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Impact of Plant-Extract Based Ethno-Medicinal-Preparation Against Keratinophilic Bacteria

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Abstract: ***Medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers worldwide since antiquity to date. About 3.4 billion people in the developing world depend on plant based traditional medicines which is used for their primary health care. According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. The science of application of these indigenous or local medicinal remedies including plants for treatment of diseases is currently called ethnopharmocology but the practice dates back since antiquity. Medicinal plants being used by the traditional herbal healers against skin diseases of local peoples of the tribal families have been documented. A total of 30 species belonging to 21 families of angiospermie plants are documented. With the increasing interest and so many promising drug candidates in the current development pipeline that are of natural origin, and with the lessening of technical drawbacks associated with natural product research, there are better opportunities to explore the biological activity of previously inaccessible sources of natural products. In addition, the increasing acceptance that the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs, there will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns.***

Keywords: Keratinophilic, Bacteria, Plant extracts, Tribes, Ethnomedical

1. Introduction

The therapeutic use of plants certainly goes back to the Sumerian and the Akkadian civilizations in about the third millennium BC. Hippocrates (ca. 460-377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. Natural products have been an integral part of the ancient traditional medicine systems, e.g. Chinese, Ayurvedic and Egyptian (Sarker & Nahar, 2007). Over the years they have assumed a very central stage in modern civilization as natural source of chemotherapy as well as amongst scientist in search for alternative sources of drugs. About 3.4 billion people in the developing world depend on plant based traditional medicines which is used for their primary health care. According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components are often referred to as photo chemicals. The science of application of these indigenous or local medicinal remedies including plants for treatment of diseases is currently called ethnopharmocology but the practice dates back since antiquity. The sample for the study purpose was collected from Sal forest of Saranda region in Singhbhum district of Jharkhand and Kaimur district in Bihar states.

2. Material and Methods

Monthly field trips were conducted in the tribal pockets of selected blocks. The traditional herbal healers, tribal heads and tribal person were contacted and enquired to gather the related information about various preparations used for the treatment of skin diseases. The ethno medicinal plant were collected and washed thoroughly 2-3 times with running water, once with sterile distilled water, and then air dried on sterile blotter under shades. Identification of plant has been made through the local name of plant and get confirmed from the relevant literature like Mudgal et al.(1997) Singh et al.(2001), Verma et al.(1993) and Oommachan et al.(1996). The ethno medicines were prepared according to the method described by herbal healers. Twenty grams of each fresh preparations comprising of leaf/bark/root/seed/flower/whole plant, fruits and other ingredients were crushed in a mortar and the medicinal paste was then extracted.

2.1 Extraction of ethno medicinal material

The ethno medicinal material was extracted with 100 ml of hot sterile distilled water. Another set of preparations was macerated and extracted in 100 ml of hexane and methanol separately for 24 hours. The extracts of the separate solvents were concentrated under reduced pressure and preserved at 5°C in air tight bottle until further use. For the extraction of oils, air dried leaves of selected plants were chopped and grinded into small pieces. Essential oil was collected dehydrated using sodium sulphate dispensed into dark bottles and stored at 4°C until used.

2.2 Procurement and maintenance of microbial culture of fungi

The three species of keratinophilic fungi viz., Aspergillus niger (MTCC 10180) Trichophyton rubrum (MTCC 7859), Candida albicans (MTCC 1637) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh and two species of fungi i.e. Rhizopus microspores and Cryptococcus neoformans were collected from the soil. The fungal cultures were subcultured on Sabouraud Dextrose Agar (SDA) medium (peptone - 10g , dextrose- 20 g , agar – 20 g , distilled water – 1000 ml) incubated at $28\pm1^{\circ}C$. The stock cultures of fungi were maintained on sabourd dextrose agar slant.

2.3 Preparation of microbial suspension for fungi

Fungal suspension were prepared from 21 days old culture of T. rubrum and R. Microspores one week old culture of A. Niger and 24 hrs old culture of C. albicans and C. neformans grown on Sabouraud dextrose agar medium. The petri dsishes were flooded with 8 to 10 ml of distilled water and conidia were scraped using sterile spatula. The spore density of each fungus was adjusted with spectophtometer ($A_{595}nm$) to obtain a final concentration of approximately 10^5 spores / ml. Bacillus subtilis, Escherichia coli and Enterococcus faecalis were pre-cultured in nutrient broth overnight in a rotary shaker at $37^{\circ}C$, centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometerically (A_{610} nm). Each of the plates was homogenized to ensure uniform distribution of the inoculums and air- dried to remove surface moisture.

