



Comparative *in vitro* study of some ethno medicines on selective keratinophilic bacteria

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Abstract

To screen hexane, methanol and water extracts of eight types of ethno medicines and four types of oils used in traditional medicines in Mandla district (Madhya Pradesh, India) against three keratinophilic bacterial species viz., *Bacillus subtilis*, *Escherichia coli* and *Enterococcus faecalis*. Disc diffusion method was used to determine the inhibition zone of different extracts. Hexane, methanol and oils showed significant zone of inhibition against Gram-positive bacteria *Bacillus subtilis* and *Enterococcus faecalis* then Gram-negative bacterium *Escherichia coli*. The hexane extracts of the studied plants were more potent than the methanol extracts. Oils showed significant activity than other medicinal preparations. The most susceptible bacterium was *B. subtilis* followed by *E. faecalis* and *E. coli* was least inhibited. Crude water, hexane and methanol extract of some preparations were not showing inhibitory activity against bacterial species. Based on the literature, this is the first study which has conducted to inhibit growth of keratinophilic bacteria. Further research also needs to purify and characterize the secondary metabolites from the selected oils for the valuable source of novel substances for future drug discovery.

Keywords: ethnomedicinal plants, disc diffusion method, keratinophilic bacteria, mandla

1. Introduction

Keratin is insoluble and hard to degrade due to extensive disulphide bond and cross linkages. Keratins are found in animal hair, nails, hoofs and feathers. Keratinolytic microbes represent an important component of soil biocenosis where they mineralize a highly resistant proteinaceous substrate keratin of vertebrate skin and adnexes. Keratinolytic bacteria, particularly from the genus *Bacillus* and actinomycetes, were most often isolated from the plumage and bird feathers^[4] feathers waste processed by fermentation^[30, 14]. In bacteria, feather keratin-degrading abilities are observed mostly in strains of *Bacillus licheniformis*^[14, 16], less frequently in populations of *Bacillus pumilis*, *B. cereus* and *B. subtilis*^[14] and non-spore forming bacteria, *Stenotrophomonas* sp.^[31], *Fervidobacterium pannavorans* and *F. islandicum*^[19].

Keratinolytic activity is well established among *Bacillus* spp. Several strains of *B. licheniformis* and *B. subtilis* are described as keratinolytic^[16, 25] and other species such as *B. pumilis* and *B. cereus* also produce keratinases^[14, 29]. *B. licheniformis* strains are often capable of completely disintegrating feathers, and their proteolytic enzymes present a broad range of activity^[17, 5]. These bacteria shared elevated homology with *B. subtilis*, *B. amyloliquefaciens*, and *B. velesensis* and produced a mixture of proteolytic activities that showed remarkable dehairing activity on bovine pelts. Some thermophilic and alkaliphilic strains of *Bacillus* have been also described to show keratin degrading activity, such as *Bacillus* sp. P-001A, *B. halodurans* AH-101^[26], *B. pseudofirmus* AL-89^[9], *B. pseudofirmus* FA30-01^[15] and *Enterococcus faecalis*^[24].

A statistically significant correlation was detected between yeasts and molds, *E. coli*, enterococci and spores of sulfite-reducing *Clostridium* and between clostridial spores and

staphylococci in an investigation on wet and dry sands in Italy. In an epidemiological study carried out on two beaches in Malaga, Spain, faecal index microorganisms, especially coliphages were highly significantly correlated with dermatophyte fungi (microscopic fungi that grow on skin and mucous membranes) on one of the beaches. Only *E. coli* showed a significant correlation with *Candida albicans* (a pathogenic fungus). At the other beach, intestinal enterococci showed the best correlation with dermatophyte fungi. Again, coliphages were the indices that best correlated with *C. albicans*^[3]. The perusal of the review of literature indicates that activity of extracts of some ethno medicines on keratinophilic bacterial species of Mandla district is still unexplored and need scientific validation.

