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Research Article

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Evaluation of antimicrobial activity from different plant extracts

Suma K and Nagesh babu R*
Department of Biochemistry
Maharani's Science College for Women, Palace Road, Bangalore, India 560001

Abstract

This work has been undertaken to study the antimicrobial efficiency of Hexane, Ethyl acetate, Ethanolic extracts of herbal plants (Anthocephaluscadamba, Allium sativum, Origanum vulgare, Ocimum sanctum) against human pathogens like Staphylococcus aureus (MTCC-3160), Escherichia coli (MTCC-1652) and fungi Aspergillus niger (MTCC-282) by using agar well diffusion method. All the plants showed significant activity against all pathogens, but the alcoholic extract of Ocimum sanctum showed maximum zone of inhibition and minimum inhibitory concentration against all the pathogens. The minimum zone of inhibition and comparatively greater inhibitory concentration were determined in hexane and ethanolic extract. The Spectrum activity of alcoholic extracts of these plants could be a possible source to obtain new and effective herbal medicines to treat various infectious diseases.

Introduction

The use of plant and its products has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine 1 . Since antiquity, many plants species reported to have pharmacological properties as they are known to possess various secondary metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids and terpenes which are utilized to combat the disease causing pathogens.

With the advancement in Science and Technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs . Antibiotics are indisputably one of the most important therapeutic discoveries of the 20th century that had effectiveness against serious bacterial Infections.

However, only one third of the infectious diseases known have been treated from these synthetic products. This is because of the emergence of resistant pathogens that is beyond doubt the consequence of years of widespread indiscriminate use, continual and misuse of antibiotics.

Antibiotic resistance has increased substantially in the recent years and is posing an ever-increasing therapeutic problem. One of the methods to reduce the resistance to antibiotics is by using antibiotic resistance inhibitors from plants.

Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant pathogens. Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world.

The different herbal plant extracts are traditionally has been used as anticancer antioxidant, antiulcer, analgesic and antidiabetic, and they also having the antiparasitic, antifungal, antibacterial, antimalarial activity, analgesic and anti-inflammatory activity.

Plants produce a diverse range of bioactive molecule, making them rich source of different types of medicines. Most of the drugs today are obtained from natura lsources or semi synthetic derivatives of natural products and used in the traditional systems of

medicine. Thus, it isa logical approach in drug discovery to screen traditional natural products.

Approximately 20% of the plants found in the worldhave been submitted to pharmaceutical or biological test and a sustainable number of new antibiotics introduced on the market are obtained from natural or semi synthetic resources. It has been reported that between the years1983 and 1994 (Cragg et al., 1999), the systematic screening of antibacterial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant bacteria. Several workers throughout the world have carried out antimicrobial studies on some medicinal plants including *Betula pendula* (Mukhtar et al., 2002) and *Ageratum houstonianum*(Bowers et al., 1976). According to World Health Organization (Santos et al., 1995) medicinal plants would be the best source to obtain a variety of drugs.

Current advancements in drug discovery technology and search for novel chemical diversity have intensified the efforts for exploring leads from Ayurveda the traditional system of medicine in India. Ayurvedic system of medicine has its long history of therapeutic potential.

The use of plant extracts and phytochemicals both with known antimicrobial properties is of great significance, inthe past few years a number of investigations have been conducted world-wide to prove antimicrobial activities from medicinal plants (Alonso-Paz et al., 1995;Nascimento et al., 1990). For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. According to World Health Organization (Santos et al., 1995)medicinal plants would be the best source to obtain avariety of drugs. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are a part of the essential oils (Jansen et al., 1987) as well as tannin(Saxena et al., 1994).There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Rojas et al., 2003). Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections (Benkeblia, 2004). Green plants represent a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides (Mahajan and Das, 2003). Biopesticides has been suggested as an effecttive substitute for chemicals (Kapoor, 2001). Reports are available on the use of several plant by-products, which posses, antimicrobialp roperties, on several pathogenic bacteria and fungi(Bylka et al., 2004; Shimpi and Bendre, 2005; Kilani,2006). Here, we evaluate the potential of several plantextracts for antibacterial activity against important human pathogenic and phytopathogenic bacteria.

