



Received on 12 November, 2013; received in revised form, 11 February, 2014; accepted, 25 March, 2014; published 01 April, 2014

## EVALUATION OF ANTIMICROBIAL ACTIVITY OF DIFFERENT HERBAL PLANT EXTRACTS

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### Keywords:

Antimicrobial activity, Human pathogens, Herbal plants, Agar well diffusion method, Minimum inhibitory concentration

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**ABSTRACT:** This work has been undertaken to study the antimicrobial efficiency of Hexane, Ethyl acetate, Ethanolic extracts of herbal plants (*Anthocephalus cadamba*, *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<sup>1</sup>.

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<sup>2, 3, 4</sup>.

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<sup>5</sup>. Antibiotics are indisputably one of the most important therapeutic discoveries of the 20<sup>th</sup> century that had effectiveness against serious bacterial infections.

However, only one third of the infectious diseases known have been treated from these synthetic products<sup>6</sup>. 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<sup>7, 8</sup>.

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<sup>9, 10</sup>.

	<p><b>QUICK RESPONSE CODE</b></p>
	<p><b>DOI:</b> 10.13040/IJPSR.0975-8232.5(4).1460-68</p>
<p>Article can be accessed online on: <a href="http://www.ijpsr.com">www.ijpsr.com</a></p>	
<p>DOI link: <a href="http://dx.doi.org/10.13040/IJPSR.0975-8232.5(4).1460-68">http://dx.doi.org/10.13040/IJPSR.0975-8232.5(4).1460-68</a></p>	

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<sup>11</sup>. 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<sup>12</sup>, and they also having the antiparasitic, antifungal, antibacterial, antimalarial activity, analgesic and anti-inflammatory activity<sup>13</sup>.

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

- *Ocimum sanctum* (Tulsi) belonging to the family *Lamiaceae*, leaves were used for the studies.
- *Anthocephalus cadamba* (Kadamba) belonging to the family *Rubiaceae*, leaves were used for the studies.
- *Allium sativum* (Garlic) belonging to the family *Liliaceae*, Pearls were used for the studies.
- *Origanum vulgare* (Oregano) belonging to the family *Lamiaceae*, Leaves were 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 plant 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.

## MATERIAL 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 *Anthocephalus cadamba* (FRLH-53753), *Allium sativum* (FRLH-53754), *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 authenticated 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 4°C before use in experiments.

**Preparation of plant extract:** In vivo leaves of *Anthocephalus cadamba*, *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 50° 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 Olurinola<sup>14</sup>,<sup>15</sup> and Minimum inhibitory concentration (MIC)<sup>16</sup>.

### 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  $1.0 \times 10^7$  CFU/ml. The inoculums were prepared and stored at 4°C 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  $1.0 \times 10^7$  in a final volume of 100 µl per well. The inoculums were stored at 4°C 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 28°C, 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  $\mu$ l into micro titer plates containing 100  $\mu$ l 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  $\mu$ l into microtiter plates containing 100  $\mu$ l of broth per well and further incubation 72 hours at 28°C. 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 *Anthocephalus cadamba*, *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 *Anthocephalus cadamba*, *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 ( $2.36 \pm 0.585$ mm,  $2.26 \pm 0.493$ mm, respectively). Similarly, Ethyl acetate extract showed maximum inhibition zone with diameter of  $1.2 \pm 0.1$ mm in *E. coli* and  $1.63 \pm 0.351$  mm in *S. aureus*. The Ethanolic extract ( $0.26 \pm 0.057$  mm) showed restrained and minimum activity, respectively (**Table 1; Fig.1 (A-D)**).

For the antifungal activity, *A. niger* ( $0.93 \pm 0.251$  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 ( $0.83 \pm 0.416$  mm) and ethanolic extract showed lowest inhibition zone ( $0.36 \pm 0.057$  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<sup>17</sup>.