2. Material and methods

Mandla district was chosen as the area because i) district has rich flora unexplored for ethno medicines used for the treatment of keratinophilic infections. The details of tribal blocks, tribal herbal healers and tribal areas were collected from tribal welfare office (near Katra Hospital) and forest office (Maharajpur road) of Mandla district. The local tribal healers /Vaidyas of fame as well as common adult inhabitants of seven selected tribal blocks, viz., Mandla, Bichhia, Ghughari, Muhgaon, Narayanganj, Niwas and Bijandanti were selected for documentation of ethno medicinal knowledge. Monthly field trips were conducted in the tribal pockets of selected blocks during the period from January 2014 to December 2014.

The traditional herbal healers, tribal heads and tribal person were contacted and enquired to gather the related information about various ethno medicines used for the treatment of

keratinophilic infections. Special attention was paid to record information from local traditional herbal healers with the help of local guides. The interviews were usually started with general questions in hindi language about the village after which more specific questions were asked about the ethno medicines used by the healers for the treatment of the skin diseases. The structured interviews were performed with the aid of a questionnaire.

Fresh preparations of selected plants free from disease were collected from various tribal villages of Mandla district (Madhya Pradesh, India) and from herbal healers. Identification of plants has been made through the local name of plant with the help of relevant literature [18, 22, 28, 20]. The ethno medicinal plant extracts were washed thoroughly 2-3 times with running water, once with sterile distilled water, then air-dried on sterile blotter under shade. The ethnomedicines 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. The ethno medicinal material was extracted with 100 ml of hot sterile distilled water. Another set of ethno medicines was macerated and extracted in 100 ml of hexane and methanol separately for 24 hours. The plant extracts were then sieved with sterile Whatman filter paper No.1. Ethno medicines which showed anti-keratinophilic activity were further extracted by Soxhlet apparatus. Fifty grams of the shade dried powder was filled in the thimble and extracted successively with hexane and methanol in soxhlet extractor for 48hrs. The extracts of the separate solvent 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. The known amount of leaves was placed in a round bottom flask (1L) together with distilled water and hydro-distillation was performed using Clevenger apparatus. Essential oil was collected, dehydrated using sodium sulphate dispensed into dark bottles and stored at 4°C until used. The three species of keratinophilic bacterial species viz., *Escherichia coli* (MTCC 77), *Bacillus subtilis* (MTCC 10110) and *Enterococcus faecalis* (MTCC 9845) were obtained from Institute of Microbial Technology (IMTECH). The bacteria obtained were first sub-cultured in a nutrient agar medium (beef extract - 3 g., NaCl - 5 g., peptone - 5 g., agar - 15 g., distilled water - 1000 ml) and incubated at 37°C for 24 hrs. The stock cultures of bacteria were maintained nutrient agar slant at 37°C.

Bacillus subtilis, *Escherichia coli* and *Enterococcus faecalis* were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometrically ($A_{610\text{nm}}$). Each of the plates was homogenized to ensure uniform distribution of the inoculums and air-dried to remove surface moisture. The filter paper disc diffusion method was used for screening the extracts for antibacterial activity [10]. Rinsed petri plates were sterilized in an oven at 120°C for three hours. Standard size Whatman filter paper discs (5.0 mm in diameter) were sterilized in an oven at 140°C for one hour, saturated with the (20mg/ml) test extract, 5 µl/disc of oil and (1mg/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 interfere with the determination.

The inoculums was evenly spread on the surface of 10cm Petri dishes containing nutrient agar medium separately. The discs were then placed on the surface of nutrient agar medium inoculated with the test bacteria. Blank disc impregnated with water, methanol and hexane at 1ml. followed by drying off was used as negative control. After the colonies grew, the zones of inhibition around the discs were measured and recorded. Standard discs of streptomycin (Abott. Health care Pvt. Ltd.) for bacteria were used as positive control. Before incubation, all the test and control petriplates were kept at 5°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. Streptomycin was used as positive control for bacteria. The antibacterial activity was tested by disc diffusion method [10]. The filter paper discs (5mm diameter) impregnated with 20mg/ml extracts 5µl/disc of oil and 1mg/ml of control were used for test bacteria-seeded plates. The diameter of inhibition zones were measured in millimeter in three replicates by using antibiotic zone scale.

