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EVALUATION OF PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF SELECTED MICROALGAE AND PLANTS AGAINST MICROBES

AGGARWAL AMONIKA,
Research Scholar,Faculty of Science,Pacific Academy of Higher Education and Research University, Udaipur, India, amonikaaggarwal@gmail.com

DR.GARIMA SAXENA
Faculty of Science, Pacific Academy of Higher Education and Research University, Udaipur, India
Corresponding Authordrgarimasaxena@gmail.com

ABSTRACT

OBJECTIVE
To study and evaluate the phytochemical and antimicrobial activity of selected algae and medicinal plant extractsagainst bacteria.

METHODS
The study was emphasised on evaluating the antimicrobial potential of the selected algae and medicinal plants against microbes The plantsselected in the study are Ficusreligiosa, Cynodon dactylon and Tinosporacordifolia. The microalgae selected in the study are Kappaphycusalvarezii (Red Algae) and Sargassum species (Brown Algae) .The extracts were made from the following solvents, Cow urine, Benzene, acetone, ethanolforfrom selected plants and algae.
The different extracts were tested for their antimicrobial activity against microbes such asbacteria, Bacillus subtilis ,Shigella flexneri, EscherichiaColi, Enterobacter cloacae which were obtained from Microbial Type Culture collection locateIndian Institute Of Microbial Technology located in Chandigarh, India

RESULT
The ethanolic extract of Cynodondactylon showed highest antimicrobial activity and29.5mm*zone of inhibition against Bacillus subtilis .The significant inhibition (23mm) against Enterobacter cloacae was shown by the ethanol extract of Cynodondactylon and Ficusreligiosa. In cow urine extract, highest zone of inhibition was shown by Sargassum species (15mm) against Bacillus subtilis.The most inhibited bacterial isolate was Bacillus subtilis .The TLC plates were used for separation with 17 extracts (85%) inhibiting its growths tested from plants and algae whereas Escherichia Coli and Shigellaflexneriexhibited resistance with only 9 extracts (45%) showing inhibition .Bacillus subtilis was also inhibited from extracts of Cow urine, Benzene, acetone, ethanol from Tinosporacordiflora.The benzene extract of Tinospora cordiflora showedhighest zone of inhibition (15.5mm) against Shigellaflexneri .The ethanol extract of Tinosporacordiflora also showed significant inhibition (13mm) against E.coli.

CONCLUSION
In the present study ,it was determined that the cow urine extract of Tinospora cordifolia, Ficus religiosa, Cynodondactylon, Kappaphycusalvarezii and Sargassumspecies revealed inhibitory activity andthis inhibitory activity can be used in the control of bacteria of various origins [2].The Kappaphycusalvarezii andSargassum speciesalso showed significant antimicrobial activity against all pathogens. The susceptibility of the bacteria to the crude extracts varied on the basis of zones of growth inhibition according to microorganism and extracting solvent.[3]. The algal extracts are a source of highly bioactive prolific secondary metabolites that might lead in the development of new innovative novel pharmaceutical

agents[4].The results emphasise on the enhanced activity of extract of cow urine in Sargassumspecies and Kappaphycusalvareziiwas due to presence of acrylic acid and dimethyl sulphide in algae [5]and antimicrobial property of cow urine due to the presence of amino acids in urinary peptides and its low pH ,which enhance the bacterial killing by increasing bacterial cell hydrophobicity exhibiting antimicrobial action against different clinical microbial strains.[6]

KEYWORDS: Ficus religiosa, Cynodon dactylon, Tinospora cordifolia. Kappaphycus alvarezii, Sargassum species, Antimicrobial activity, phytochemical activity, cow urine extract.

INTRODUCTION

The new innovative and inventive methods for the development of novel drugs vast potential lie in use of medicinal plants and marine species as rich source and of antimicrobial agents.These seaweeds are classified based chemical composition and their nutrition such as Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae) [7]. Among marine algae ,red algae contains phycoerthyrin and phycocyanin as pigments responsible for red colour [8].Brown algae are a group of algae with possession of pigment called fucoxanthin[9].The phlorotannin contents as marine phenolic compounds have reported in good amount in brown algae.[10].The phyto constituents such as flavanoids, phenols and tannins are present in algae indicating a possibility that the extracts may have antioxidant property[11].The phytochemical in algae are extensively used in textiles, gelling, thickening and as stabilising agents .