Ethyl acetate extracts of *Anthocephalus cadamba* and *Ocimum sanctum* showed least MIC values 0.9  $\mu$ g/ml and 0.9  $\mu$ g/ml against *S. aureus* and *E. coli* while hexane extract 1.1  $\mu$ g/ml and 2.3  $\mu$ g/ml against *S. aureus* and *E. coli* showed comparatively efficient MIC values of hexane 1.5  $\mu$ g/ml and 2.5  $\mu$ g/ml and hexane extracts of *Origanum vulgare* and *Ocimum sanctum*, showed least MIC values 0.5  $\mu$ g/ml and 0.7  $\mu$ g/ml against *S. aureus* and *E. coli* while ethyl acetate extracts 0.9  $\mu$ g/ml and 0.9  $\mu$ g/ml against *S. aureus* and *E. coli* showed comparatively efficient MIC value of ethyl acetate are 1.6  $\mu$ g/ml and 1.2  $\mu$ g/ml and in ethanol extract



showed 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

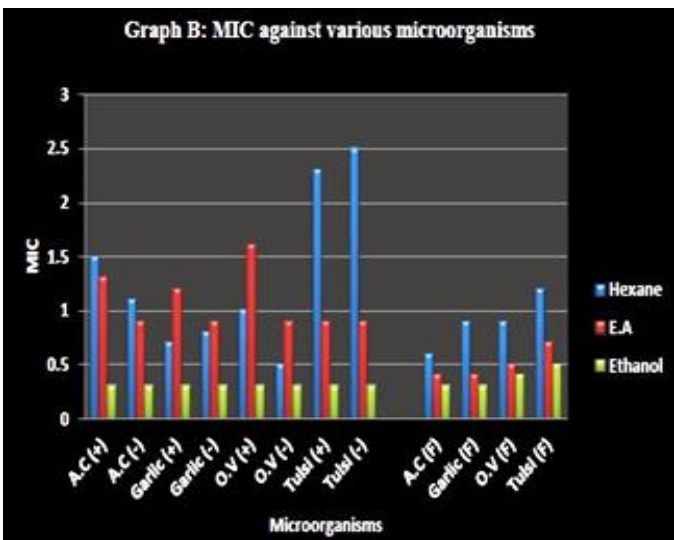
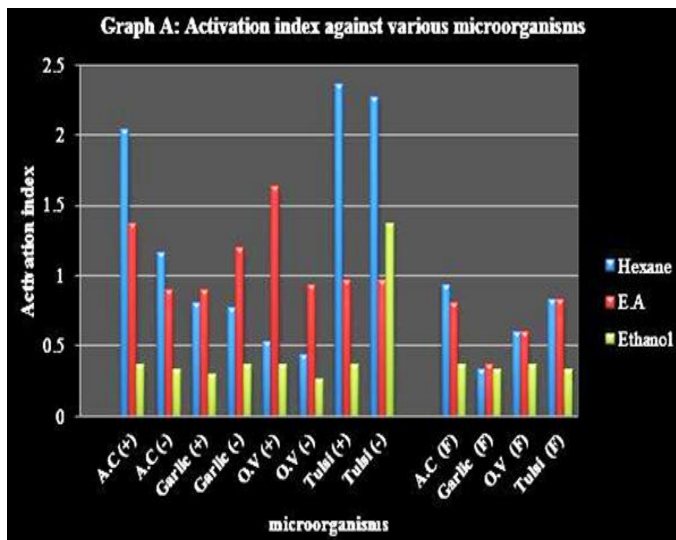
Ethyl acetate extract respectively. The least MBC and MFC value 0.3 µg/ml and 0.4 µg/ml was observed in ethanolic extracts against *A. niger* respectively (Table 2).

