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RESEARCH ARTICLE

MICROBIOLOGY

SCREENING OF ANTIGONORRHOEAL ACTIVITY OF SOME MEDICINAL PLANTS IN NEPAL.

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ABSTRACT

In view of the wide spread emergence of antibiotic resistant *Neisseria gonorrhoeae* isolates, the antigonococcal activity of ten Nepalese folk medicinal plants commonly used by the ethnic groups of peoples was evaluated. Among ten plant extracts, the ethanolic extracts of four plants and hexane extracts of one plant were very sensitive to *N. gonorrhoeae.* The maximum mean zone of inhibition by agar well diffusion method was seen for *Eupatorium odoratum* and the minimum was for *Syzygium cumini*. Similarly, the minimum inhibitory concentration by test tube dilution method for *Eupatorium odoratum* was the least followed by *Ocimum sanctum, Sapindus mukorossi*, *Allium sativum* and *Syzygium cumini*. Qualitative phytochemical analysis of the extracts reveals the presence of bioactive components. Thus, the result of this study justified the folkloric usage of the studied plants and concluded that these plants extract have great potential in finding new clinically effective antigonorrhoeal compounds.

KEY WORDS

antigonorrhoeal activity; gonorrhoea; *Neisseria gonorrhoeae*; medicinal plants, plant extracts, phytochemical analysis.

INTRODUCTION

Gonorrhoea is one of the classical sexually transmitted disease (STD) with human as the host for the causative agent, *Neisseria. gonorrhoeae*. According to a global estimate from World Health organization (WHO, 1995), around 62 million new cases occurred in 1995 and the highest rate was found in South and Southeast Asia, Sub Saharan Africa and South and Central America [1]. It is estimated that more than 340 million new episodes of curable sexually transmitted diseases occur each year and gonorrhoea is one of the most prevalent infections [2]. According to Family Health Division, Ministry of Health, Nepal, gonorrhoea is one of the prevalent STD in this country also [3]. The problem is further compounded by the emergence of resistance to antimicrobial agents that are commonly used against *N. gonorrhoeae,* making the treatment expensive and prolonged [4].

In the context, countries like Nepal the prohibitively expensive cost of efficacious antibiotics and the emergence of single and multiple antibiotic resistant *N. gonorrhoeae* strains call for the search of alternative agents with possible antibacterial effects from natural resources. This situation forced scientists to search for alternate antimicrobial substances, from plants which are cheap, readily available for the population, and have minimum side effects. The World Health Organization also supports the use of medicinal plants provided it is proven to be efficacious and safe [5]. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases [6].

Nepal is rich in all the 3 levels of biodiversity, namely species diversity, genetic diversity and habitat diversity. In Nepal thousands of species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments. In Nepalese traditional medicine, there is a rich local ethnobotanical bibliography describing the species most frequently used by the population to cure gastrointestinal, respiratory, urinary and skin infections [7, 8]. However, there is a lack of experimental scientific studies confirming the possible antibiotic properties of a great number of these remedies.

In the present paper an attempt has been made to screen the *in vitro* antigonorrhoeal properties of ten traditional plants, which are used by local communities for the treatment of skin and venereal diseases.

MATERIALS AND METHODS

The leaves of 10 plant samples were collected from different sites of Parsa (Birgunj), Bara (Simra) and Makwanpur (Hetauda) districts (altitude about 1500ft from above the sea level), and processed at Clinical Microbiology laboratory of National Medical College and Teaching Hospital, Birgunj Nepal. These plants were identified according to various literatures [7, 8] and in the Department of Botany, Vidyasagar University, India.

Preparation of Plant Extracts:

The leaves of the plants were air dried separately at room temperature and grounded to fine powder by using Mortar and Pistol. The extracts were prepared by dissolving10 grams of plant leaves in 90 ml of solvent and kept at room temperature for 5 days with daily agitation. The extract was

separated by filtration using Whatman paper No. 2 [9]. Different solvents like water, methanol (50%, v/v), ethanol (50%, v/v), acetone (50%, v/v) and hexane (50%, v/v) were used for extracting the antimicrobial compounds from the plant.

