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ANTIMICROBIAL ACTIVITY OF *NIGELLA SATIVA, ACORUS CALAMUS, MYRISTICA FRAGRANS* AND *HEMIDESMUS INDICUS* AND ITS SYNERGISTIC EFFECT WITH ANTIBIOTICS

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ABSTRACT

Methanol and aqueous extracts of four plant species namely *Nigella sativa, Acorus calamus, Myristica fragrans* and *Hemidesmus indicus* traditionally used in Indian folklore medicine for the treatment of various bacterial and fungal infections were investigated for antimicrobial activity against pathogens viz *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus* sp., *Escherichia coli* and *Candida albicans* by well diffusion and minimum inhibitory concentration (MIC) methods. Comparative study of the antimicrobial activity of the plant extracts and its synergistic effect with antibiotics was carriedout. Maximum zone of inhibition was observed against *S. aureus*, *E.coli*, *Bacillus* sp and *P. aeruginosa* by methanol extracts of *Nigella sativa* and *Hemidesmus indicus*. *C albicans* showed susceptibility to methanol extracts of *Acorus calamus* and *Hemidesmus indicus*. Phytochemical analysis of methanol and aqueous extracts of *Nigella sativa* and *Hemidesmus indicus* revealed the presence of alkaloids, flavonoids, triterpenes, polyphenols and sterols. Both the active extracts were separated by adopting analytical thin layer chromatography. Totally two spots were observed for *Hemidesmus indicus* and *Nigella sativa* showed only one spot, when chloroform: methanol (70:30) used as a solvent system. The active fraction was determined by bioautography by using *S. aureus* as test organism. Only *Nigella sativa* extract alone showed synergistic effect with antibiotics and the positive results were showed by *S. aureus* and *E. coli*. The results provide justification for the use of the plants in folk medicine to treat various infectious diseases.

Key words: *Nigella sativa, Hemidesmus indicus*, Antimicrobial Activity, Synergistic effect, Bioautography

INTRODUCTION

India is endowed with a rich wealth of medicinal plants which have been a valuable source of natural products for maintaining human health. A large number of these medicinal plants are used in several formulations for the treatment of various diseases caused by microbes. According to World Health Organization, medicinal plants would be the source of obtaining a variety of drugs. Various societies across the world have shown great interest in curing diseases using plants/ plant based drugs. Microbes are closely associated with the health and welfare of human beings. Some are beneficial and some are detrimental. As preventive and curative measures, plants and their products are used in the treatment of infections for many centuries ago. WHO estimated that 80% of the people worldwide rely on plant based medicines for their primary healthcare[1,2] and India happens to be the largest user of traditional medical cure, using 7000 plant species.

The increasing failure of chemotherapies and antibiotic resistance exhibited by pathogenic microbial infections agents have led to the screening of several medicinal plants for their potential antimicrobial activity [1,3, 4]. Antibacterial properties of various plants parts, such as leaves, seeds and fruits have been well documented for some of the medicinal plants for the past two decades[1,5]. Antibiotic principles are distributed widely among angiospermic plants. A variety of compounds are accumulated in plant parts accounting for their constitutive antimicrobial activities [1,6].

Within the recent years, infections have increased to a great extent and antibiotic resistance effects become an ever-increasing therapeutic problem. Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms of action. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Therefore, it is of great interest to carry out a screening of some plant materials in order to validate their use in folk medicine and to reveal the active principles by isolation and characterization of their constituents[7].

The present study aimed to screening and evaluating the antimicrobial assay of *Nigella sativa*, *Acorus calamus*, *Myristica fragrans* and *Hemidesmus indicus* against five pathogenic microorganisms.

MATERIALS AND METHODS

Collection of plant Material

Nigella sativa seeds, Rhizome of *Acorus calamus*, Root of *Hemidesmus indicus* and *Myristica fragrans* seeds were collected from nearby areas in Vellore (Latitude '12° 55' N' North: Longitude 79° 11' E' East) Tamilnadu.

Preparation of plant extracts

Plant samples were air dried at room temperature and dried samples were coarsely powdered with pestle and mortar. Each 20 gms of powdered material were completely soaked in 100ml of sterile water and 70% methanol and then covered with aluminum foil. The Extraction was allowed to proceed for 48h. The extract was decanted and the solvents were removed by evaporation in vacuom by rotary evaporator. The air dried extracts were used for the antimicrobial and phytochemical analysis[8].

