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Research Article

Screening of various leaf extracts of *Chromolaena odorata* L. for biochemical constituents and antimicrobial sensitivity

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ABSTRACT

Background: Ethanolic, aqueous, petroleum ether and ethyl acetate extracts of leaves of *Chromolaena odorata* were studied for their antimicrobial activity. Twelve bacterial species, including six Gram-positive and six Gram-negative bacteria viz. *Vibrio cholerae*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Salmonella paratyphi A*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Staphylococcus citreus*, *methicillin-resistant S. aureus*, nonpathogenic *Mycobacterium gordonae* and *Mycobacterium fortuitum* along with fungi *Candida albicans* and *Aspergillus niger* were studied.
Methods: This study involved antibiotic sensitivity testing of extracts against a wide spectrum of micro-organisms, to determine the antimicrobial activity of the plant.
Results: Initial phytochemical testing unveiled the chemical constituents of *C. odorata* as saponins, flavonoids, tannins, steroids and proteins. The zone of inhibition for the respective extracts was compared with the zone of the standard antibiotic and was found to be quite effective.
Conclusions: The ethanolic extract of *C. odorata* exhibited significant antimicrobial activity. The plant extracts could be used to treat resistant form of prevailing infections. Successful antimicrobial drugs can be developed out of these extracts if specific compounds are isolated and purified.

Keywords: Antibacterial, Antifungal, *Chromolaena odorata*, *Eupatorium odoratum*

INTRODUCTION

Antimicrobials are one of the most widely used and misused among all drugs. As a result, of which there is an increase in antibiotic-resistant pathogens, rendering an increased need for newer drugs. Development of antimicrobials has slowed dramatically each year.[1] Although antibiotic-resistance is well-known due to its misuse, overprescribing continues, mainly because of patient demand, need for quick relief from diseases, time constraint on clinicians and diagnostic dubiousness.[2] Selective use of antimicrobials agents is the need of the hour.

Natural products are receiving more attention nowadays as drug sources because they have been effective in the past. Remedies that are not yet discovered may be present in plants; as yet not discovered and that may never be discovered or described.[3] Herbal medicines play an important role in the healthcare system worldwide.[4] Observations that natural substances controlled the symptoms of certain diseases: quinine, obtained from the bark of the cinchona tree to treat malaria and emetine, an alkaloid obtained from ipecacuanha root for amoebic dysentery.[5] These compounds were introduced in the seventeenth century, and both have survived into present day use. Otherwise, the only useful antibacterial compounds were topical antiseptics that were far too toxic for systemic use. More than 60 years of use have prompted a more sober assessment of the limitations of antimicrobial therapy. Microbes have shown amazing

versatility in avoiding, withstanding or repelling the antibiotic onslaught while parallel medical advances have provided a large and increasing group of vulnerable patients for them to attack.[6,7] Antimicrobial agents are essential tools of modern medicine, but the battle against infection is far from won.[8] The challenge now is to preserve the remarkable achievements of the twentieth century by learning to use these powerful drugs more judiciously.

Infections emerging from drug resistant species usually fail to respond to standard therapy resulting in an increase in the time period for recovery. Irrational prescribing along with inappropriate use of antimicrobials makes way for resistant organisms to grow and multiply. Ciprofloxacin is currently recommended by WHO for Shigella induced bloody diarrhea since a good deal of resistance has emerged to previously used antibiotics.[9] However increasing resistance to ciprofloxacin is reducing the treatment options of shigellosis mainly for children. Newer broad spectrum antimicrobials are needed. Newer resistance mechanisms can render strong antibiotics ineffective those of which are usually the last line of defense against drug resistant organisms. The rise in antimicrobial resistance (AMR) is of global concern, and quick response is needed to fight the emergence of AMR.[10]

Methicillin resistant *S. aureus* (MRSA) are endemic in hospitals[11] along with multidrug resistant strains and high level of resistance to vancomycin has been reported.[12] There are now resistant strains of pseudomonas, enterococci and enterobacter that are resistant to standard antibiotics. Of particular concern now and one of public health importance is the emergence of MRSA. These are resistant to methicillin, cephalosporins, imipenem, nafcillin, oxacillin and or other beta-lactam antibiotics.[13] There is a need to discover newer antimicrobial drugs. The wide spread resistance alarms an increase in the need for new and re-emerging traditional medicine and necessity to investigate medicinal plants.