Isolation of Neisseria gonorrhoeae:

N. gonorrhoeae was isolated from 30 male patients with acute gonococcal urethritis attending the Skin and Venereal disease department of National Medical College and Teaching Hospital, Birgunj, Nepal, which were direct smear-positive cases. For isolation of *N. gonorrhoeae*, urethral swabs were inoculated onto Modified Thayer Martin agar media (Hi Media, India) with vancomycin, colistin, nystatin and trimethoprim (VCNT) supplement (Hi Media, India) [10]. The inoculated culture plates were incubated at 36 - 37°C in a moist atmosphere containing 5% CO_2 in CO_2 incubator for 24 – 48 hours. These consecutive clinical isolates were identified on the basis of colony morphology, gram staining, oxidase, superoxol and rapid carbohydrate utilization tests [11].

Preparation of the Inoculum:

3 to 5 colonies grown on Modified Thayer Martin agar media were obtained. These colonies were inoculated in 2.5 ml Muller-Hinton broth in a test tube by rotating the straight wire at least ten times with the tip touching the bottom of the test tube. The turbidity is also matched with 0.5 McFarland Standard.

Antigonorrhoeal activity of plant extracts:

Muller Hinton chocolate agar plates with 5% sheep red blood cells were swabbed all over the surface with freshly prepared inoculum, using sterile cotton swab. Five wells (6 mm. diameter) were bored in the medium with the help of sterile cork-borer and were labeled properly. Fifty micro-liters (µl) of each solvent extracts of a medicinal plant were added in each well. Plates were left for 5 minutes till the extract diffuse in the medium and then incubated at 37°C in a moist atmosphere containing 5-10% CO_2 for 48 hours. The antigonorrhoeal activity of the plant extracts were recorded by measuring the inhibition zones in millimeters with a measuring scale. The whole process was repeated in triplicate. Tested plant extract having more than 8 mm zone of inhibition were selected for further study.

Determination of Minimum Inhibitory Concentration (MIC):

The selected plant extracts were used for the determination of minimum inhibitory concentration (MIC) by the test tube dilution method [12]. Appropriate dilutions of 45 – 500 µg/ml were made to give a final volume of 1ml in the tubes. One drop equivalent to 0.02 ml of organisms (prepared as previously described) was added to each test tube. A tube was set up without the extract as a control. The test tubes were incubated at 36°-37°C in a moist atmosphere containing 5% CO_2 in CO_2 incubator for 24 – 48 hours. The MIC was regarded as the lowest concentration that inhibited visible growth.

Phytochemical evaluation of the plant extracts:

The phytochemical evaluation of the plant extracts for the major constituents was undertaken as per the standard methods [13, 14]. It was conducted only for those plants which show antigonorrhoeal activity on screening test.

Detection of alkaloids: The plant extracts were dissolved individually in dilute HCL and filtered. The filtrates were used to test for the presence of alkaloids.

(a) Mayer's test: Filtrates were treated with Mayer's reagent (Potassium mercuric iodide). Formation of yellow cream precipitate indicates the presence of alkaloids.

(b) Dragendroff's test: Filtrates were treated with Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

***Detection of Carbohydrates*:** The plant extracts were dissolved individually in 5ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

(a) Molisch's test: The filtrates were treated with 2 drops of alcoholic α- napthol solution in a test tube and 2 ml of concentrated H_2SO_4 was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.
(b) Benedict's test: The filtrates were treated with Benedict's reagent and heated on water bath. Formation of orange red precipitate indicates the presence of reducing sugar.

Detection of Glycosides: The plant extracts were hydrolysed with diluted HCl and then subjected to test for glycosides.

Modified Borntrager's test: Extracts were treated with Ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose pink colour in the ammoniacal layer indicates the presence of glycosides.

Detection of tannins:

Gelatin test: 1% gelatin solution containing sodium chloride was added to the filtered extract. The presence of tannins was indicated by the presence of white precipitate.

Detection of Saponins:

(a) Froth test: Extracts were dilutes with 20 ml distilled water and this was shaken in a graduated cylinder for 15 minutes. Formation of 1cm. layer of foam indicates the presence of saponins.
(b) Foam test: Small amount of the extracts were shaken with little quantity of water. If the produced foam persists for ten minutes it indicates the presence of saponins.