Microorganisms used

A total of 5 clinical isolates of bacteria and a fungi belonging to 5 genera comprising of *S. aureus*, *P. aeruginosa*, *Bacillus sp., E .coli* and *C. albicans* isolated from various clinical samples were obtained from Government hospital, Vellore, Tamilnadu. The clinical isolates were identified by biochemical methods as recommended by Bergey's Manual of Systemic Bacteriology[9].

SCREENING FOR ANTIMICROBIAL ACTIVITY

(i)Well diffusion method

The 18 hrs bacterial and fungal suspensions were prepared with help of McFarland standard. The aliquot was spread evenly on Muller Hinton agar and Sabouraud's Dextrose agar. On each plate, equidistant wells were made with a 6 mm diameter with help of sterilized cork borer. Then fifty micro liter of each plant extract containing 0.25mg was aseptically introduced into a respective agar well. Ciprofloxacin (5 μg/ml) and

amoxicillin (25 µg /ml) were used as positive controls and nystatin (5 µg/ml) and amphotericin B (25 µg /ml) were used for fungal isolates. The methanol and distilled water were used as negative controls and incubated at 37 ^{o}C for 24-48 hrs for bacteria and fungus. The formations of clear inhibition zone of ≥12 mm diameters around the wells were regarded as significant susceptibility of the organisms to the extract. The experiment was performed with duplicate[10].

(ii) Phytochemical Analysis

The solvent extracts which showed maximum antibacterial activity was subjected to qualitative test for the identification of various plant constituents such as carbohydrates, proteins, phytosterols and flavonoids[11].

(iii) Separation of active compound by TLC

The methanol extract of both plants which showed promising activity against the test pathogens were subjected to separation by thin layer chromatography (TLC) using silica gel coated TLC plates [12]. About 3µl of active crude extract was spotted at the bottom of the sheet using capillary tube. To find out the better solvent system for separation, the sheet was placed in a beaker containing the different solvent systems, such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water with following proportions: n-butanol: acetic acid: water (60:30:10), (70:20:10), (75:20:5) chloroform: methanol (70:30), (60:40), (40:60), (50:50), and n-hexane : chloroform (60:40). After running the chromatogram the sheet was kept in a developing chamber containing potassium iodide crystals. The compound present in the crude extracts were appeared as brown spots [13].

(iv) Detection of active compound by bioautography:

The bioautography method used for the detection of active compound separated in TLC[14]. Chromatogram developed as described above was placed in a sterile bioassay petridish and overlaid with 10ml of molten nutrient agar seeded with 0.2ml of *S aureus* as test organism and incubated overnight at 37^{0}C for 24 hours.

(v) Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the extracts was estimated for each of the test organisms in triplicates. 0.5ml of varying concentrations of the extracts (50-800µg/ml) are added with 2ml of nutrient broth and Sabouraud's Dextrose broth's and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard for bacterial isolates and 10^6 cfu/ml for fungal isolates were introduced to the tubes. The procedure was repeated on the test organisms using the standard antibiotics (ciprofloxacin and cotrimoxazole for bacteria and nystatin and ampothericin B for fungal isolates). A tube containing nutrient broth and Sabouraud's Dextrose broth only were seeded with the test organisms as described above to serve as control. Tubes containing bacterial cultures were then incubated at 37^{o}C for 24 h while tubes containing fungal spore cultures were incubated for 48 h at room temperature (30 – 32^{o}C). After incubation the tubes were then examined for microbial growth by observing for turbidity[15].

(vi) Evaluation of synergistic effect of antibiotics and plant extracts on resistant bacterial samples

Aliquots of 100 µl of resistant bacterial cultures (0.5 MacFarland Standard) grown in 10 ml of nutrient broth for 6 h were inoculated in nutrient broth supplemented with the respective antibiotics (50µg/ml) and 10^6 cells/ml fungal cultures grown in Sabouraud's Dextrose broth supplemented with 100 µg /ml coltrimazole with different concentrations of plant extracts. The concentration for plant extracts ranged from 10 to 500 µg/ml, based on MIC values that had previously been evaluated. Only streptomycin or gentamicin was used as the sub-inhibitory concentration (50µg / ml) and incubated at 37^{o}C for 48 hours. After 48 h, the optical density of each sample was recorded and compared to those of MIC to verify the synergistic effect of the tested compounds[16].