This study involved antibiotic sensitivity testing of extracts against a wide spectrum of micro-organisms, to determine the antimicrobial activity. The zone of inhibition for the respective extracts was compared to the zone of the standard antibiotic which is done by agar well diffusion technique. *Chromolaena odorata* (*Eupatorium odoratum*) belongs to the family *Asteraceae* (sunflower family). The plant material of interest in this study was the freshly dried leaves of *C. odorata* Linn.[14] The parts of the plant which have been used in ethnic medicine include leaves and flowers. No formulations containing *C. odorata* are available.

METHODS

The leaves of *C.odorata* post collection were identified by Dr. Janarthanam a Botanist from Goa University. The collected leaves (Figure 1) were washed thoroughly under running water. They were chopped into small pieces and dried in (Figure 2) the shade for 17 days. The pieces were then the ground into a coarse powder with the help of an electric blender. The coarse powder of (Figure 3) the leaves was soaked in the respective solvents and subjected to maceration every 4 days with occasional shaking. After 4 days, the solvent layer was decanted off. The process was repeated 4 times with fresh solvent. A rotary evaporator was used to evaporate the solvent from total extract to give the dry extract. The extract was then stored in the refrigerator at 6-8°C until used (Figures 4-7). Solvents and chemicals of analytical grade were used. Biochemical reagents were freshly prepared.

Microorganisms were supplied by the Microbiology Department of Goa Medical College. Bacteria used included: *S. aureus ATCC 25923*, *Escherichia coli ATCC 25922*, *Pseudomonas aeruginosa ATCC 27853*, *Salmonella paratyphi A*, *Klebsiella pneumoniae*, *Shigella sonnei*, *Vibrio cholera (ogawa)*, *Enterococcus faecalis*, *Staphylococcus citreus*, MRSA, nonpathogenic *Mycobacterium gordonae* and *Mycobacterium fortuitum*. Fungi used in the study were: *Aspergillus niger* and *Candida albicans*.

Sub culturing

The slants of bacterial cultures were sub cultured every 2 weeks by transferring loop full of cultures from the original slants into the prepared and sterilized slants using sterilized loop. One extra set of cultures was always maintained as stock. The *Mycobacterium* strains were sub cultured every 2 weeks by transferring them into freshly prepared sterilized broth using sterilized pipettes. One extra set of cultures was always maintained as a stock.

Maintenance of cultures

The slants of the bacterial cultures on nutrient agar were maintained separately in the refrigerator at 2-8°C. The *Mycobacterium* cultures were maintained in LJ medium in the incubator at 37°C throughout the experiment. All the apparatus used in the experiment were autoclaved at 121°C at 15 lbs pressure for 15 mins.

Preparation of stock solutions

Dimethyl sulfoxide (DMSO) was used as a solvent. It is neutral and is used as a universal solvent in most of the antibacterial sensitivity procedures to dissolve compounds. 5 mg of the dried extract was weighed and added to a mortar. 1 ml of DMSO was added drop by drop and the mixture was triturated gently till a uniform solution was obtained. This solution was then purged through a syringe to obtain a lump free solution. It was then subjected to a sonicator for 30 mins where thorough mixing of the extract was undertaken. The stock solutions were then used to prepare dilutions and stored at 6-8°C until used.

Figure 1: Fresh leaves of *Chromolaena odorata*.

Figure 2: Dried leaves of *Chromolaena odorata.*

Figure 3: Powdered leaves of *Chromolaena odorata.*

Preparation of nutrient agar plates

A total of 28 g of nutrient agar was weighed on the precision balance and was dissolved in 1000 ml of distilled water and heated to boiling. Plates were prepared by pouring 20 ml molten nutrient agar in each Petri dish. This was then autoclaved at 121°C at 15 lbs pressure for 15 mins. The plates were wrapped and stored at 4°C until used within 15 days.

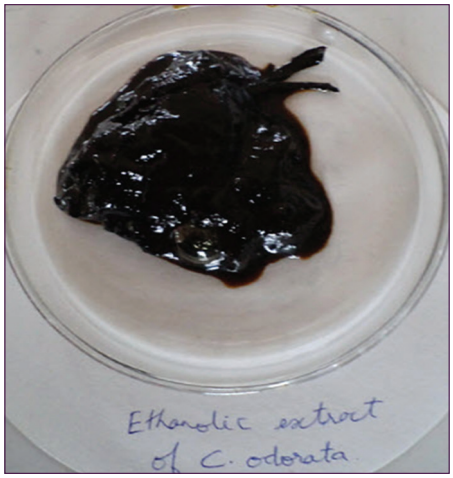

Figure 4: Ethanolic extract of leaves.