Detection of Oils and Fats: Small quantities of the extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oil.

Detection of Flavonoids:

(a) Alkaline reagent test: The extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.
(b) Zinc hydrochloric acid reduction test: To the alcoholic solution of extracts, small amount of zinc dust and concentrated HCl was added. After few minutes appearance of magenta colour indicates presence of flavonoids.
(c) Lead acetate test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Statistical Analysis:

To ensure consistency of all findings, antigonorrhoeal activity of plant extracts was performed in triplicate under aseptic condition. Data were statistically analyzed and expressed as mean ± Standard deviation (SD) (Figure-1)

RESULTS

Ten medicinal plants (as shown in Table 1) used in this study are widely used in folkloric medicine of Nepal in treating different diseases [7, 8].

TABLE 1
LIST OF MEDICINAL PLANTS USED IN ANTIGONORRHOEAL ASSAY

Sl. No.	Botanical Name	Vernacular Name	Family	Parts used	Month of collection	Location/ District
1.	*Curcuma longa*	Haledo	Zingiberaceae	Leaves	May,2009	Birgunj
2.	*Emblica officinalis*	Amlaki	Euphorbiaceae	Leaves	June,2009	Simra
3.	*Ocimum sanctum*	Tulsi	Lamiaceae	Leaves	June,2009	N.M.C.T.H. campus, Birgunj and Hetauda
4.	*Sapindus mukorossi*	Reetha	Sapindaceae	Leaves	July,2009	Pathlaiya, Birgunj and Hetauda,
5.	*Origanum majorana*	Ramtulsi	Lamiaceae	Leaves	August,2009	N.M.C.T.H Campus
6.	*Syzygium cumini*	Jamun	Myrtaceae	Leaves	August,2009	Birgunj
7.	*Eupatorium odoratum*	Banmara	Asteraceae	Leaves	July,2009	N.M.C.T.H Campus and Hetauda
8.	*Justicia adhatoda*	Asuro	Apocynaceae	Leaves	July,2009	Birgunj
9.	*Rauvolfia serpentina*	Sarpagandha	Apocynaceae	Leaves	August,2009	Simra
10.	*Allium sativum*	Lasun	Liliaceae	Leaves	August,2009	Birgunj

In this study, aqueous, ethanolic, methanolic, hexane and acetone extracts from the leaves of these ten plants were studied. Among ten different plants, organic extract of *Ocimum sanctum, Sapindus mukorossi, Syzygium cuminii, Eupatorium odoratum* and *Allium sativum.* (Table - 2) were showed more sensitive against *N. gonorrhoeae*. The maximum mean zone of inhibition was observed for *Eupatorium odoratum* (12.5 ± 0.5 mm). and the minimum was for *Syzygium cumini* (8.8 ± 0.3 mm) (Fig. 1).