3. Results

Activity of hexane, methanol and water extracts of eight types of ethno medicines and four types of oils against *Bacillus subtilis*, *Escherichia coli* and *Enterococcus faecalis* is presented in (Table 1). Hexane root extract of *Argemone mexicana* Linn produced highest 13mm zone of inhibition against *Escherichia coli* and 12mm against *Bacillus subtilis* while no activity was observed against *Enterococcus faecalis*. Methanol extract produce inhibition zone of 12mm against *Escherichia coli* and 6mm against *Bacillus subtilis* while *Enterococcus faecalis* remain unaffected in methanol extract. *A. mexicana* leaf and seeds extracts showed considerable antibacterial activity [1]. Hexane leaf extract of *Azadirachta indica* A. Juss produced highest zone of inhibition 18mm against *Bacillus subtilis* followed by 15mm against *Escherichia coli*, while the methanol leaf extract was ineffective against bacterial species. Hexane and methanol extract of *Azadirachta indica* bark showed poor antibacterial activity as compared to leaf extract. Hexane seed extract of *Cassia tora* Linn. also showed poor antibacterial activity with inhibition zone of 10mm against *Bacillus subtilis* and 7mm against *Enterococcus faecalis*.

Hexane seed extract of *Centratherum anthelminticum* also produced highest inhibition zone of 13mm against *Bacillus subtilis* while methanol extract remain ineffective against bacterial species. *Cymbopogon citratus* produced highest inhibition zone of 27 mm against *Bacillus subtilis*, 18mm against *Escherichia coli* and 22mm against *Enterococcus faecalis* while *Cymbopogon nardus* oil inhibited the growth of *Escherichia coli* 32 mm. Extracts for activity against *Enterococcus faecalis*, *Escherichia coli* and other bacterial species using an agar dilution method at concentrations of 2.0% (v/v) [11]. Antimicrobial activity of essential oils of lemongrass against *Escherichia coli* and other bacterial

species in fruit juices [21]. Lemongrass, at concentration of 2 µL/mL inactivated the pathogens in apple and pear juice. They concluded that lemongrass essential oil had strong antibacterial activity than *Cinnamomum verum* and *Helianthus anus* essential oils.

Mentha piperita oil exhibited antibacterial activity 40 mm against *Bacillus subtilis*, 13mm against *Escherichia coli* but it showed no activity against *Enterococcus faecalis*. *Ocimum gratissimum* leaf oil produced highest zone of inhibition 22mm against *Bacillus subtilis* 20mm against *Enterococcus faecalis* and 13mm for *Escherichia coli*. *Ocimum sanctum* hexane leaf extract produced inhibition zone of 15mm against *Escherichia coli* while methanol extract produce inhibition zone 15mm against *Bacillus subtilis*. The other bacterial species remain unaffected in both the extracts. In a similar study, reported the antimicrobial activity of *Ocimum gratissimum* leaf extract against bacteria and yeast [13]. The diameter of inhibition zone recorded in *E.coli* was 18 mm at 22 µl of oil, while in the present study 5µl of oil/disc was used. These differences may be attributed due to difference in concentration of oil and presence of antibacterial component in high concentration in local variety enhancing the medicinal importance of indigenous essential oil.

The hexane leaf extract of *Plumbago zeylanica* Linn. produced inhibition zone of 10mm against *Bacillus subtilis*, 8mm against *Escherichia coli* and 13mm against *Enterococcus faecalis*. The methanol extract of leaf was found to ineffective against tested bacterial species. Hexane and methanol extract of *Pongamia pinnata* Pierre leaf and *Ipomoea pestigridis* root produced inhibition zone of 8mm and 15mm respectively against *Enterococcus faecalis* while no activity was observed against *Bacillus subtilis* and *Escherichia coli* in both the solvents.