Figure 5: Ethyl acetate extract.

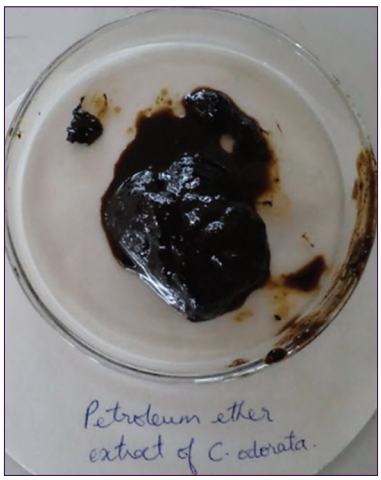

Figure 6: Petroleum ether extract.

Preparation of culture suspension

A volume of 5 ml of Mueller Hinton Broth was dispersed in previously sterilized tubes and a loop full of each organism was transferred in the tubes separately using Nichrome loop. These tubes were then incubated for 24 hrs in an incubator at 37°C.

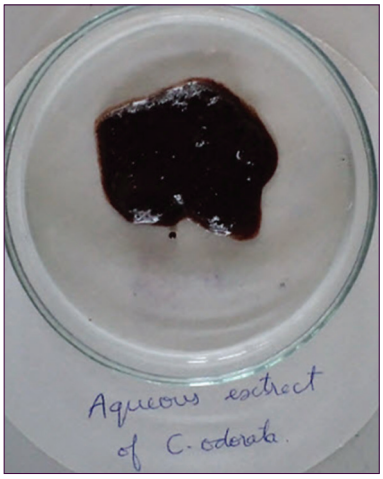

Figure 7: Aqueous extract.

Preparation of the broth

LJ medium for *Mycobacterium* - 37.24 g of the powder in 600 ml distilled water containing 12 ml glycerol and mixed thoroughly. To this was added 1000 ml sterile whole egg emulsion and mixed gently to obtain a uniform mixture. This was then autoclaved at 121°C for 15 mins. Medium was then dispersed in stopper tubes.

Mueller Hinton Broth for bacteria - 21 g of the Mueller Hinton powder was suspended in 1 L of purified water mixed thoroughly and heated with frequent agitation to dissolve powder. The media was autoclaved at 121°C for 15 mins. Care was taken not to overheat the broth. Immediately after autoclaving the prepared medium was cooled to 40°C and the pH of 7.1 was ensured.

Preparation of inoculum

For *Mycobacterium* - 2 sets of 80 ml of LJ medium were prepared and 2 ml from each set was pipetted out and stored to serve as the blank. *M. gordonae*: 780 mg of the freeze dried *M. gordonae* was weighed and added to the first set of 78 ml of sterilized LJ medium. *M. fortuitum*: 780 mg of the freeze dried *M. fortuitum* was weighed and added to the 2nd set of 78 ml of sterilized LJ medium.

For rest of the bacterium: Streaking of the culture was done 24 hrs prior to the experimentation on freshly prepared slants to obtain young cultures. A loopful of culture was transferred from the slant into 5 ml sterile Mueller Hinton Broth aseptically. The tube was then shaken well on a cyclonic mixer for uniform distribution of the culture and was then incubated for 24 hrs at 37°C. This was used as the culture suspension.

Identification tests for Bacteria were carried out which is tabulated in Table 1, along with the microscopic characters of bacteria in Table 2.

Table 1: Identification tests for bacteria (The tests were performed to confirm identity of culture and the results are indicated as follows).