The below mentioned plants were used to study the antimicrobial activity.

1. Ocimum sanctum (Tulsi) belonging to the family Lamiaceae, leaves wereused for the studies.
2. Anthocephaluscadamba (Kadamba) belonging to the family Rubiaceae,leaves were used for the studies.
3. Allium sativum (Garlic) belonging to the family Liliaceae, Pearls were used the studies.
4. Origanum vulgare (Oregano) belonging to the family Lamiaceae, Leaveswere used for the studies.

After scrutiny of published literature showing its medicinal importance, the present protocol has been outlined regarding the antimicrobial activity on these selected plants using different extracts. It is in view of this, that the present research was set up to evaluate the antibacterial activity of different herbal plant extractions against some pathogenic bacteria and fungi.

Materials and Methods

Chemicals:

The following ingredients were used for the preparation of nutrient agar media and Potato dextrose media: Agar, Peptone, Sodium chloride, Beef extract, Potato, dextrose, water. All other chemicals and analytical reagents were purchased from Hi-media, India, unless stated otherwise. Mature plants of Anthocephaluscadamba (FRLH-53753), Allium sativum (FRLH53754), Origanum vulgare (FRLH-53755) and Ocimum sanctum (FRLH- 53756), were used for this study was collected from Institute of Ayurveda and Integrative Medicine-Bengaluru and authentified by Mrs. S. Noorunnisa Begum.

Culture and Maintenance of microorganisms:

Pure cultures of all experimental bacteria; Staphylococcus aureus (MTCC No.3160), Escherichia coli (MTCC No.1652) and fungi Aspergillus niger (MTCC No.282) were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The pure bacterial cultures were maintained on nutrient agar medium and fungal culture on potato dextrose agar (PDA) medium. Each bacterial and fungal culture was further maintained by

sub culturing regularly on the same medium and stored at 4ºC before use in experiments.

Preparation of plant extract:

In vivo leaves of Anthocephaluscadamba, Allium sativum, Origanum vulgare, Ocimum sanctum were washed for 2-3 times with tap water and finally with distilled water and then allowed to dry at 50º C for overnight and finally milled to a coarse powder (Sieve no 80). 100 gm of powdered material was soxhlet extracted with different solvents like, Hexane, Ethyl acetate, Ethanol 80% and water 20% (12 hours each). All the extracts were evaporated in vacuum under reduced pressure. All extracts were stored in sterile glass bottles at room temperature until screened.

Microbiological screening:

Antimicrobial activities of different extracts were evaluated by the agar well diffusion method modified by Olurinola14, 15 and Minimum inhibitory concentration (MIC).

Media Preparation and Its Sterilization: Antimicrobial susceptibility was tested on solid Agar-agar media (gm/l: beef extract, 3g; peptone, 5g; sodium chloride, 5g; agar, 20g) and for fungus PDA(39 gm/l) was used for developing surface colony growth.

The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values were determined by serial micro dilution assay.

The suspension culture, for bacterial cells growth was done by preparing 2% Lauria Broth (w/v), and for fungus cells growth, 2.4% (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min.

Agar well diffusion method:

Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 24 hr old -broth culture of respective bacteria and fungi.

Wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork-borer. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extracts viz. Hexane, Ethyl acetate, Ethanol (80%) and water (20%). About 100 µl of different concentrations of plant solvent extracts were added sterile syringe into the wells and allowed to diffuse at room temperature for 2hr.

Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 hr for bacterial pathogens and 28°C for 48 hr fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded.