The Ficusreligiosa is a sacred fig tree whose leave contains tannic acid and stem rich in vitamin K .This herb found as a relieve for many ailments like gout ,constipation,swollen glands ,skin disease ,dehydration etc[12].Cynodondactylon is a Bermuda grass and is of good nutritional value ,is beneficial in wounds ,skin problems ,diabetes ,epilepsy ,gynaecological problemsetc[13].Tinosporacordifolia ,also known as guduchi or Amrita is an immunity boosting herb and is a promising anti cancer herb and found use in diabetes[14].Shigellaflexneri**,** a most common bacteria of a group ,gram-negative, recognized as the etiologic agents of bacillary dysentery or shigellosis. [15]Enterobacter, a gram-negative bacteria classified as facultative anaerobes, can cause eye and skin infections, meningitis, bacteraemia (bacterial blood infection), pneumonia, and urinarytractinfections [16]. Bacillus subtiliscells are rod-shaped, Gram-positive bacteria that are found naturally in soil and vegetation [17].Bacillus subtilisbacteria are non-pathogenic [18]. They can contaminate food and seldom result in food poisoning. Bacillus subtilisstrains can cause rots in potatoes.E.coliis a Gram-negative, facultative anaerobic virulent strain that can cause gastroenteritis, urinary tractinfections, and neonatal meningitis [19].Plants and Seaweeds are considered as source of bioactive compounds and produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities [20].Seaweeds have become a recognized potential natural product in pharmaceutical industries and an important resource to combat serious diseases in the world. The medicinal value of these plants lies in some bio active substances that produce a definite physiological action on the human body. Plants and algae are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial growth and have a great potential for producing new drugs for human benefit.The most important of these secondary metabolites of plants are flavanoids alkaloids, tannin, and phenolic compounds.. Therefore, it is of great attraction to carry out a systematic way of screening in order to utilise these plants in folk medicines and disclose the active principle forisolation and characterization of their constituents which may result in the discovery of novel active compounds Therefore, the present investigation aims to detect the bactericidal efficacy of Ficusreligiosa, Cynodondactylon and Tinosporacordifolia. Kappaphycusalvareziiand Sargassum species.

MATERIAL AND METHODS

PLANT MATERIAL

The three selected plants, Cynodondactylon, Ficusreligiosa, Tinosporacordifolia, were collected from forest region and wild population around Udaipur district andidentity was confirmed at Maharana Pratap University of Agriculture and Technology, Udaipur.

The Sea weeds, Kappaphycusalvareziiwas gathered from the seacoast of Rameshwaram, Tamilnadu, IndiaSargassum species was gathered from sea coast of kanyakumari district ofTamilnadu, India. Their bioactivity and identity was confirmed at Maharana Pratap University of Agriculture and Technology Udaipur. The Kappaphycusalvarezii and Sargassum species were carried in sterile bags. The selected algae samples were washed with water and dried to remove any waste which was stuck on the surface .The sample was then powdered in a mixer grinder.

BACTERIAL CULTURES

The test bacterial organisms:B1-Bacillus subtilis-MTCC 441, B2-Shigella flexneri-MTCC 1457, B5-Escherichia Coli-MTCC 739, B6-Enterobacter cloacaewere obtained from Microbial Type Culture collection, Indian Institute of Microbial Technology located in Chandigarh, India.

The purity of the bacterial culture was ensured and checked by their growth in selective media .Then they were further utilised for studying antimicrobial property of the samples.

