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Ethonomedicinal, Antibacterial and Antifungal Potentiality of *Centella asiatica, Nerium indicum* and *Cuscuta reflexa* - Widely Used In Tiwa Tribe of Morigaon district of Assam, India.

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Abstract

In Assam, Approximately 17 different types of Tribes are residing from immemorial times. They have lots of knowledge to remedy disease by using various medicinal plants. The aim of our study is to extract out the knowledge of those plants having the medicinal values and examine the laboratory based antibacterial and antifungal property. The selection of these three plants was based on highly uses by the Tiwa Tribes in gastrointestinal troubles and skin diseases. Aquous and 50% ethanolic extract was prepared from the plants and screened against enteropathogenic bacteria *E.coli* (MTCC723), *Bacillus subtilis* (MTCC10619) and *Staphyllococcus aureus* (MTCC96). Antifungal activity were tested against *Aspergillus niger* and *Candida albicans*. *Centella asiatica* and *Nerium indicum* showed good results against *E.coli* and *Bacillus subtilis* and *Cuscuta reflexa* showed higher activity against *S. aureus*. In case of antifungal activity, *Centella asiatica* results were found fruitful in comparison to the others.

Keywords: Antibacterial, Antifungal, E.coli, Staphyllococcus aureus, Aspergillus niger, MTCC

Introduction

After the development of comparatively effective and safe antibiotics in the 1940's, medical treatment had been revolutionaries leading to the low rate of morbidity and mortality previously induced by microbial diseases [1]. With the evolutionary process that enables microbes to adapt genetically to changes in their environment, the unwise use of antibiotics makes the microbes resitant [2]. Therefore consistent development of new drugs are required to counteract the development of resistance and cost effective to control the disease [3].

Medicinal plants are granary of various hidden chemicals . These chemicals have capacity to cure human and animal diseases caused by bacteria,

fungus, virus, protozoa etc. As well as these chemicals are used to treat some other kind metabolic disorder of human and animals also. These chemicals are extracted out from the particular plant for the treatment. These are used as juice, infusion, powered, decoction and paste. These chemical constituents posses pharmacological activities which leads to new era of drug discovery from plant origin.

India is rich country in case of medicinal plants and have a good knowledge of their usage to heal regular diseases generally caused by microbes. Tribes of India are very good practitioner of these kind of medicinal plants from the ancient time. Assam is a state in North-East India, makes it a biological hotspot with many rare and endemic plant and animal species. Assam is situated from 89° 42′ E to 96° E longitude and 24° 8′ N to 28° 2′ N latitude, has an area of 78,438 km². With the 'Tropical Monsoon Rainforest Climate, Assam is a temperate region with heavy rainfall and humidity. The minimum temperature is 6 to 8°C.Assam is one of the richest biodiversity zone in the world. Morigaon district is located between 26.15°C to 26.5°C Northern latitude and 92°C to 95.5°C Eastern longitude. Tiwa is the only tribes residing in the Morigaon district from ancient time. The people of Tiwa tribes uses different medicinal plants to treat various caused by microbes.

The methanolic extract and different fractions of *S. nigrum* leaves showed the different degree of antimicrobial activity. Leaf extract along with fractions of *S. nigrum* were mildly potent as antibacterial

agent. In the antifungal activity test, *S. nigrum* leaves extract/fractions was poor. The components of leaves extract/fractions of *S. nigrum* may serve as a potential source of industrial drugs useful in chemotherapy against some bacterial infections. [4]. The leaves of the medicinal plant *Lantana camara* were extracted out by using different solvents and antibacterial and analgesic activities was screened out. The chloroform and methanolic extract of this plant has showed the presence of four alkaloid compounds each. Antibacterial activity was evaluated using MIC method against bacterial pathogens[5]. Interest in ethno pharmacy as a source of active natural compounds has increased worldwide, particularly in the search for drugs to multi-resistant microorganism. Plants with antimicrobials activities have become more interesting because many people aware of problems associated with the over prescription and misuse of traditional antibiotics. Nevertheless, only approximately 20% of the plants found in the world have been submitted to pharmacological or biological testing [6]. Antibiotic resistance is a major problem in hospitals as well as in community settings. Gram positive pathogens such as *Staphylococcus aureus, Enterococcus faecium, Enterococcus faecalis* and *Streptococcuspneumoniae* are becoming resistant to most of the existing antibiotics [7].

Due to the permanent resistance of the microorganisms to available drugs, continuous search for new antimicrobials is a scientific challenge. Natural products continue to play a most significant role in the drug discovery and development process [8], and plants are recognized as a useful sources of highly active antimicrobials metabolites [9,10]. As their medicinal purposes, most of the people uses the plant parts. To detect their antimicrobial activity, several methods are available and but not on the basis of same principles. All results are depend on the method , microorganisms, extraction method and the degree of solubility of each test-compound [11,12]. The plant extracts having the properties of antimicrobial activity are used as flavouring agents in foods, due to their essential oil fraction [13].