Test	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. Paratyphi A*	*V. cholerae*	*S. sonnei*	*S. aureus*	*S. citreus*	*E. faecalis*	MRSA
Gram test	–	–	–	–	–	–	+	+	+	+
Motility test	+	+		+	+	–	–	–	–	–
Sugar fermentation test			–							
Glucose production	+	+	+	+	–	–	+	+	+	–
Lactose production	+	–	+	–	–	–	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	–	+
Oxidase test	–	+	–	–	+	–	–	–	+	–
In dole test	+	–	–	–	–	–	–	–	–	–
Methyl red test	+	–	–	+	–	+	+	+	–	–
BSA test	–	–	–	+	–	–	–	–	–	–
McConkeys test	+	–	–	–	–	–	–	–	–	–

M. fortuitum: Gram-positive, nonmotile and acid-fast rods, *M. gordonae*: Gram-positive, nonmotile and moderate to long acid-fast rods. *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, K. pneumonia: Klebsiella pneumonia, S. paratyphi: Salmonella paratyphi, V. cholera: Vibrio cholera, S. sonnei: Shigella sonnei, S. aureus: Staphylococcus aureus, S. citreus: Staphylococcus citreus, E. faecalis: Enterococcus faecalis*, MRSA: Methicillin-resistant *Staphylococcus aureus, M. gordonae: Mycobacterium gordonae, M. fortuitum: Mycobacterium fortuitum*

Table 2: The microscopic characters of bacteria.

Culture	Gram staining	Morphology
E. coli	Gram-negative	Rod-shaped bacterium
P. aeruginosa	Gram-negative	Motile, non-sporing rods
K. pneumonia	Gram-negative	Non-motile, rods
S. paratyphi A	Gram-negative	Motile, aerobic rods
V. cholera	Gram-negative	Anaerobic nonspore forming rods
S. sonnei	Gram-negative	Nonspore forming, non-motile, rod-shaped bacteria
S. aureus	Gram-positive	Non motile, anaerobic cocci
M. gordonae	Gram-positive	Nonmotile and moderate to long acid-fast rods
M. fortuitum	Gram-positive	Nonmotile and acid-fast rods
S. citreus	Gram-positive	Non motile, anaerobic cocci
E. faecalis	Gram-positive	Anaerobic, nonmotile cocci
MRSA	Gram-positive	Anaerobic cocci

E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, K. pneumonia: Klebsiella pneumonia, S. paratyphi: Salmonella paratyphi, V. cholera: Vibrio cholera, S. sonnei: Shigella sonnei, S. aureus: Staphylococcus aureus, S. citreus: Staphylococcus citreus, E. faecalis: Enterococcus faecalis, MRSA: Methicillin-resistant *Staphylococcus aureus*, *M. gordonae: Mycobacterium gordonae, M. fortuitum: Mycobacterium fortuitum*

Reading and interpretation

The results for bacteria were read after 48 hrs of incubation at 37°C. The results for the *M. fortuitum* and *M. gordonae* were read after 3 days of incubation at 37°C.

RESULTS

Preliminary phytochemical screening[15] of the ethanolic, aqueous, Pet. ether and ethyl acetate extract of leaves of *C. odorata* L. were carried out, the results of which are tabulated in Table 3.

The ethanolic, ethyl acetate and petroleum ether extract of *C. odorata* leaves showed antimicrobial activity against all the microorganisms. The aqueous extract revealed variable degree of antibacterial activity (Table 4). DMSO (control) showed no inhibition. The standard ciprofloxacin, terbinafine and pyrazinamide exerted their effects some of which were better than the extracts.

The ethanolic extract proved effective than the standard in case of *E. coli*, *K. pneumoniae*, *V. cholerae*, *S. aureus* and *S. citreus*. The ethyl acetate extracts proved effective than the standard in case of *M. gordonae*, *S. citreus*, *E. faecalis* and *K. pneumoniae*. The petroleum ether extracts proved effective than the standard in case of *M. gordonae*, *M. fortuitum* and *S. citreus*.

Antifungal activity of the plant extracts

The petroleum ether extract of leaf revealed promising antifungal activity against *C. albicans* (Figure 8). The negative control exerted no effect against the microorganisms which proves the effectiveness of the extracts. Zone of inhibition was studied and the organism was found to be resistant, intermediate or susceptible based on the zones obtained, results of which are tabulated in Table 4. Standard for antibacterial used was ciprofloxacin 100 µg/ml and drug extract was 100 µg/ml and for antifungal was terbinafine 30 µg/ml and drug extract was 30 µg/ml and for mycobacterium was pyrazinamide 100 µg/ml and drug extract was 100 µg/ml.

Based on Kirby Bauer disk diffusion organisms are considered resistant ≤15 mm, intermediate 16-20 mm, sensitive ≥21 mm.