Minimum Inhibitory concentration:

The minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. This was determined from readings on the culture plates after incubation. The most commonly employed methods are the tube dilution method and agar dilution methods. Serial dilutions are made of the products in bacterial and fungal growth media. The test organisms are then added to the dilutions of the products, incubated, and scored for growth. This procedure is a standard assay for antimicrobials.

Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism.

Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents.

Preparation of Inoculum:

Test for antibacterial activity:

The antibacterial assay was carried out by micro dilution method in order to determine the antibacterial activity of compounds tested against the pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 X 107 CFU/ml.

The inoculums were prepared and stored at 4ºC until use. Dilutions of the inoculums were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculums. All experiments were performed in duplicate and repeated three times.

Test for antifungal activity:

In order to investigate the antifungal activity of the extracts, a modified micro dilution technique was used.

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v).

The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0× 10-7 in a final volume of 100 µl per well. The inoculums were stored at 4ºC for further use.

Dilutions of the inoculums were cultured on solid potato dextrose agar to verify the absence of contamination and to check the validity of the inoculums.

Determination of MIC:

The minimum inhibitory concentrations (MIC), MBC and MFCs were performed by a serial dilution technique using 96- well micro titer plates. The different plant extracts viz. Hexane, Ethyl acetate, Ethanol (80%) and water (20%), were taken (1 mg/ml) and serial dilution of the extract with Luria Broth for bacterial culture and for fungus, potato dextrose broth medium with respective inoculum were used.

The microplates were incubated for 72 hours at 28ºC, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

Determination of MBC:

The MBCs were determined by serial sub-cultivation of 2 µl into micro titer plates containing 100 µl of broth per well and further incubation for 72 hours.

The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. All experiments were performed in duplicate and repeated three times.

Determination of MFC:

The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of a 2 µl into microtiter plates containing 100 µl of broth per well and further incubation 72 hours at 28ºC.

The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. All experiments were performed in duplicate and repeated three times.

Observation and Result

In the present investigation, the inhibitory effect of different extracts (viz. Hexane, Ethyl acetate, Ethanol (80%) and water (20%) of in vivo leaves from Anthocephaluscadamba, Allium sativum, Origanum vulgare and Ocimum sanctum were evaluated against both fungicidal and bacterial strains.

The antimicrobial activity was determined using agar well diffusion method and micro dilution method summarized in Table 1-2. The activity was quantitatively assessed on the basis of inhibition zone and their activity index was also calculated along with minimum inhibitory concentration (MIC).

Measurement of antimicrobial activity using Agar well diffusion Method:

The antimicrobial potential of both the experimental plants was evaluated according to their zone of inhibition against various pathogens and the results (zone of inhibition).

The results revealed that all the extracts are potent antimicrobials against all the microorganisms studied. Among the different solvents extracts studied Hexane and Ethyl acetate showed high degree of inhibition followed by Ethanol (80%) and water (20%) extract.

For all the tested microorganisms Hexane and Ethyl acetate showed maximum antibacterial activity in from Anthocephaluscadamba, Allium sativum, Origanum vulgare and Ocimum sanctum.

In Hexane extract, maximum inhibition zone diameter was obtained in S. aureus and in E. coli with diameter (2.36 ± 0.585mm, 2.26 ± 0.493mm, respectively. Similarly, Ethyl acetate extract showed maximum inhibition zone with diameter of 1.2 ± 0.1mm in E. coli and 1.63 ± 0.351 mm in S. aureus. The Ethanolic extract (0.26 ± 0.057 mm) showed restrained and minimum activity, respectively (Table 1; Fig.1 (A-D)).

For the antifungal activity, A. niger (0.93 ± 0.251 mm) showed efficient antifungal activity for hexane plant extract and comparing to the ethyl acetate and ethanolic extracts. Ethyl acetate extract showed maximum inhibition zone with diameter of (0.83 ± 0.416 mm) and ethanolic extract showed lowest inhibition zone (0.36 ± 0.057 mm) against all pathogenic fungal strains, respectively (Table 1; Fig. 2(A-D).