Oils showed significant activity than other ethno medicines. Some ethno medicines were not showing inhibitory activity against bacterial species. Hexane, methanol and oils showed significant zone of inhibition against Gram-positive bacteria *Bacillus subtilis* and *Enterococcus faecalis* then Gram-negative bacterium *Escherichia coli*. The hexane extracts of the studied plants were more potent than the methanol extracts. The most susceptible bacterium was *B.subtilis* followed by *E.faecalis* and *E. coli* was least inhibited. These results are consistent with previous reports on related plants regarding "Gram-positive" bacteria [6].

Crude water, hexane and methanol extract of *Aegle marmelos* (bark) + *Cicer arietinum* (flour), *Annona squamosa* (fruit)+*Ricinus communis* (leaf),*Boswellia serrata* (bark), *Caesalpinia sepiaria* (leaf), *Cymbopogon winterianus* (leaf), *Datura innoxia* (root), *Eclipta alba* (leaf), *Elaeodendron glaucum* (bark), *Ficus arnottiana* (fruits) + *Brassica cernua* (ash), *Holarrhena antidysenterica* (bark), *Holoptelea integrifolia* (leaf), *Mangifera indica* (flower), *Selaginella bryopteris* (whole plant),*Tectona grandis* (leaf) and *Terminalia alata* (leaf) did not show activity against keratinophilic bacteria. However, negative results do not mean absence of bioactive constituents nor is that the extract is inactive. Active compound (s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed [27]. Lack of activity can thus only be proven by using large doses. Alternatively, if the active principle is

present in high quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents [12]. The extracts may be active against other bacterial species which were not tested (Shale *et al.*, 1999) [23].

Table 1: Activity of ethnomedicinal extracts (20mg/ml) and control (1mg/ml) against keratinophilic bacteria

S. No	Botanical name	S	Inhibition zone (mm)		
			<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Enterococcus faecalis</i>
1	<i>Argemone mexicana</i> (root)	H	12	13	0
		M	6	12	0
		W	0	0	0
2	<i>Azadirachta indica</i> (leaf)	H	18	15	0
		M	0	0	0
		W	7	0	0
3	<i>Azadirachta indica</i> (bark)	H	8	12	0
		M	11	0	6
		W	0	0	0
4	<i>Cassia tora</i> (seed)	H	10	0	7
		M	0	0	0
		W	0	0	0
5	<i>Centratherum anthelminticum</i> (seed)	H	13	0	0
		M	8	0	0
		W	0	0	0
6	<i>Cymbopogon citratus</i> (leaf)	O	27	18	22
7	<i>Cymbopogon nardus</i> (leaf)	O	0	32	0
8	<i>Mentha piperita</i> (leaf)	O	40	13	0
9	<i>Ocimum gratissimum</i> (leaf)	O	22	13	20
10	<i>Ocimum sanctum</i> (leaf)	H	8	15	0
		M	15	0	6
		W	6	0	0
11	<i>Plumbago zeylanica</i> (leaf)	H	10	8	13
		M	0	0	10
		W	0	0	0
12	<i>Pongamia pinnata</i> (fruit) + <i>Ipomoea pestigridis</i> (root)	H	0	0	8
		M	0	0	15
		W	0	6	0
	Streptomycin	C	13	12	12

T=Type of extract, H=Hexane extract, M=Methanol extract, W=Water extract, O=Oil and C=Control

4. Conclusion

From the present study it is concluded that keratinophilic infection was the most common in and around Mandla district. Oils of the tested ethno medicines showed potential inhibitory activity against keratinophilic bacterial species than other tested plant extracts. Present work emphasizes the use of such herbal ethno medicines, to reduce the occurrence of keratinophilic bacterial infection.

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6. References

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