The reported investigation aims to highlight *Centella asiatica, Nerium antidysentericum* and *Cuscuta reflexa,* the traditional flora(eastern Himalayan belt) as a prospective medicinal plant in the realm of phyto chemistry and to make a comparative assessment of its medicinal properties in relation to available ethno botanical knowledge.

Meterials and Methods

Collection and Identification of plant material

The plant leaves were collected during end of summer from Gova hill of Morigaon District, Assam during their full growing season and were identified with name tags through literature available in the Department of Botany, Morigaon College as well as internet based information. The voucher specimen numbers of the plants were preserved. Leaves of the plants were dried under shed, for 2-3 weeks until they were completely dried. Shed drying is done, because volatile constituents may be lost due to the evaporation and degradation of constituents mainly the ones like glycosides and amino acids which are essential of medicinal use, may be noticed shade dried leaves were powdered.

15 gm of the powdered plant leaf extract was subjected to the Soxhlet apparatus (Riviera) successive extraction method (60-80˚C) using 200ml 50% of ethanol in the order of increasing polarity of solvent for a period of 30 hour. The extracts obtained were completely dried by using vacuum rotary evaporator.

Preparation of cultures

Bacterial strains

For antibacterial test, bacterial test organisms are used *E. coli (*Gram negative, MTCC723) and *Staphylococcus aureus* (Gram positive, MTCC96) are collected from Institute of Microbial Technology, IMTECH, Chandigarh, India. These two bacteria are very important hospitally aquired infection. Fresh bacterial cultures were prepared by adding a loopful of old culture of bacteria to the sterilized nutrient broth. The medium was kept shaker incubator at 37˚C for 18 – 24 hrs. The incubated culture is used for checking antibacterial activity.

Fungal species

To test the antifungal properties of plant extract, *Aspergillus niger* and *Candida albicans* was obtained from the IMTECH, Chandigarh, India. The fungus is grown in Potato Dextrose Agar by adding spore suspension and kept in 37^0 C in incubator for 48hrs.

Antibacterial tests of plant extracts by disc diffusion method.

To carry out antibacterial activity of plant, disc diffusion method as described by [14], was performed. In this, Muller Hinton agar medium was prepared and sterilized. Then media were poured into plate and kept for solidification. 0.1ml of bacterial culture was inoculated through spread plate technique. Whatman's filter paper no.1 discs (5mm diameter) were soaked in 1% DMSO extracts. The soaked discs were placed on the surface of inoculated plate and allowed to dry in laminar air flow. These plates were incubated at 37^oC for 24 hour in inverted position. The streptomycin (20µg/ml) and 1% DMSO were taken as positive and negative control respectively. The experiment was carried out in triplicates and average ZOI (zone of inhibition) was recorded. The ZOI of plants extract were compared with ZOI showed by streptomycin (20µg/ml) in same bacteria.

Antibacterial tests of plant extracts by agar well method.

The antibacterial assay was done by using the agar well diffusion method as described by (15). The assay was done to find out if the plant extracts had any antibacterial activity. Bacterial cultural was adjusted to 0.5 McFarland standard before the tests. The media used for the assay was Muller Hinton Agar. The standard solution of the extracts were prepared in 1% DMSO (Merck). The sterile liquid culture medium (20 ml) was poured into each petriplate. The solidified plates were inoculated by spread plate method with an inoculums corresponding to 0.5 McFarland standard. Four well of 5mm diameter were punched into the agar with the sterilized well puncture with 5mm diameter size and 50µl of plant extract were

added into two of the wells. Streptomycin (20µg/ml) and 1% DMSO was taken as the as positive control and negative control respectively. The plates were then sealed with paraffin and kept for incubation at 37ºC for 18hours. The antibacterial activity was evaluated by measuring the ZOI diameter observed using zone scale (mention Hi Antibiotic Zone Scale). The test was conducted in triplicates.

Determination of Minimum Inhibitory Concentration (MIC)

The determination of MIC was done following the protocol of [16]. With little modification. MIC activity was determined using 96 well micro plates. Stock solution of the extracts of plants was prepared at a concentration of 10mg/ml. various concentrations of the extract were prepared by serial dilution. 100µL of extract of each concentration was added in each of the wells. After this 100µL of the bacterial inoculums corresponding to 0.5McFarlnd Standard was added into each well. Streptomycin was taken as the positive control while DMSO 1% was the negative control. The plates were then incubated at 37ºC for 16 hours in an incubator. After the incubation period was over, 40µL of MTT solution (0.2 mg/ml) was added into each well and then further incubated at 37ºC for 30 – 45 minutes. The bacteria – formazan complexes were collected using a pipette. The complexes were transferred into glass test tubes added 1ml of 1% DMSO and 1ml of distilled water. The tubes were mixed manually to accelerate the dissolution process. The absorbance of the mixtures were recorded at 550nm.