2.4 Preparation of impregnated paper discs

Standard size Whatman filter paper discs (5.0 mm in diameter) kept on a sterilized petriplates was sterilized in an oven at $140^{\circ}C$ for one hour. The sterilized discs were saturated with the (20 mg/ ml) test extract, 5 μl/ disc of oil and (1 mg/ ml) for control. For enhancing the essential oil solubility , Tween-80, 0.5%(v/v) was added. Plates with Tween-80 but without any plant essential oil were used as control. Discs were then dried at room temperature to remove any residual solvent that might interface with the determination.

2.5 Assessment of antifungal activity

The antifungal activity was tested by discs diffusion method (Gould and Bowie, 1952). The inoculums of the test organism was evenly spread on the surface of the Sabouraud dextrose agar/ nutrient agar medium. The impregnated discs were then placed on the surface of sterilized medium inoculated with the test fungi. Blank disc impregnated with water, methanol, hexane and Tween 80 at 1 ml. Followed by drying off was used as negative control. After the colonies grew, the zones of inhibition around the discs were measured and recorded.

Different standard discs of nystatin (Dr Reddy Laboratories Ltd ketoconazole (Jhonson Jhonson Ltd.) and fluconozole (Cipla Pharmaceuticals Ltd.) for different fungi were used as positive control. Before incubation, all the test and control petriplates were kept at $5^{\circ}C$ for one hour to allow the diffusion of the substance from the disc into the agar medium plate. All the experiments were done in three replicates to determine activity.

The antibiotics known to be effective against their respective test micro-organisms were used as reference for comparison of the antifungal activity of the test samples. Ketoconozole (1000 μg/ml) was used against Trichophyton rubrum, nystatin (1000 μg/ml) against Candida albicans, fluconozole (1000 μg/ml) against Cryptococcus neoformans, Aspergillus niger and Rhizopus microspores. Streptomycin was used as positive control against Bacillus subtilis, Escherichia coli and Enterococcus faecalis. The filter discs (5mm diameter) impregnated with 20 mg/ml extracts, $5\mu l/disc$ of oil and 1 mg/ml of control were for separate fungal species seeded plates. These were incubated at $28^{\circ}C$ for 7-24 days for T. Rubrum, R. Microspores and A. Niger 2 days for Candida albicans and Cryptococcus neoformans in an incubator and were looked for the development of clearance / inhibition zone around the disc. The diameters of the inhibition zones were measured in three replicates by using antibiotic zone scale.

2.6 Gas chromatography- mass spectrometry (GC-MS) of oil

The chemical constituents of Cymbopogon citratus leaf oil identified by GC-MS analysis was carried out on a Shimadzu 2010 Gas Chromatograph equipped with an FID and 25 m x 0.25 mm x 0.25 μm WCOT column coated with diethylene glycol (AB-Innowax, 7031428, japan) at Indian institute of integrative Medicine (CSIR lab) Jammu. Both injector and detector (FID) temparatures were maintained at $260^{\circ}C$. Helium was used as carrier gas at a flow rate of 3.0 ml/min at a coloumn pressure of 152 kPa. Samples (0.2μl) were injected into the column with a split ratio of 100 : 1. Component separation was achieved following in a linear temperature program of 60 - $260^{\circ}C$ at $3^{\circ}C$/ minutes and then held at $260^{\circ}C$ for 10 minutes, with a total run time of 20 minutes. The percentage composition was calculated using peak normalization method assuming equal detector response. The samples were then analysed on same Shimadzu instrument fitted with the same column and following the same temperature program as above. MS parameters used were; lionisation Voltage (EI) 70 eV, peak width 2 s, mass range 40-600 amu and detector voltage 1.5 volts. Results are based on GC-FID; MS acquisition started after 4 minutes. Peak identification was carried out by comparison of the mass spectra with mass spectra available on database of NISTO5 and WILEY8 libraries and co-injection of available pure standards. The compound identification was finally confirmed by

comparison of their relative retention indices with literature values (Davies, 1990).