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

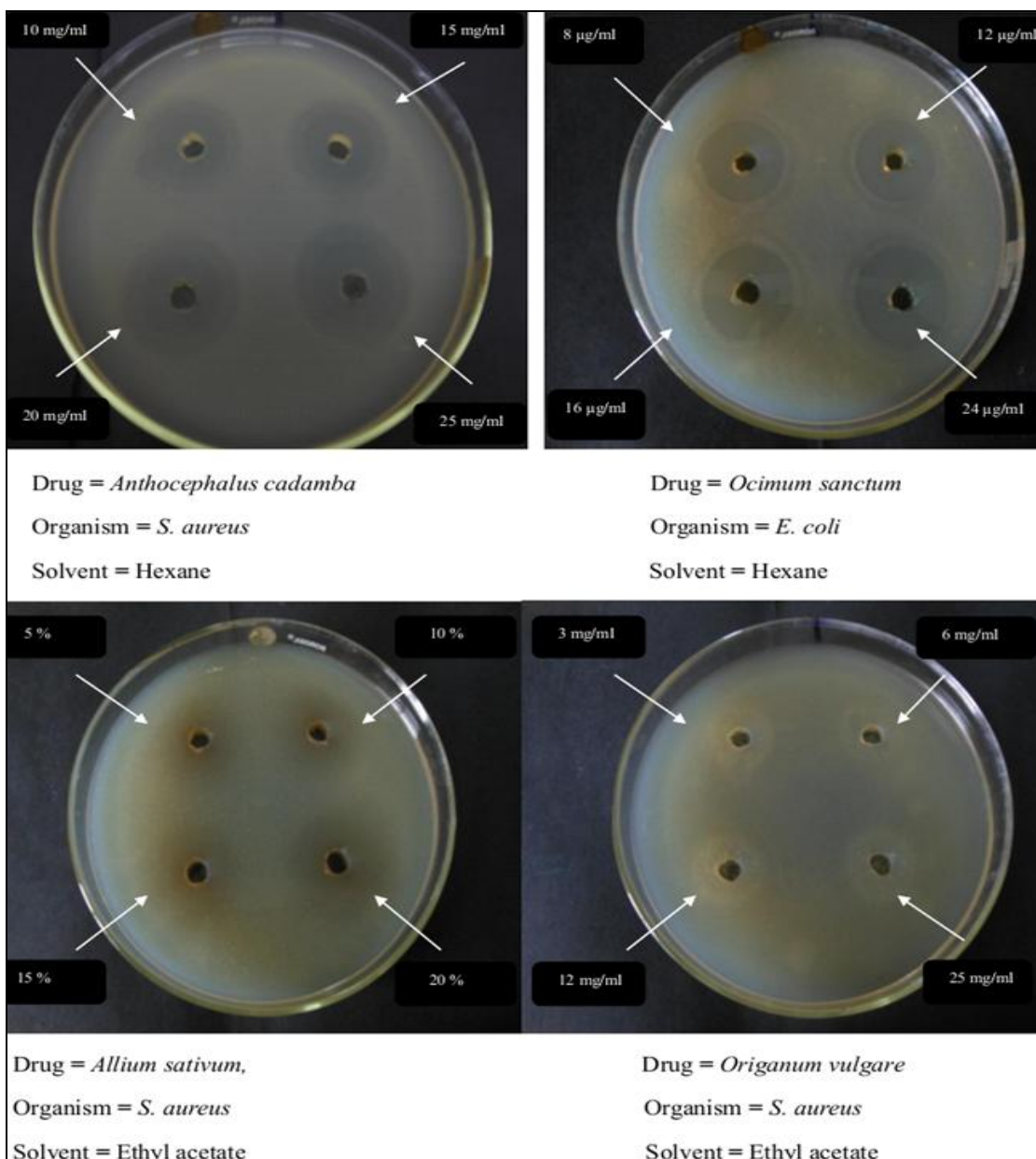
S. No.	Plant extract	Micro. Org	Hexane	E.A	Ethanol
<b>Bacteria</b>					
1	A.C	<i>S. aureus</i>	2.03 ± 0.416	1.36 ± 0.152	0.36 ± 0.057
2	A.C	<i>E. coli</i>	1.16 ± 1.069	0.90 ± 0.11	0.33 ± 0.057
3	Garlic	<i>S. aureus</i>	0.8 ± 1.360	0.90 ± 0.173	0.3 ± 0.1
4	Garlic	<i>E. coli</i>	0.76 ± 0.057	1.2 ± 0.1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