The ethanolic, petroleum ether and ethyl acetate extract of *C. odorata* leaves showed antimicrobial activity against all the microorganisms (Figures 9-12). The aqueous extract revealed variable degree of antibacterial activity (Table 4). DMSO (control) showed no inhibition. The standard

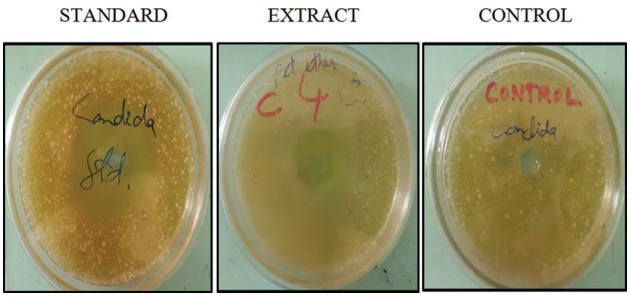

Figure 8: Zone of inhibition of *Candida albicans*.

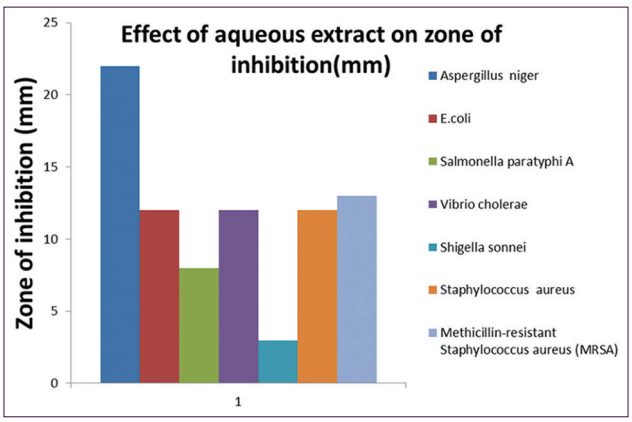

Figure 9: Effect of aqueous extract on zone of inhibition.

Table 3: Preliminary phytochemical screening of the ethanolic, aqueous, Pet. ether and ethyl acetate extract of leaves of *C. odorata L.*

Biochemical tests	Ethanolic	Aqueous	Pet. ether	Ethyl acetate
Tests for carbohydrates				
Molisch test (general test) Test soln+α naphthol+conc H_2SO_4→Violet ring	+	+	+	+
Test for reducing sugars Fehlings test→Yellow to brick Red ppt	+	+	+	+
Benedicts test→Green/Yellow/Red ppt	+	+	+	+
Test for monosaccharide's Barfoed's test→Red ppt	–	–	–	–
Test for hexose sugar Cobalt chloride test→greenish Blue color (glucose) Purple color (fructose)	+	+	–	–
Test for non-reducing polysaccharides (starch): Iodine test→Blue color	–	–	–	–
Tannic acid test for Starch→ppt	–	–	–	–
Tests for proteins				
Biuret test (general)→Violet\Pink color	+	+	+	+
Millon' s test (for proteins)→Red color	+	+	+	+
Xanthoprotein test (for tyrosine/tryptophan)→White ppt	+	+	+	+
Tests for amino acids				
Ninhydrin test	+	+	+	+
Test for tyrosine	+	+	+	+
Test for tryptophan	+	+	+	+
Test for cystein	+	+	+	+
Tests for steroids and triterpenoids				
1.Salkowski's reaction →chloroform layer – Red (steroids present)	+	+	+	+
→acid layer – Green Yellow (triterpenoids)	+	+	+	+
2.Liebermann burchard reaction Brown ring→steroids	+	+	+	+
3.Liebermann reaction →Blue colored solution	–	–	–	–
Tests for glycosides				
Tests for cardiac glycosides Baljet test	+	+	+	+
Legal test	+	+	+	+
Keller killiani test	+	+	+	+
Liebermann test for bufadienolides	–	–	–	–
Test for saponin glycosides Foam test	+	+	+	+
Test for anthraquinone glycosides Borntragers test	–	–	–	–
Modified borntragers test	–	–	–	–
Biochemical tests for flavonoids				
Shinoda test→Pink to Magenta Red color	+	+	+	+
Alkaline reagent test→Intense Yellow color	+	+	+	+
Zinc HCl test→Red color after few minutes	+	+	+	+
Tests for alkaloids				
Dragendorff's test	+	+	+	+

Contd...