3. Results and Discussion

Out of thirty four preparations used in the district for skin diseases fourteen preparations were active against fungal species. The presence of active ingredients in herbal medicines is directly linked to their ability to prevent or treat keratinophilic infections. Moreover, the anti-keratinophilic action of the plants on the tested bacteria responsible for superficial mycoses, confirms their therapeutic potency and this appraisal may authenticate their use in traditional medicines by different tribes for certain skin infectious. Amongst the plant species investigated, oil of *C. Citrates* leaf showed the most remarkable activity. This plant oil can be further subjected to isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation. Data for ethno medicinal preparation extracted from different part of plants viz., Argemone Mexicana (root) , Azadirachta indica (leaf) , Azadirachta India (bark) , Cassia tora (seed), Centratherum anthelminticum (seed) , Ocimum sanctum (leaf) , Plumbago zeylanica (leaf), Pongamia pinnata (fruit) + Ipomoea pestigridis (root) in the form of Hexane (20mg/ml), Methanol (20mg/ml) and water (20mg/ml) against keratinophilic bacteria were transformed in square root and analysed using Randomised Block Design for two factors (Parts of plant and different plant extracts) and their interaction to study the variation against different inhibition zone (mm) namely Bacillus Subtilis , Escherichia Coli, Enterococcus Faecalis (Table 1.1 to 3.2). The analysis of variance result shows significant variation in all the parts of plants (fruit, leaf, bark, seed, root collected from different medicinal plants at $p<0.01$ level. The mean ± standard error indicated in all tables 1.1, table 2.1 to table 3.1 shows significant variation within and between factors at $p<0.05$ critical difference. The graphical representation for original data collected from sample for the purpose in different inhibition zone depict the level of hexane extract, methanol extract and water against different parts of plant. The level of occurrence in Hexane and Methanol was found in most of the parts of plants. On the other hand oils (5μl/disc) extracted from leaf of different medicinal plants indicated in table 4.1 and its one way analysis of variance result in table 4.2 shows significant different in all inhibition zone against keratinophilic bacteria. (Table 1-4).

Table 1.1: Activity of plant extracts (20mg/ml), and control (1mg/ml) against keratinophilic bacteria for Inhibition Zone (mm) : Bacillus Subtilis

Plant Parts	Chemical extract			Mean
	Hexane extract (H)	Methanol extract (M)	Water (W)	
Argemone Mexicana (root)	12.00 (3.535±0.045)	6.00 (2.547±0.082)	0.00 (0.707±0.001)	6.00 (2.263±0.415)
Azadirachta indica (leaf)	18.00 (4.300±0.063)	0.00 (0.707±0.082)	7.00 (2.737±0.057)	8.33 (2.582±0.521)
Azadirachta India (bark)	8.00 (2.915±0.040)	11.00 (3.391±0.017)	0.00 (0.707±0.057)	6.33 (2.338±0.414)
Cassia tora (seed)	10.00 (3.240±0.039)	0.00 (0.707±0.017)	0.00 (0.707±0.057)	3.33 (1.551±0.422)
Centratherum anthelminticum (seed)	13.00 (3.674±0.042)	8.00 (2.915±0.040)	0.00 (0.707±0.057)	7.00 (2.432±0.445)
Ocimum sanctum (leaf)	8.00 (2.902±0.200)	15.00 (3.936±0.073)	6.00 (2.544±0.114)	9.67 (3.127±0.220)
Plumbago zeylanica (leaf)	10.00 (3.240±0.036)	0.00 (0.707±0.073)	0.00 (0.707±0.114)	3.33 (1.551±0.422)
Pongamia pinnata (fruit) + Ipomoea pestigridis (root)	0.00 (0.707±0.036)	0.00 (0.707±0.073)	0.00 (0.707±0.114)	0.00 (0.707±0.001)
Mean	9.88 (3.064±0.207)	5.00 (1.952±0.271)	1.63 (1.191±0.175)	
CD at 5%	Plant Parts : 0.90, Chemical conc. : 0.055 , Plant x Chemical : 0.156			

Streptomycin (Control): Mean (13.00 ±0.577)

- Parenthesis value (Mean ±SE) is based on Square Root Transformation of original data,
- Critical difference at 5% calculated for transformed data