RESULTS

(i) Well diffusion method

In the present study among the 4 plants screened, the methanolic extracts of *N.sativa* and *H.indicus* showed promising activity and found to be more effective than aqueous extract except for *Nigella sativa*. Maximum zone of inhibition were observed against *S.aureus, P.aeruginosa* and *E.coli* by aqueous and methanol extracts of *N.sativa* and *H.indicus*. *Bacillus sp.* Showed susceptibility to *N. sativa, M. fragrans* and *H.indicus* extract. *C.albicans* inhibited by methanol extracts of

A.calamus and *H.indicus*. The results of the antimicrobial activity of plant extracts tested against microorganisms by well diffusion method are shown in Table -1 & 2,2A.

(ii) Separation of active compound by TLC

Methanol extract of *H.indicus* has showed two clear spots, when chloroform: methanol (70:30) used as solvent system. Their Rf values are calculated as 0.56 and 0.88. Aqueous extract of *N. sativa* showed only one clear spot, when chloroform: methanol (70:30) used as a solvent system and its Rf value was calculated as 0.75.

(iii) Detection of active compounds by bioautography:

In bioautography, two spots of *H.indicus* and one spot of *N sativa*, totally three different spots separated in TLC were taken. In this the first spots of *H.indicus* and *N sativa* showed good activity against *S. aureus* with 15-26 mm of inhibition zone.

(iv) Phytochemical Analysis

Methanolic extract of *H indicus* showed presence of flavonoids, triterpenes, polyphenols and sterols. Aqueous and methanolic extracts of *N.sativa* only comprised of alkaloids (Table 3). The result will help in identification of various compounds through gas chromatography in the further studies.

(v) Minimum inhibitory concentration method

Aqueous extract of *N. sativa* and methanol extract of *H. indicus* was taken for MIC assay. *N.sativa* extract showed minimum inhibitory concentration to *S aureus, E coli, Bacillus sp.* and *P. aeruginosa* at the concentrations of 20µg/ml, 80µg/ml, 20µg/ml and 40µg/ml respectively (Table 4). *A. calamus* extract showed minimum inhibitory concentration to *C. albicans* and *Bacillus sp.* at concentrations of 40 µg/ml and 80 µg/ml respectively (Table 5). The extract of *M fragrans* exhibited MIC to *Bacillus sp* alone at concentration of 20mg/ml (Table 6). *H. indicus* extract showed minimum inhibitory concentration to *S aureus, Bacillus sp, E coli, P. aeruginosa* and *C. albicans* at concentrations of 40µg/ml, 80µg/ml, 20µg/ml 160µg/ml and 80µg/ml respectively (Table 7).

(vi) Synergistic effects of antibiotics with plant extract

The comparative studies of the synergistic effect of antibiotics with plant extracts alone were studied. The effect of association of *N.sativa* extract with streptomycin and gentamicin on *S aureus* and *E coli* were shown in figure 1. *S aureus* showed MIC at concentration of 8µg/ml, when *N sativa* extract combined with streptomycin and Gentamicin, whereas *N.sativa* alone showed MIC only at 20µg/ml. *E coli* showed MIC at 40µg/ml concentration, When *N sativa* extract combined with streptomycin and gentamicin, whereas *N.sativa* extract alone showed MIC only at 80µg/ml. No other plant extracts showed synergistic effects with antibiotics to any other test organisms.

DISCUSSION

In the last few decades, there has been particular interest in the use of abundant naturally occurring antimicrobials (herbs, spices and plants) [17]. *N.sativa, A.calamus, M. fragrans* and *H. indicus* are traditionally used in Indian folklore medicine. So far only a limited data is available regarding its efficacy against pathogenic microorganisms. Hence the present study was therefore designed to evaluate it's antimicrobial activity.