PREPARATION OF LEAF EXTRACT

The healthy leaf samples of selected plants, Ficusreligiosa, Tinosporacordiflora, Cynodondactylon were gathered and washed with tap water. The leaves were dried under sunlight for 5 days by covering them with a dry newspaper. The dried leaves were converted to a powder form using a mixer. The total weight of each sample was noted and they were stored in a dry container. Equal amount of each powdered sample (4g) was dissolved in 4 selected (40ml)– ethanol, acetone, benzene and distilled cow's urine.The dissolved ratio (1:10) for the powder to solvent was taken in a wide mouth test tube. The solutions weremixedand kept aside for 3 days to provide enough time for extraction. This process was repeated thricefor ample extraction of the samples in the same test tube .After 3 days ,the supernatant of the powder and solvent solutions were pipette out and placed in glass bottles. The extracts in the bottles were then exposed for evaporation to get powder form of the extract.

PREPARATION OF AQUEOUS SEA WEED EXTRACT

4g of powdered sample was soaked in 40 ml of the solvents cow urine, benzene, acetone and ethanol for 3 days for the preparation of aqueous extract. The remaining extracts were filtered and concentrated in a rotator evaporator .The vacuum pump was used to remove the residual water .The weighted crude extract were suspended in dimethyl sulfoxide (DEMSO) to a final concentration of 50mg/ml and stored in a refrigerator.

ANTIMICROBIAL ASSAY

PROCEDURE

1. PREPARATION OF PLATES

i. NAM for bacteria was prepared according to the accurate composition and immediately after autoclaving, it was cooled in a 45 - 50°C.The freshly prepared and cooled medium was poured into petri plates.

ii. The agar medium was cooled to room temperature unless the plate is used the same day; andstored in a refrigerator (4°C).

2. SPREADING OF BACTERIA ON THE PLATES

i. 100 µL of bacteria from freshly prepared culture was taken in the pipette and poured in the middle of the respective petri plate.
ii. Using a cotton swab that has already put in UV light, the bacteria wasspread evenly on the surface of the plate so that bacteria were spread in each corner of the plateand dried for 4-5 minutes.

3. ANTIMICROBIAL DISKS

i. Using a flame - sterilized forceps the disc was dipped in the sample or antibiotics for 5-10 seconds.Each disc was then gently placed on the agar plate to ensure that the disc was attached into the agar.The plate was kept in laminar air flow for 30 minutes so that the drug was properly absorbed in the gel.

4. INCUBATION OF PLATES

i. Plates were inverted and incubated at 24h at 30°C for bacteria.

5. MEASUREMENT OF DIAMETER

i. Zone of inhibition is measured with the help of the scale and noted down.

RESULT

The ethanol, acetone benzene, cow urine extracts of Ficusreligiosa, Cynodondactylon, Kappaphycusalvarezii (Red Algae), Sargassum species (Brown Algae), Tinosporacordifolia were tested against the pathogenic microbes.Out of plants and algae tested for antimicrobial activity, plant species and algae showed antibacterial activity by inhibiting one or more microorganisms. The results of the antimicrobial activity of plant extracts tested against bacteria by disc diffusion method shown in Table 2. Among the plants screened, the ethanolic extract of Ficusreligiosa, Cynodondactylon,Kappaphycusalvarezii (Red Algae), Sargassum species (Brown Algae), Tinosporacordifolia showed significant inhibition of all tested bacteria whereas ethanolic extract of Kappaphycusalvarezii and Sargassum species (Brown Algae), showed less inhibition activity. Cow urine extract of marine species Kappaphycusalvarezii and Sargassum species showed significant inhibition compared to less inhibition activity of Ficusreligiosa and Cynodondactylon. Acetone extracts of Ficusreligiosa, Cynodondactylon, and Tinosporacordifolia showed very less activity as compared to good inhibition activity of ethanolic extracts against Bacillus subtilis .Benzene extract showed good activity in both algae extracts as well as plant extracts.The antimicrobial activity of Bacillus subtilisagainst Ficusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum speciesof ethanol, acetone, cow urine and benzene extracts was maximum for Cynodondactylon (29.5) mm in ethanol extract.
Tinospora cordiflorashowed antibacterial activity against all bacteria used in this study Cow urine extracts of Kappaphycus (Red Algae) Sargassum species showed good inhibition activity against Bacillus subtilis and Shigellaflexneri. Escherichia Coli, Enterobacter cloacae showed minimal activity against cow urine extracts of all the selected algae and plants. Ethanol extract showed a maximum activity against pathogens like Bacillus subtilis(29.5 mm), Enterobacter cloacae (23mm). The antimicrobial activity of Ficusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum species of ethanol, acetone, cow urine and benzene extracts was highest for Tinosporacordifolia (15.5) mm in benzene extract against Shigellaflexneri.
The antimicrobial activity of E.coli againstFicusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum species of ethanol, acetone, cow urine and benzene extracts was highest for Tinosporacordifolia **(**13) mm in ethanol extract. The antimicrobial activity of Enterobactercloacae against Ficusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum species of ethanol, acetone, cow urine and benzene extracts was highest for Ficus religiosa and Cynodon dactylon (23) mm in ethanol extract. Analysis of plant extracts revealed the presence of flavanoids, glycosides, phenols, saponins, steroids and tannins in most of the selected plants which could be responsible for the observed antimicrobial property[21].Flavanoids, tannins and saponins are present in all plant and algae