Table 1.2: Analysis of variance for Bacillus Subtilis

Source of Variations	DF	Sum of Squares	Mean Squares	F Ratio	Probability	h^2	hp^2	w^2
Replicates	2	0.0965	0.0483	5.36	0.0079 **	0.001	0.182	0.001
Control vs Treatments	1	7.4063	7.4063	821.99	0.0000 **	0.056	0.945	0.053
Treatments	23	123.3163	5.3616	595.06	0.0000 **	0.940	0.997	0.484
Plant parts (P)	7	36.1340	5.1620	572.91	0.0000 **	0.275	0.988	0.215
Chemical extract (C)	2	42.6122	21.3061	2364.68	0.0000 **	0.325	0.990	0.245
P*C	14	44.5702	3.1836	353.33	0.0000 **	0.340	0.990	0.253
Error (B)	48	0.4325	0.0090					
Total	71	131.2516	1.8486					

** - significant at $p<0.01$ level, DF: Degree of freedom

Table 2.1: Activity of plant extracts (20mg/ml), and control (1mg/ml) against keratinophilic bacteria for Inhibition Zone (mm) : Escherichia Coli

Plant Parts	Chemical extract			Mean
	Hexane extract (H)	Methanol extract (M)	Water (W)	
Argemone Mexicana (root)	13.00 (3.674±0.043)	12.00 (3.535±0.050)	0.00 (0.707±0.001)	8.33 (2.639±0.484)
Azadirachta indica (leaf)	15.00 (3.935±0.078)	0.00 (0.707±0.050)	0.00 (0.707±0.001)	5.00 (1.783±0.539)
Azadirachta India (bark)	12.00 (3.535±0.043)	0.00 (0.707±0.050)	0.00 (0.707±0.001)	4.00 (1.650±0.471)
Cassia tora (seed)	0.00 (0.707±0.043)	0.00 (0.707±0.050)	0.00 (0.707±0.001)	0.00 (0.707±0.001)
Centratherum anthelminticum (seed)	0.00 (0.707±0.043)	0.00 (0.707±0.050)	0.00 (0.707±0.001)	0.00 (0.707±0.001)
Ocimum sanctum (leaf)	14.00 (3.808±0.015)	0.00 (0.707±0.050)	0.00 (0.707±0.001)	4.67 (1.741±0.020)
Plumbago zeylanica (leaf)	8.00 (2.915±0.020)	0.00 (0.707±0.050)	0.00 (0.707±0.001)	2.67 (1.443±0.021)
Pongamia pinnata (fruit) + Ipomoea pestigridis (root)	0.00 (0.707±0.020)	0.00 (0.707±0.050)	6.00 (2.544±0.114)	2.00 (1.320±0.022)
Mean	7.75 (2.499±0.295)	1.50 (1.061±0.195)	0.75 (0.937±0.127)	
CD at 5%	Plant Parts : 0.063, Chemical conc. : 0.039 , Plant x Chemical : 0.110			

Streptomycin (Control): Mean (13.00 ±0.577)

- Parenthesis value (Mean ±SE) is based on Square Root Transformation of original data,
- CD: Critical difference at 5% calculated for transformed data

Table 2.2: Analysis of variance for Escherichia Coli

Source of Variations	df	Sum of Squares	Mean Squares	F Ratio	Probability	h^2	hp^2	w^2
Replicates	2	0.0010	0.0005	0.11	0.8931	0.000	0.005	0.000
Control vs Treatments	1	5.7538	5.7538	1291.98	0.0000 **	0.048	0.964	0.046
Treatments	23	114.1374	4.9625	1114.29	0.0000 **	0.95	0.998	0.487
Plant parts (P)	7	24.7499	3.5357	793.91	0.0000 **	0.206	0.991	0.171
Chemical extract (C)	2	36.1796	18.0898	4061.93	0.0000 **	0.301	0.994	0.231
P*C	14	53.2079	3.8006	853.39	0.0000 **	0.443	0.996	0.307
Error (B)	48	0.2138	0.0045					
Total	71	120.1060	1.6916					

** - significant at p<0.01 level, DF: Degree of freedom

Table 3.1: Activity of plant extracts (20mg/ml) and control (1mg/ml) against keratinophilic bacteria for Inhibition Zone (mm): Enterococcus Faecalis