Results and Discussion

The results of antibacterial activity by measuring the diameter of the zone of inhibition are shown in the table- 1 and table- 2

Table1:-Plants and plant's part used for treatment of wounds and gastrointestinal problem in Assam

Scientific Name	Vernacular name	Family	Part used	Indication	Mode of application
Acacia nilotica	Khoira goch	Mimosaceae	Leaf	Skin infection	Paste
Adhatoda vasica	Tita bahak	Acanthaceae	Root and leaves	Chronic diarrhea.	Decoction
Centella asiatica.	Manimuni	Apiaceae	Leaves and stem	Dysentery,	2-3 spoon juice
Cuscuta reflexa	Akashi lota	Convolvulaceae	Stems	Skin diseases	Paste
Eclipta prostrata Linn.	Kenhraj	Asteaceae	Leaf	dysentery	Infusion
Emblica officinalis Gaenth.	Amlokhi	Euphorbiaceae	Seed	dysentery	Paste
Ficus religiosa Linn.	Ahot	Moraceae	Bark	Wound	Paste
Hemidesmus indicus R. Br.	Anantamul	Asclepiadaceae	Root	Wound	Decoction
Mangifera indica Linn.	Aam	Magnoliaceae	Immature fruit	Skin diseases	Juice
Nerium indicum	Korobi	Apocynaceae	Leaves and bark	Dysentery	1 spoon juice
Oroxulum indicum (L)	Bhat ghila	Bignoniaceae	Root	Dysentery and diarrhea	Juice
Plumbago zeylanica Linn.	Agyasit	Plumbaginaceae	Root	Wound	Paste
Ranunculus indicus	Jal dhania	Ranunculaceae	Leaves	Skin diseases	Paste
Rubus ellipticus Sm.	Jutuli poka	Rosaceae	Leaves, fruit	Diarrhea	Decoction
Solanum myriacanthum Dunal.	Kota Bengena	Solanaceae	Friut	Skin diseases	Paste
Spondias pinnata(L.f.) Kurz.	Amora	Anacardiaceae	Seed	Amoebic Dysentery, Itching of skin	Juice
Terminalia arjuna Roxb.	Arjun	Combretaceae	Bark	Skin sore	Paste
Vitex negundo (L)	Pachatia	Verbenaceae	Flower	Diarrhea	Decoction
Wrightia tinctoria R&S.	Boga kutraj	Apocynaceae	Leaves	Skin disease	Paste
Xanthium strumerium(L)	Agora	Asteraceae	Leaves	Skin disease	Paste.
Zingiber officinale Rose.	Ada	Zingiberaceae	Rhizome	Dysentary	Juice

Table 2: Antibacterial activity of different plant extracts

Plant species	Extract	Test organisms	ZOI(mm)	MIC values(mg/ml)
Centella asiatica	Aquous	*E. coli MTCC723*	35	4.21
	Aquous	*Staphylococcus aureus* MTCC96	10	1.22
	Aquous	*Bacillus subtillis* MTCC10619	31	3.11
Nerium indicum	50% Ethanol	*E. coli* MTCC723	42	5.01
	50% Ethanol	*Staphylococcus aureus* MTCC96	15	2.12
	50% Ethanol	Bacillus subtillis MTCC10619	17	2.34
Cuscuta reflexa	50% Ethanol	*E. coli* MTCC723	13	1.74
	50% Ethanol	*Staphylococcus aureus* MTCC96	37	4.47
	50% Ethanol	Bacillus subtillis MTCC10619	22	2.21

Table 3: Antifungal activity of different plant extracts.

Plant species	Extract	Test organisms	ZOI(mm)
Centella asiatica	Aquous	*A. niger*	30
	Aquous	*C. albicans*	26
Nerium indicum	50% Ethanol	*A. niger*	10
	50% Ethanol	*C. albicans*	13
Cuscuta reflexa	50% Ethanol	*A. niger*	22
	50% Ethanol	*C. albicans*	19