Methanolic extracts, hot and cold water extracts of *N. sativa* showed excellent antibacterial activity against pathogenic microbes[18]. Chloroform extract of *H.indicus* showed promising activity against the clinical isolates of *Helicobacter pylori*[19]. In the present study the methanolic extracts of *N.sativa* and *H.indicus* also showed promising activity than the standard antibiotics and methanol extracts found to be more effective than aqueous extract except for *N.sativa*.

In TLC Petroleum-ether extract of *N.sativa* using benzene: ethyl acetate (6:1), showed five spots. In the chloroform extract, using benzene: ethyl acetate (4:1), five spots and in ethanol extract, using chloroform: methanol (93:7), six spots were observed[13]. Methanol extract of *H.indicus* has showed two clear spots and *N. sativa* showed only one clear spot, when chloroform: methanol (70:30) used as a solvent system.

The assay for bioautography demonstrated that the strong inhibition zones of *H. indicus* and *N.sativa* against the growth of *S aureus*. The clear zones were

located in separate places on the TLC plate, suggesting the potent antimicrobial effect.

In phytochemical analysis, *H indicus* showed presence of flavonoids, triterpenes, polyphenols and sterols. Aqueous and methanolic extracts of *N. sativa* only comprised of alkaloids. the antimicrobial activity of black pepper is due to the presence of essential oil (3%), whose aroma is dominated by monoterpenes hydrocarbons: sabinene, β-pinene and limonene. The mechanism of action of terpene is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds[20].

In MIC assay, *N.sativa* extract showed minimum inhibitory concentration against *S aureus* and *Bacillus sp.* at the concentrations of 20 µg/ml respectively. *H.indicus* extract showed minimum inhibitory concentration against *S aureus* and *E.coli* at the concentrations of 40µg/ml and 20 µg/ml respectively. This antimicrobial activity may be due to the presence of alkaloids in plant extracts.

Synergistic effect was found by *S aureus* and *E coli* in the combination of *N sativa* with streptomycin. and gentamicin. No other plant extracts showed synergistic effects with antibiotics to any other test organisms.

In earlier studies, *N. sativa* seed extract has antimicrobial activity against MRSA[21] and it was found to be active against ESBL producers[22]. *H.indicus* showed good activity against *Propionibacterium acnes* [23]. The antimicrobial activity and phytochemical analysis also carried out for *N. sativa* and *H.indicus* extracts .But the present study focused on purification, bioautography and comparative study of *N.sativa* and *H. indicus* synergestic effects.

The results of present investigation clearly indicate that the antibacterial and antifungal activity vary with the species of the plants and plant material used. Thus, the study ascertains the value of plants used in folk medicine, which could be of considerable interest to the development of new drugs.

CONCLUSION

Based on the results obtained in this study, it may be concluded that out of 4 plants screened, plant extracts of *N. sativa* and *H. indicus* have a stronger and broader spectrum of antimicrobial activity against pathogenic microorganisms and the extracts may be used to discover bioactive natural products that may serve as basic source for the development of new antimicrobial compounds to overcome the problem of increasing resistance to known traditional antibiotics. The further purification through HPLC and spectral analysis will be worthy study to identify the nature of compound present.

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Table – 1: Antimicrobial activity of 4 plants against clinical pathogens

Name of the organism	*Nigella sativa*		*Acorus calamus*		*Myristica fragrans*		*Hemidesmus indicus*	
	Zone of inhibition (mm in diameter)							
	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol
Staphylococcus aureus	15	12	-	-	-	-	-	20
Pseudomonas aeruginosa	-	14	-	-	-	-	-	12
Bacillus spp.	26	19	6	-	-	12	-	14
Escherichia coli	14	-	-	-	-	-	-	13
Candida albicans	-	-	-	15	-	-	-	12

Table – 2: Susceptibility pattern of 4 plants against pathogens

MICROORGANISMS	*Nigella sativa*	*Acrous calamus*	*Myristica fragrans*	*Hemidesmus indicus*
Staphylococcus aureus	+	-	-	+
Pseudomonas aeruginosa	+	-	-	+
Bacillus spp.	+	-	+	+
ESCHERICHIA COLI	+	-	-	+
CANDIDA ALBICANS	-	+	-	+