TABLE 2
ANTIGONORRHOEAL ACTIVITY OF PLANTS DIFFERENT SOLVENT EXTRACTS THROUGH AGAR DIFFUSION METHOD

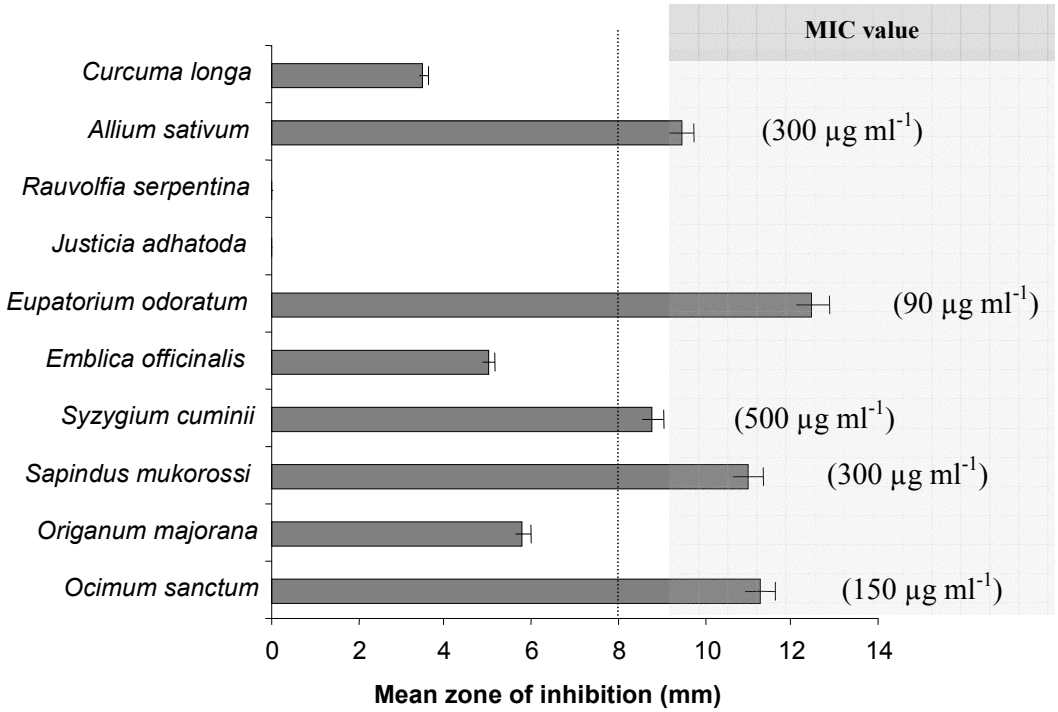
Plants Name	Aqueous extract	Ethanol extract	Methanol extract	Hexane extract	Acetone extract
Curcuma longa	-	-	-	-	-
Emblica officinalis	-	-	-	-	-
Justicia adhatoda	-	-	-	-	-
Ocimum sanctum	-	-	-	+++	+
Sapindus mukorossi	-	++	-	-	-
Origanum majorana	-	-	-	-	-
Syzygium cumini	-	++	+	-	-
Eupatorium odoratum	-	+++	+	-	-
Rauvolfia serpentine	-	-	-	-	-
Allium sativum	-	++	-	-	-

(Note: +++ = highly sensitive, ++ = moderately sensitive, + = less sensitive and - = not sensitive)
Extraction Solvent: (50µl)
Diameter of well (6mm)

Evaluation of antigonorrhoeal activities was further assessed by measuring the MIC values. The MIC test was performed for only those plant extracts showing mean zone of inhibition more than 8 mm. MIC of *Eupatorium odoratum* was 90µg/ml followed by *Ocimum sanctum*(150µg/ml), *Sapindus mukorossi* (300µg/ml), *Allium sativum* (300µg/ml) and *Syzygium cumini* (500µg/ml) (Fig.1)

FIGURE 1

Antigonorrheal activity of different plant extracts against N. gonorrhoeae.
(MIC value was given only for the cases, which have mean zone of inhibition higher than 8 mm)



Each histogram is a mean zone of inhibition and horizontal line on side represents Standard Deviation (SD).

Preliminary phytochemical analysis conducted for the five plants extracts (four ethanolic and one hexane) reveals the presence of flavonoids, alkaloids and saponins as the major constituents (table 3). The other secondary metabolites like tannins, glycosides and carbohydrates were present in trace amount in some of the plant extracts. (table 3).

Table 3

Phytochemical analysis of the five plant extracts

Components → Plant extracts ↓	Alkaloids	Flavonoids	Saponins	Tanins	Carbohydrate	Glycosides	Oils and Fats
Eupatorium odoratum	++	+++	-	-	-	-	-
Ocimum sanctum$	++	-	-	+++	-	+	-
Sapindus mukorossi	+	-	+++	-	+	-	-
Syzygium cuminii	++	-	-	+++	+	-	-
Allium sativum	-	-	-	-	-	+	+++

$: Ocimum sanctum: Hexane extract, rest all are ethanol extract
'+': Presence of phytoconstituents '-': Absence of phytoconstituents
'+++': Highly intense colour; '++': Modarately intense colour; '+': Less intense colour

DISCUSSION

The present study was conducted for the first time to obtain preliminary information on the antigonorrhoeal activity of ten traditional medicinal plants of Nepal. Information on medicinal plants used for the treatment of gonorrhoea and reports on antigonorrhoeal activity of plants is very scarce [9]. Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. Through this *in vitro* study, we could see that five plant extracts inhibited the growth of *N. gonorrhoeae* but their effectiveness varied. Out of the five plants extracts, ethanolic extracts of four and hexane extract of one was showed significant antigonorrhoeal activities (FIG. 1). The susceptibility of *N gonorrhoeae* towards the limited number of plant extracts may be due to its gram negative nature and the permeability barrier provided by the outer membrane or the membrane accumulation mechanism. Earlier, it was also reported that gram positive organisms are more sensitive to plant extract than gram negative [15, 16].