Table 3: Contd...

Biochemical tests	Ethanolic	Aqueous	Pet. ether	Ethyl acetate
Mayer's test	+	+	+	+
Wagner's test	+	−	+	+
Hager's test	+	+	+	+
Tests for tannins (phenolic compounds)				
Test solution+5% $FeCl_3$→deep bluish Black color	+	+	+	+
Test solution+lead acetate→White ppt	+	+	+	+
Test solution+iodine→transient Red color	+	+	+	+
Test solution+bromine water→discoloration of bromine water	+	+	+	+
Test solution+$KMnO_4$→discoloration of $KMnO_4$	+	+	+	+
Test solution+dil HNO_3→Reddish Yellow color	+	+	+	+

C. odorata: Chromolaena odorata

Table 4: Zone of inhibition of drug extracts on microorganisms.

Organism	Ethanolic	Zone of inhibition		Diameter (mm)		
		Aqueous	Ethyl acetate	Pet. ether	Standard	Control
C. albicans	6	No inhibition	10	12	25	No inhibition
A. niger	28	17	18	22	34	No inhibition
E. coli	19	14	14	12	15	No inhibition
P. aeruginosa	15	No inhibition	12	11	16	No inhibition
K. pneumoniae	13	No inhibition	18	7	11	No inhibition
S. paratyphi A	10	7	8	8	19	No inhibition
V. cholerae	22	13	12	12	15	No inhibition
S. sonnei	16	10	10	3	17	No inhibition
S. aureus	20	11	12	12	14	No inhibition
S. citreus	14	No inhibition	13	9	7	No inhibition
E. faecalis	12	No inhibition	18	10	15	No inhibition
MRSA	14	9	8	13	32	No inhibition
M. gordonae	13	No inhibition	21	23	15	No inhibition
M. fortuitum	12	No inhibition	16	25	18	No inhibition

C. albicans: Candida albicans, A. niger: Aspergillus niger, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, K. pneumonia: Klebsiella pneumonia, S. paratyphi: Salmonella paratyphi, V. cholera: Vibrio cholera, S. sonnei: Shigella sonnei, S. aureus: Staphylococcus aureus, S. citreus: Staphylococcus citreus, E. faecalis: Enterococcus faecalis, MRSA: Methicillin-resistant *Staphylococcus aureus, M. gordonae: Mycobacterium gordonae, M. fortuitum: Mycobacterium fortuitum*

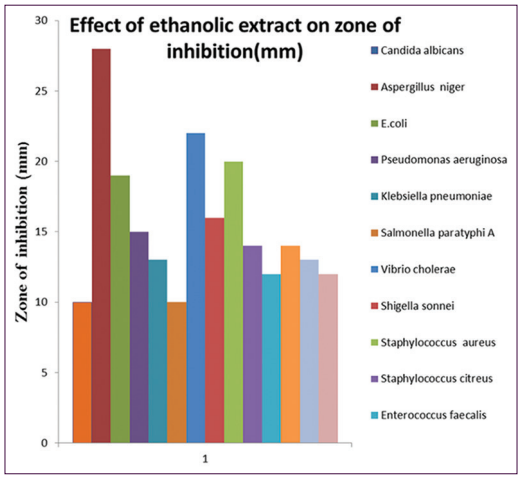

Figure 10: Effect of ethanolic extract on zone of inhibition.

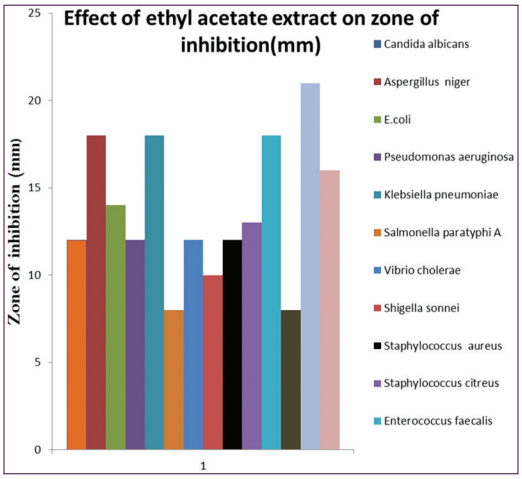

Figure 11: Effect of ethyl acetate extract on zone of inhibition.