Plant Parts	Chemical extract			Mean
	Hexane extract (H)	Methanol extract (M)	Water (W)	
Argemone Mexicana (root)	0.00 (0.707±0.001)	0.00 (0.707±0.001)	0.00 (0.707±0.001)	0.00 (0.707±0.001)
Azadirachta indica (leaf)	0.00 (0.707±0.001)	0.00 (0.707±0.001)	0.00 (0.707±0.001)	0.00 (0.707±0.001)
Azadirachta India (bark)	0.00 (0.707±0.001)	6.00 (2.547±0.083)	0.00 (0.707±0.001)	2.00 (1.320±0.923)
Cassia tora (seed)	7.00 (2.736±0.086)	0.00 (0.707±0.083)	0.00 (0.707±0.001)	2.33 (1.383±0.339)
Centratherum anthelminticum (seed)	0.00 (0.707±0.086)	0.00 (0.707±0.083)	0.00 (0.707±0.001)	0.00 (0.707±0.001)
Ocimum sanctum (leaf)	0.00 (0.707±0.086)	6.00 (2.549±0.045)	0.00 (0.707±0.001)	2.00 (1.321±0.307)
Plumbago zeylanica (leaf)	13.00 (3.674±0.047)	10.00 (3.238±0.089)	0.00 (0.707±0.001)	7.67 (2.540±0.463)
Pongamia pinnata (fruit) + Ipomoea pestigridis (root)	2.67 (1.443±0.736)	15.00 (3.936±0.073)	0.00 (0.707±0.001)	5.89 (2.029±0.533)
Mean	2.83 (1.424±0.238)	4.63 (1.887±0.261)	0.00 (0.707±0.001)	
CD at 5%	Plant Parts : 0.251, Chemical conc. : 0.153, Plant x Chemical : 0.434			

Streptomycin (Control): Mean (13.00 ±0.577)

- Parenthesis value (Mean ±SE) is based on Square Root Transformation of original data,
- CD: Critical difference at 5% calculated for transformed data

Table 3.2: Analysis of variance for Enterococcus Faecalis

Source of Variations	DF	Sum of Squares	Mean Squares	F Ratio	Probability	h^2	hp^2	w^2
Replicates	2	0.1259	0.0629	0.9	0.4132	0.001	0.036	0
Control vs Treatments	1	13.8679	13.8679	198.34	0.0000 **	0.139	0.805	0.121
Treatments	23	82.4140	3.5832	51.25	0.0000 **	0.826	0.961	0.444
Plant parts (P)	7	28.0571	4.0082	57.33	0.0000 **	0.281	0.893	0.216
Chemical extract (C)	2	16.9667	8.4834	121.33	0.0000 **	0.17	0.835	0.144
P*C	14	37.3901	2.6707	38.2	0.0000 **	0.375	0.918	0.265
Error (B)	48	3.3561	0.0699					
Total	71	99.7639	1.4051					

** - significant at p<0.01 level, DF: Degree of freedom

Table 4.1: Activity of plant extract oils (5µl/disc) against keratinophilic bacteria

Plant parts	Inhibition zone (mm)		
	Bacillus Subtilis	Escherichia coli	Enterococcus Faecalis
Cymbopogon citrates (leaf)	27.00 (5.244±0.034)	18.00 (4.300±0.041)	22.00 (4.742±0.049)
Cymbopogon nardus (leaf)	0.00 (0.707±0.001)	32.00 (5.700±0.057)	0.00 (0.707±0.001)
Mentha piperita (leaf)	40.00 (6.363±0.068)	13.00 (3.673±0.040)	0.00 (0.707±0.001)
Ocimum gratissimum (leaf)	22.00 (4.741±0.075)	13.00 (3.669±0.096)	20.00 (4.525±0.078)
CD at 5%	0.159	1.886	0.139

- Parenthesis value (Mean ±SE) is based on Square Root Transformation of original data,
- CD : Critical difference at 5% calculated for transformed data

Table 4.2: Analysis of Variance for plant extract oils (5µl/disc) against keratinophilic bacteria in different inhibition zone (mm)

Source of Variation	DF	Mean Sum of Square		
		Bacillus Subtilis	Escherichia coli	Enterococcus Faecalis
Plant extract oil from different parts	3	30.4042**	4.5746**	25.7362**
Error	16	0.0141	0.0198	0.0107
Total	19	4.8125	0.7390	4.0726

** - significant at p<0.01 level, DF: Degree of freedom

4. Conclusion

With the increasing interest and so many promising drug candidates in the current development pipeline that are of natural origin, and with the lessening of technical drawbacks associated with natural product research, there are better opportunities to explore the biological activity of previously inaccessible sources of natural products. In addition, the increasing acceptance that the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs, there will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns.

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