Determination of MIC, MBC and MFC values:

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The MBC and MFC was determined by sub culturing the test dilution (used in MIC) on to a fresh solid medium

and incubated further for 24 h. The concentration of plant extract that completely killed the Bacteria and fungi was taken as MBC and MFC, respectively.

Moreover, it was noted that most of the antimicrobial properties in different plant part extractions shows, MBC value that is almost two fold higher than there corresponding MICs.

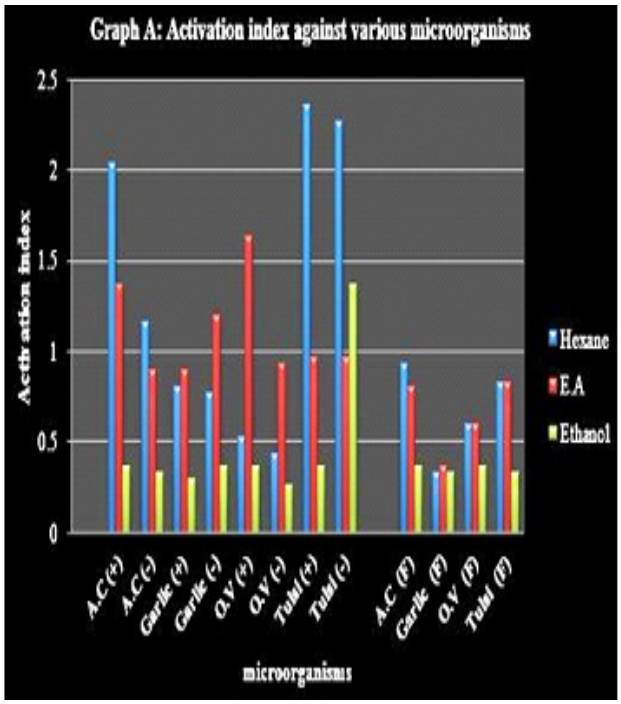
Ethyl acetate extracts of Anthocephaluscadamba and Ocimum sanctum showed least MIC values0.9 µg/ml and 0.9 µg/ml against S. aureus and E. coli while hexane extract 1.1 µg/ml and 2.3 µg/ml against S. aureus and E. coli showed comparatively efficient MIC values of hexane1.5 µg/ml and 2.5 µg/ml and hexane extracts of Origanum vulgare and Ocimum sanctum, showed least MIC values0.5 µg/ml and 0.7 µg/ml against S. aureus and E. coli while ethyl acetate extracts 0.9 µg/ml and 0.9 µg/ml against S. aureus and E. coli showed comparatively efficient MIC value of ethyl acetate are 1.6 µg/ml and 1.2 µg/ml and in ethanol extractshowed least MIC value 0.3 µg/ml comparing to the hexane and ethyl acetate extracts respectively (Table 2). A. niger was proved to have highest activity 1.2 µg/ml and 0.7 µg/ml in hexane and Ethyl acetate extract respectively. The least MBC and MFC value 0.3 µg/ml and 0.4 µg/ml was observed in ethanolic extracts against A. niger respectively (Table 2).