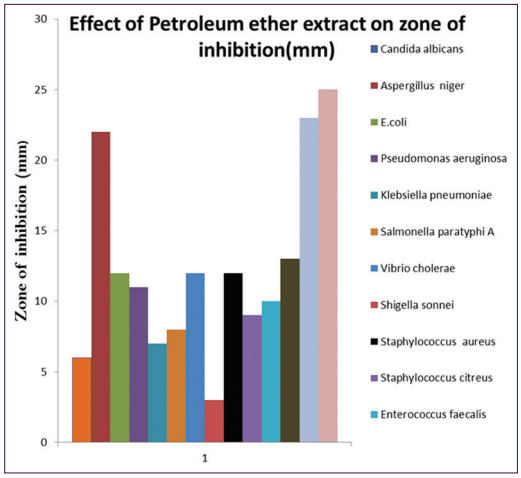

Figure 12: Effect of petroleum ether extract on zone of inhibition.

ciprofloxacin, terbinafine and pyrazinamide exerted their effects some of which were better than the extracts.

DISCUSSION

Disc diffusion on agar plates was used to determine the antibacterial and antifungal activities of *C. odorata* extracts. The ethanolic, ethyl acetate and petroleum ether extract of *C. odorata* showed antimicrobial activity against *E. coli*, *P. aeruginosa*, *S. paratyphi* A, *K. pneumoniae*, *S. sonnei*, *V. cholerae*, *S. aureus*, *E. faecalis*, *S. citreus*, MRSA, *A. niger*, *C. albicans*, *M. gordonae* and *M. fortuitum* (Figure 13). The aqueous portion (Figure 14) failed to show much of antibacterial activity although inhibition of growth was seen on MRSA, *E. coli*, *S. paratyphi A*, *S. sonnei*, *V. cholerae*, *S. aureus* and *A. niger*. Antibiotics are known to diffuse better with MHA, hence used for antibiotic sensitivity test.

The petroleum ether extract of leaf revealed promising antimicrobial activity against *C. albicans*. The solvents, on the other hand, used as negative control exerted no effect against the microorganisms in the broth medium. This implies the effectiveness of the plant extracts. The use of this plant may offer a new source of antifungal agent against the pathogenic *C. albicans* since this fungus is not easily inhibited by other drugs.

It was also seen from the study that plant extracts were slightly better than ciprofloxacin since the former inhibited micro-organisms, such as *K. pneumoniae*, *S. aureus* which were inhibited by the antibiotic to a lesser extent. Phytochemical screening has revealed that triterpenoids and alkaloids are present in the plant. Hence antimicrobial activity in this plant could be attributed to triterpenoids and alkaloids present in the plant. These findings indicate there is a potential for these plants for future work on isolation of active constituents responsible for antimicrobial activity so that they can be developed into antimicrobial drugs.

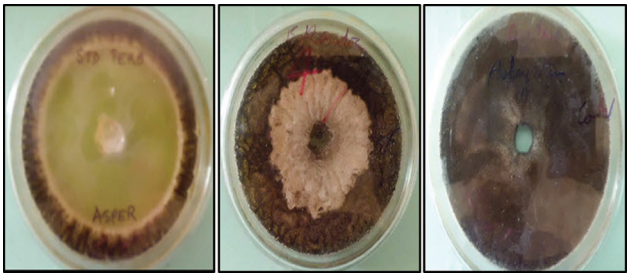

Figure 13: Zone of inhibition of *Aspergillus niger.*

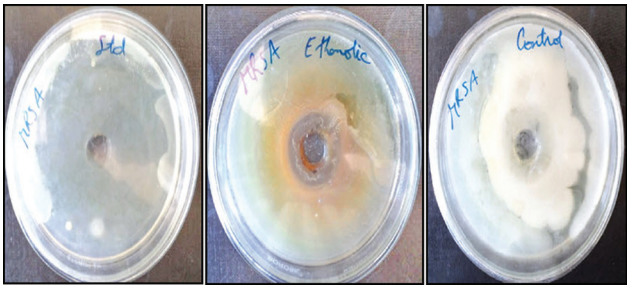

Figure 14: Zone of inhibition of Methicillin-resistant *Staphylococcus aureus.*

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Conflict of interest: None declared
Ethical approval: *The study was approved by the Institutional Ethics Committee*

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