Note: (+) Susceptibility (inhibition zone ≥12mm
(-) Absence of Susceptibility

Table: 2A Antibacterial Effect of Standard Antibiotics

Test organisms	Ciprofloxacin / Zone of inhibition (mm in dia)	Amoxicillin / Zone of inhibition (mm in dia)	Nystatin/ Zone of inhibition (mm in dia)	Amphoteracin B/ Zone of inhibition (mm in dia)
Staphylococcus aureus	14	10	-	-
Pseudomonas aeruginosa	14	10	-	-
Bacillus spp.	10	9	-	-
ESCHERICHIA COLI	10	8	-	-
CANDIDA ALBICANS	-	-	10	10

Table-3: Phytochemical Analysis of *Nigella sativa* and *Hemidesmus indicus* extracts

Test	*N.Sativa*		*H.indicus*
	Aqueous	**Methanol**	**Methanol**
Alkaloid	+	+	-
Steroid	-	-	-
Terpene	-	-	-
Triterpenes	-	-	+
Flavonoid	-	-	+
Tannin	- -		-
Saponin	- -		-
Sterols	-	-	+
Polyphenols	-	-	+

Table – 4 Minimum inhibitory concentration of test organisms with *Nigella sativa* extracts

S. No.	Organisms	MIC µg/ml
1	**Staphylococcus aureus**	20
2	*Escherichia coli*	80
3	*Bacillus spp.*	20
4	***PSEUDOMONAS AERUGINOSA***	40

Table – 5: Minimum inhibitory concentration of test organisms with *Acorus calamus*

S. No.	Organisms	MIC (µg/ml)
1	*Candida albicans*	40
2	*Bacillus spp.*	80

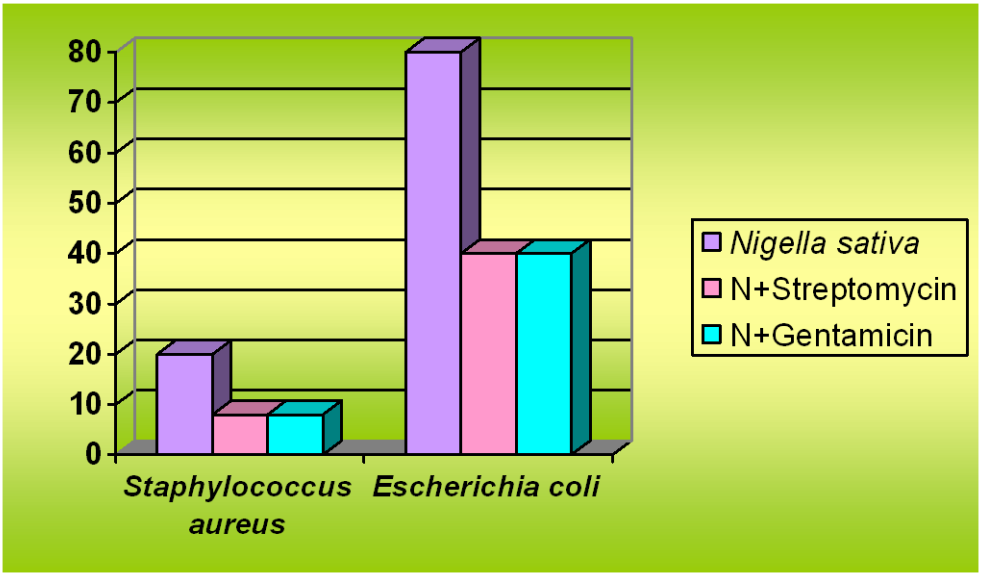
Table – 6: Minimum inhibitory concentration of test organisms with *Myristica fragrans*

S. No.	Organism	MIC µg/ml
1	*Bacillus spp.*	20

Table – 7: Minimum inhibitory concentration of test organisms with *Hemidesmus indicus*

S. No.	Organisms	MIC µg/ml)
1	***Staphylococcus aureus***	40
2	*Bacillus spp.*	80
3	*Escherichia coli*	20
4	*Pseudomonas aeruginosa*	160
5	*Candida albicans*	80

Figure – 1: Evaluation of synergistic effect of *Nigella sativa* with antibiotics against test organism



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