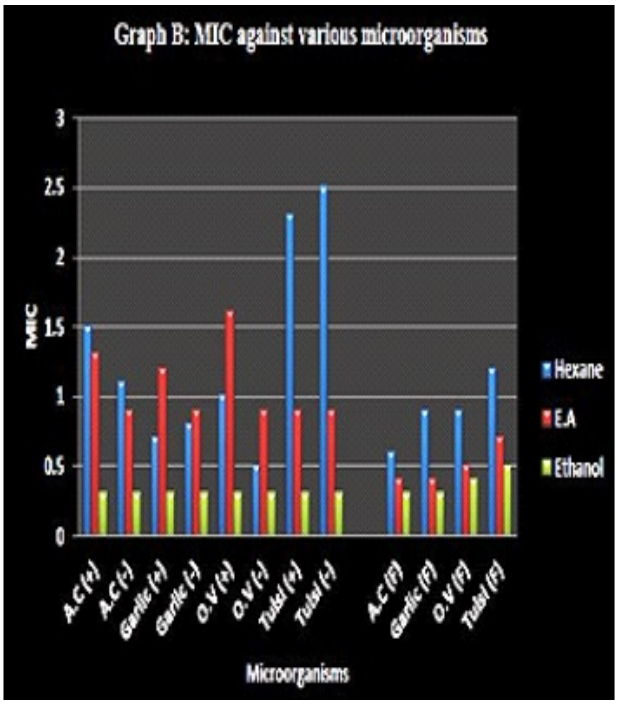
TABLE 1: ANTIMICROBIAL ACTIVITY (ZONE OF INHIBITION, MM) OF VARIOUS PLANT EXTRACTS ANTHOCEPHALUS CADAMBA, ALLIUM SATIVUM, ORIGANUM VULGARE AND OCIMUM SANCTUM AGAINST CLINICAL PATHOGENS.

Sl. No.	Plant extract	Plant extract	Hexane	E.A	Ethanol
Bacteria					
1	A.C	S. aureus	2.03 ± 0.416	1.36 ± 0.152	0.36 ± 0.057
2	A.C	E. coli	1.16 ± 1.069	0.90 ± 0.11	0.33 ± 0.057
3	Garlic	S. aureus	0.8 ± 1.360	0.90 ± 0.173	0.3 ± 0.1
4	Garlic	E. coli	0.76 ± 0.057	1.2 ± 0.1	0.36 ± 0.057
5	O.V	S. aureus	0.53 ± 0.057	1.63 ± 0.351	0.36 ± 0.057
6	O.V	E. coli	0.43 ± 0.057	0.93 ± o.152	0.26 ± 0.057
7	TULSI	S. aureus	2.36 ± 0.585	0.96 ± 0.115	0.36 ± 0.057
8	TULSI	E. coli	2.26 ± 0.493	0.96 ± 0.115	1.36 ± 0.152
Fungi					
9	A.C	A. niger	0.93 ± 0.251	0.8 ± 0.2	0.36 ± 0.057
	Garlic	A. niger	0.33 ± 0.057	0.36 ± 0.057	0.33 ± 0.057
11	O.V	A. niger	0.6 ± 0.264	0.6 ± 0.2	0.33 ± 0.057
12	TULSI	A. niger	0.83 ± 0.351	0.83 ± 0.416	0.36 ± 0.057

TABLE 2: MIC (µg/ml), MBC AND MFC PERFORMANCE OF DIFFERENT EXTRACTS OF ANTHOCEPHALUS CADAMBA, ALLIUM SATIVUM, ORIGANUM VULGARE AND OCIMUM SANCTUM AGAINST PATHOGENIC ORGANISMS.

Sl. No.	Plant extract	Micro. Org	Hexane	E.A	Ethanol
Bacteria					
1	A.C	S. aureus	1.5	1.3	0.3
2	A.C	E. coli	1.1	0.9	0.3
3	Garlic	S. aureus	0.7	1.2	0.3
4	Garlic	E. coli	0.8	0.9	0.3
5	O.V	S. aureus	1	0.6	0.3
6	O.V	E. coli	0.5	0.9	0.3
7	TULSI	S. aureus	2.3	0.9	0.3
8	TULSI	E. coli	2.5	0.9	0.3
Fungi					
9	A.C	A. niger	0.6	0.4	0.3
10	Garlic	A. niger	0.9	0.4	0.3
11	O.V	A. niger	0.9	0.5	0.4
12	TULSI	A. niger	1.2	0.7	0.5





Graph A:Activation index against Various microorganisms.

Graph B:MIC against various microorganisms.

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