Table 3: Antibacterial and antifungal activity of *Hemidesmus indicus* root extract and isolated compounds (2H-4-MB).

S. No.	Test microorganisms	2-Hydroxy-4-methoxy-benzaldehyde(µg/ml)	
		MBC*/MFC**	MIC***
1.0.	*Staphylococcus aureus MTCC 737*	150	80
2.0.	*E.coli MTCC 1687*	200	200
3.0.	*Pseudomonas aeruginosa MTCC 1688*	250	250
4.0.	*Salmonella enterica MTCC 3858*	200	150
5.0.	*Candida albicans MTCC 3017*	200	150

*Minimum Bactericidal Concentration; **Minimum Fungicidal Concentration; ***Minimum Inhibitory Concentration

Table 4: MIC of 2-hydroxy-4-methoxy-benzaldehyde.

Concentration of sample extracts (200 mg/ml)	Hemolysis
1:1	**-**
1:10	**-**
1:100	**-**
1:1000	**-**
Negative Control	**-**
Positive Control	**+**
Solvent Control	**-**

Table 5: Cellular toxicity testing of plant extracts against fresh sheep erythrocytes.

control exhibited the strong hemolysis. Whereas the negative control containing only phosphate buffered saline exhibited no hemolysis (Table 5).

The herb *Hemidesmus indicus* showed broad spectrum of action and is non-cytotoxic, it seems to have a promising application in modern medicine as antiseptics and disinfectant. The antibacterial activities of the herb are particularly noteworthy, considering the importance of these organisms in nosocomial infections.

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