Based on the mean zone of inhibition and MIC, the test organism *N. gonorrhoeae* was found to be most susceptible to *Eupatorium odoratum* extract and least susceptible to *Syzygium cumini* among the five plant extracts considered to be effective against the organism. The results obtained indicate the existence of antimicrobial compounds in the crude ethanolic extracts of four plants and crude hexane extract of one plant under study and showed a good correlation between the reported medicinal uses of these plants against different diseases in local communities of Nepal. The inhibitory effect of these five plants against *N. gonorrhoeae* might be attributed to the presence of some active constituents in the plant as in other herbal plants.

The phytochemical analysis of the study reveals that the extract of the five plants (four ethanolic and one hexane) contained bioactive compounds (Flavonoids, Alkaloids, Saponins, tannins, carbohydrates, glycosides & oils). These bioactive compounds are believed to be responsible for the observed antigonorrhoeal effects. Some workers have observed the antimicrobial effects of plant extracts to the presence of these bioactive compounds [17, 18]. The presence of these bioactive compounds is an indicator that the five plants can be a potential source of precursors in the development of synthetic drugs against gonorrhoea.

It is notable that despite the several studies on the antimicrobial properties of herbs and spices [19-21], the reports on antigonorrhoeal activity of plant extracts are limited. An ethnobotanical survey conducted in Rwanda and Guatemala found that plants extracts are effectively used against STDs [9, 22]. Likewise, it has been reported in 2005 that the extracts of plants are inhibitory to the clinical isolates of *N. gonorrhoeae* [23].

Other five plants namely *Curcuma longa*, *Emblica officinalis*, *Justicia adhatoda*, *Origanum majorana*, *Rauvolfia serpentina*, though were selected on the basis of their use in diarrhea, dysentery, fever and other enteric bacteria, *Curcuma longa*, *Emblica officinalis* and *Origanum majorana* showed remarkably small zone of inhibition and *Justicia adhatoda* and *Rauvolfia serpentina* showed no zone of inhibition at all against *N. gonorrhoeae.* This might be attributed to the unavailability of the active constituents against the test organism or the plant extracts may have contained the active constituents, just not in sufficient concentration so as to be effective.

In recent years, antibiotic resistance against *N. gonorrhoeae* have increased dramatically in Nepal and in our previous study we reported that out of the 30 isolates, 18 (60.0%) were resistant to penicillin, 10 (33%) were resistant of tetracycline and 6 (20%) were resistant to Ciprofloxacin [4]. Thus, the treatment of gonorrhoeae has become very difficult reducing the therapeutic options. Likewise, it is also very dangerous to use

large dose of most synthetic drugs due to their toxicity while the body system can still accommodate some plant extracts at relatively high doses [24]. Therefore, bioactive substances from the plants under study can be employed in the formulation of antimicrobial agents for the treatment of gonorrhoea.

The result of the present study provides a scientific proof for the local use of the five medicinal plants studied and ushers for the selection of plants with antigonorrhoeal activities for further phytochemical work in the isolation and identification of the active compounds.

CONCLUSION

The plant extracts under study demonstrated great potential against *N. gonorrhoeae* and supported their folkloric use against the disease caused by the organism. Further, it has opened the avenues for the discovery of new clinically effective antigonorrhoeal compounds. Chemical analysis of these five plant extracts reveals the presence of several alkaloids, flavonoids, saponins, glycosides and tanins responsible for the antigonorrhoeal activity, possibly due to their synergistic effect on the test organisms [25,26].Therefore, the future investigations should be directed towards the determination of chemical structure of the active principle and toxicological evaluation with the aim of formulating novel chemotherapeutic agents to cope up with increasing prevalence of drug resistant gonorrhoea.

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