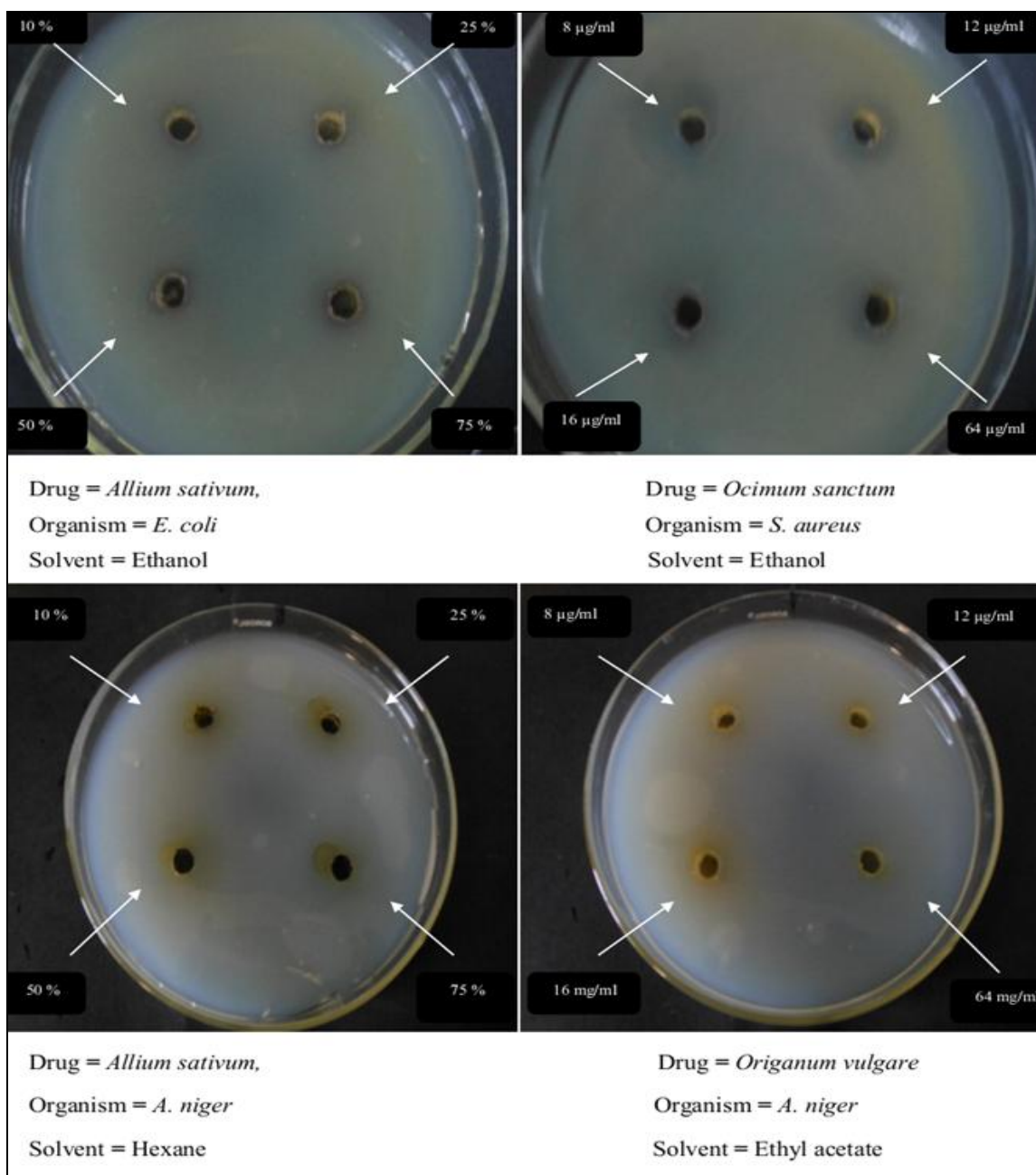
**TABLE 2: MIC (µg/ml), MBC AND MFC PERFORMANCE OF DIFFERENT EXTRACTS OF ANTHOCEPHALUS CADAMBA, ALLIUM SATIVUM, ORIGANUM VULGARE AND OCIMUM SANCTUM AGAINST PATHOGENIC ORGANISMS**

S. No.	Plant extract	Micro. Org	Hexane	E.A	Ethanol
<b>Bacteria</b>					
1	A.C	<i>S. aureus</i>	1.5	1.3	0.3
2	A.C	<i>E. coli</i>	1.1	0.9	0.3
3	Garlic	<i>S. aureus</i>	0.7	1.2	0.3
4	Garlic	<i>E. coli</i>	0.8	0.9	0.3
5	O.V	<i>S. aureus</i>	1	1.6	0.3
6	O.V	<i>E. coli</i>	0.5	0.9	0.3
7	TULSI	<i>S. aureus</i>	2.3	0.9	0.3
8	TULSI	<i>E. coli</i>	2.5	0.9	0.3
<b>Fungi</b>					
9	A.C	<i>A. niger</i>	0.6	0.4	0.3
10	Garlic	<i>A. niger</i>	0.9	0.4	0.3
11	O.V	<i>A. niger</i>	0.9	0.5	0.4
12	TULSI	<i>A. niger</i>	1.2	0.7	0.5



A.C = *Anthocephalus cadamba*, Garlic = *Allium sativum*, O.V = *Origanum vulgare*, Tulsi = *Ocimum sanctum*, (+) = *Staphylococcus aureus*, (-) = *Escherichia coli* and F = Fungi (*Aspergillus niger*), MIC = Minimum Inhibitory Concentration, E.A = Ethyl acetate





**DISCUSSION:** The search for antimicrobials from natural sources has received much attention and efforts have been put in to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones.

Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism<sup>18, 19</sup>. These compounds have significant therapeutic application against human pathogens including bacteria, fungi or virus. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds<sup>20, 21</sup>.

Therefore, medicinal plants are finding their way into pharmaceuticals, nutraceuticals and food Supplements.

In the present investigation, different solvent extracts of *Anthocephalus cadamba*, *Allium sativum*, *Origanum vulgare* and *Ocimum sanctum* was evaluated for exploration of their antimicrobial activity against certain Gram negative and Gram positive bacteria, fungus which was regarded as human pathogenic microorganism.

Susceptibility of each plant extract was tested by serial microdilution method (MIC) and agar well diffusion method was determined.

Our preliminary investigation showed that all Hexane, Ethyl acetate and Ethanolic extracts of *Anthocephalus cadamba*, *Allium sativum*, *Origanum vulgare* and *Ocimum sanctum* were active against the locally isolated human pathogens like *Escherichia coli* and *Staphylococcus aureus*. This analysis of using several extracts so as to study the efficacy of plant for antimicrobial activity has also been realized by many scientists in many plant species like *Adhatoda zeylanica*, *Medic*<sup>22</sup>, *Trianthema decandra* L.<sup>23</sup>, *Argemone mexicana* L.<sup>24</sup>, *Tinospora cordifolia* and *Cassia fistula*<sup>25</sup>.

The alcoholic extracts of herbal plant showed significant antimicrobial activity against multi-drug resistant clinically isolated microorganisms (Graph 1(A-B)). Though, the mechanism of the action of these plant constituents is not yet fully known it is clear that the effectiveness of the extracts largely depends on the type of solvent used. This observation clearly indicates that the existence of non-polar residues in the extracts which have higher both bactericidal and bacteriostatic abilities.

Cowan<sup>26</sup> mentioned that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily solubilized in organic solvents. Similar results showing that the alcoholic extract having the best antimicrobial activity is also reported by Preethi<sup>27</sup> in *Leucas aspera*, *Holarrhena antidysenterica*.

Suree<sup>28</sup> also studied the antibacterial activity of crude ethanolic extracts and essential oils of spices against salmonellae and other enterobacteria and observed that this difference in the activity between different plant extracts is due to the difference between extract compounds.

The study also revealed that ethanolic extract shows minimum antimicrobial activity. However, Ali Mirzaei<sup>29</sup> showed that ethanolic extract of plant *Origanum Vulgare* shows significant antimicrobial activity.

Furthermore, Chloroform and acetone extract from the leaves of *Anthocephalous cadamba* had been reported to have prominent antimicrobial activity against several gram positive and gram negative human pathogenic bacteria<sup>30</sup>.

The antimicrobial analysis using the agar well diffusion method and MIC value is been used by many researchers<sup>31, 32, 33</sup>.

In the present study, the MIC value of the active plant extracts obtained in this study were lower than the MBC values (Table 1-2, Graph 1 (A-B)) suggesting that the plant extracts were bacteriostatic at lower concentration but bactericidal at higher concentration<sup>34</sup>.

In conclusion, of the present investigation of these herbal plants contain potential antimicrobial components that may be of great use for the development of pharmaceutical industries as a therapy against various diseases. The hexane, ethyl acetate, ethanolic extracts of *Anthocephalus cadamba*, *Allium sativum*, *Origanum vulgare* and *Ocimum sanctum* possess significant inhibitory effect against tested pathogens.

**ACKNOWLEDGEMENT:** The authors would like to thank the chairman Acharya Institutes Sri B. Premnath Reddy for providing laboratory facilities and supporting this research work.

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**How to cite this article:**

Rajesh T, Venkatanagaraju E, Goli D and Basha SJ: Evaluation of antimicrobial activity of different herbal plant extracts. *Int J Pharm Sci Res* 2014; 5(4): 1460-68. doi: 10.13040/IJPSR.0975-8232.5(4).1460-68

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