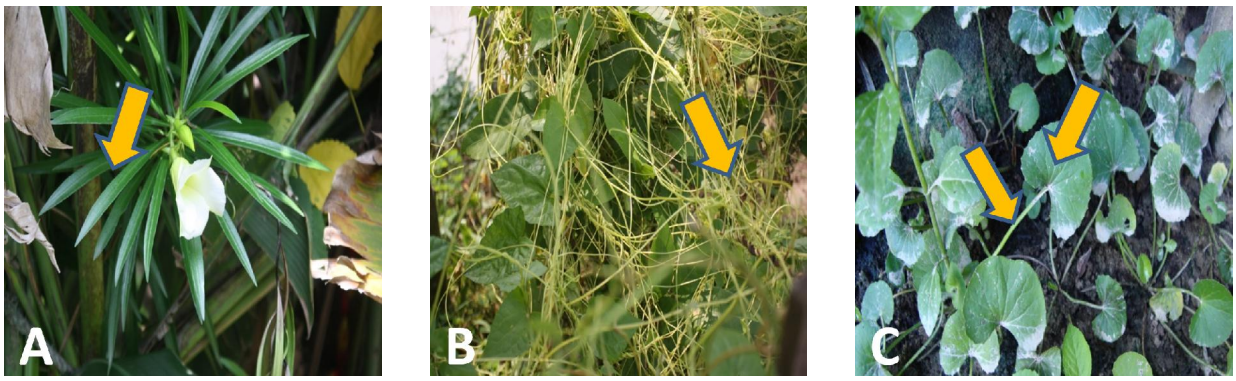

Figure 2: Photographs of medicinal plants. Arrow indicates the part used as medicine. A: Leaves of *Nerium indicum* B: Stems of *Cuscuta reflexa* . C: Leaves and stems of *Centella asiatica.*

respectively. The water extract of *Centella asiatica* showed maximum inhibition zone against *E. coli* (35mm) and *Bacillus subtillis* (31mm) and moderate against *Staphylococcus aureus* (10mm). In case of *E. coli* and *Bacillus subtillis*, ZOI was found better when it was compared to ZOI of standard antibiotic Streptomycin but ZOI of *Staphylococcus aureus* was less compare to antibiotic. The 50% ethanol extract of *Nerium indicum* showed inhibition zone 42mm,17mm and 15mm for *E. coli*, *Bacillus subtillis* and *Staphylococcus aureus* respectively. In case of *Cuscuta reflexa*, the results shows a different angle. 50% ethanol extract of *Cuscuta reflexa* reduces the growth of *S. aureus*. The

ZOI against *E. coli, Staphylococcus aureus* and *Bacillus subtillis* was 13mm, 37mm and 22mm accordingly.

From this research, it can be concluded that *Centella asiatica* and *Nerium indicum* was found to be most active against the growth of *E. coli.* Likewise *Cuscuta reflexa* was found fruitful against the growth of *Staphylococcus aureus.* It was clearly observed that all the two leaf extracts possessed the potentiality against the growth of *E.coli* but the *Staphylococcus aureus* strain*s* showed bit resistive to these leaf extracts. These medicinal plants can be explored in the production of the useful drugs as they are commonly available in Assam.

Antimicrobial agents are generally chemical compounds which inhibit microbial growth or cause microbial death and are used as food additives. In the last decades, there has been particular interest in the use of abundant naturally occurring antimicrobials (herbs, spices and plants)in food stuffs [17]. The ability to compare the results for antimicrobial plant compounds or extracts is limited because of different methodologies used and definitions of MIC [17]. It is also necessary to screen out the new antimicrobials and the evaluation of biological activity is essential for the assessment of susceptibility to antibiotics [18]. Several antimicrobial compounds are extracted out from the easily available sources, like agricultural and horticultural crops or medicinal plants such as pine, sage, rosemary, and many others [19,20]. The antimicrobial nature is determined by its chemical properties, such as pKa value, hydrophobicity/lipophilicity ratios, solubility, and volatility [21]. The pH and polarity influences the effectiveness of a food antimicrobial. Polarity is related to both the ionization of the molecule and the contribution of any alkyl side groups or hydrophobic parent molecules [22].

The combined effects of adsorption of polyphenols to bacterial membranes leads the membrane disruption and subsequent leakage of cellular contents [23,24], and the generation of hydroperoxides from polyphenols [25]. Experiment shows that Garlic reduces the growth of *E. coli* in ground meat and addition of fresh garlic and garlic powder controls microbial contamination and preserved chicken sausages [26, 27]. Species of the genus Mentha (family Lamiaceae) are a granary of polyphenolic compounds, flavonoids, terpenoids, and other volatile compounds, which has strong antimicrobial properties against most of the pathgens [28, 29]. It is also reported that more than 8 log reductions in the artificially inoculated pasteurized tomato juice when mint was used as a preservative [30]. It is a long tradition by the local tribal people of using pepper to fight against several ailments, which is still in the practice mood, in many parts of the rural India. Therefore, utilizing the tribal knowledge for this medicinal plant, an attempt was made to isolate some novel bioactive compounds having the potential activity against multidrug resistant (MDR) *Mycobacterium*. A bioassay guided fractionation of Pippali (*Piper longum* L.) was performed in five different organic solvents and their activities were examined against different pathogenic bacteria including MDR *Mycobacterium*(31).

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