extracts[22].These bioactive compounds are known to exert antimicrobial action and act by different mechanism[23].Tannins bind interfere protein synthesis by binding to proline rich proteins[24].Flavanoids are synthesized by plants in response to microbial infection, arehydroxylatedphenolic substance and effective antimicrobial substances against microorganisms[25] Antimicrobial property of saponin is due to its ability to cause leakage certain enzymes and proteins from the cell[26].

DISCUSSION

The cow urine extracts of Kappaphycusalvarezii Sargassum speciesand Tinospora cordiflora are among alternatives for bacterial disease management .It has been found cow urine extracts of plants possesses significant antibacterial activity against pathogenic bacteria. In the early reported papers ,there are a number of reports which evaluated the antimicrobial properties of marine algaeand medicinal plants but very few work was done on cow urineextracts of selected algae species and plants against Bacteria subtilis,E.coli, Enterobactercloacae,Shigellaflexneri.In the present investigation, the high susceptibility was recorded for cow urine extracts of Kappaphycusalvarezii and Sargassum species,Tinosporacordiflora against Bacillus subtilis.In astudy ,Tiwari and Das .[27] showed inhibitory effects of cow urine extracts against medicinal plants In another study Venkatesh et al.[28] found inhibitory effect of cow urine extract of red algae against Xanthomonasoryzae.Rakesh et al. [29] showed inhibitory effects of cow urine extract of plants aginstF.axysporum, F.spzingiberi and P.aphanidermatum causative agent or rhizome rot of ginger.Venkatesh Ret al. [30] studiedAntixanthomonas activity, phytochemical analysis and characterisation of antimicrobial compounds from Kappaphycus alvarezii. It has been experimentally shown that cow urine extract possesses inhibitory activity against phytopathogens [31].The results provide justification for the use of these algae in folk medicine to treat various infectious diseases. When four bacterial organisms were compared it was found that, maximum suppression was observed against Bacillus subtilis followed byEnterobacter cloacae, E.coli and Shigellaflexneri against all selected extracts. The enhanced activity of extract of cow urine inSargassumspecies and Kappaphycusalvarezii was due to presence of acrylic acid and dimethyl sulphide and antimicrobial property of cow urine itself wasdue to its low pH, presence of amino acids in urinary peptides which enhance the bacterial killing by increasing bacterial cell hydrophobicity exhibiting antimicrobial action against different clinical microbial strains. This synergistic effect was responsible for inhibition activity .The inhibition activity of ethanol extracts of Cynodondactylon and Ficusreligiosa was due to the presence of secondary metabolites like saponins ,flavonoids etc.From the results it dictates that the greateractivity resides in benzene, cowurine extractsof Tinospora cordifolia since other extracts of acetonewhich did not effectivelyinhibit the growth of the bacteria. This maydue to the chemical constituents responsiblefor the antibacterial activity are more solublein ethanol extracts. [32]It can be interpreted thatthe antibacterial activity againstmicroorganisms is due to any one or more alkaloids of the plants. Further work isnecessary to isolate and purificationcompounds in Tinospora cordifolia which will allow the scientificcommunity to recommend their utilization asan accessible alternative to syntheticantibiotics.Further research in this direction will help to develop medicinal plant extracts by making proper formulations of cow urine and to counter various infections caused by microbes, thus boosting the human immune system. It was concluded that cow urine itself has antimicrobial property and this inhibitory activity in combination with Kappaphycusalvarezii, Sargassum speciesand Tinospora cordiflora extracts can synergistically can be used in the control of bacteria.

Table1. Shows the antimicrobial activity, Ethanol extract showed a maximum activity against pathogens like Bacteria subtilis (29.5 mm), Enterobacter cloacae (23mm). Benzene extract against Enterobacter cloacae showed promising activity.

Selected Plants and algae	Bacteria	Ethanol	Acetone	Benzene	Cow Urine	Positive Control	Negative Control
Ficus religiosa	Bacillus subtilis	27	6	7.5	10	31.5	6
	Shigella flexneri	8.5	6	6	6	28.5	6
	E.coli	10.5	6	7	6	10	6
	Enterobacter cloacae	23	12.5	15	6	15.5	6
Cynodon dactylon	Bacillus subtilis	29.5*	6	13	8.5	31.5	6
	Shigella flexneri	8	6	6	6	27.5	6
	E.coli	6	6	6	6	13.5	6
	Enterobacter cloacae	23	17.5	18.5	6	18.5	6
Tinospora cordifolia	Bacillus subtilis	15.5	8	12	13.5	18	6
	Shigella flexneri	14	12	15.5	6	34	6
	E.coli	13	12.5	9.5	6	18	6
	Enterobacter cloacae	8.5	6	6	8	26.5	6
Kappaphycus alvarezii	Bacillus subtilis	6	15	8	12	50	-
	Shigella flexneri	9	9	11.5	13	46	-
	E.coli	6	11.5	6	6	13	-
	Enterobacter cloacae	6.5	6.5	6.5	6	16	-
Sargassum species	Bacillus subtilis	9	9	10	15	48	-
	Shigella flexneri	9	9	10.5	11	44	-
	E.coli	8	12	11.5	6	14	-
	Enterobacter cloacae	8.5	6	10	11	10	-

Table2a. The antimicrobial activity of Bacillus subtilis against Ficusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum species. Of ethanol, acetone, cow urine and benzene extracts. In these four extracts Cynodon dactylon showed highest antimicrobial activity.

Bacillus subtilis	Ethanol	Acetone	Benzene	Cow Urine	+ Control	- Control
Ficus religiosa	27	6	7.5	10	31.5	6
Cynodon dactylon	29.5*	6	13	8.5	31.5	6
Tinospora cordifolia	15.5	8	12	13.5	18	6
Kappaphycus alvarezii	6	15	8	12	50	-
Sargassum species	9	9	10	15	48	-

Table 2b shows the antimicrobial activity of Shigellaflexneriagainst Ficusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum species of ethanol, acetone, cow urine and benzene extracts. In these four extracts Tinosporacordifolia showed highest antimicrobial activity against Shigellaflexneri (15.5)mm in benzene extract

Shigella flexneri	Ethanol	Acetone	Benzene	Cow Urine	+ Control	- Control
Ficus religiosa	8.5	6	6	6	28.5	6
Cynodon dactylon	8	6	6	6	27.5	6
Tinospora cordifolia	14	12	15.5*	6	34	6
Kappaphycus alvarezii	9	9	11.5	13	46	-
Sargassum species	9	9	10.5	11	44	-

Table2c.The antimicrobial activity of E.coliagainst Ficus religiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycusalvarezii, Sargassum species of ethanol, acetone, cow urine and benzene extracts. In these four extracts Tinosporacordiflora showed highest antimicrobial activity against (13) mm in ethanol extract.

E.coli	Ethanol	Acetone	Benzene	Cow Urine	+ Control	-Control
Ficus religiosa	10.5	6	7	6	10	6
Cynodon dactylon	6	6	6	6	13.5	6
Tinospora cordifolia	13*	12.5	9.5	6	18	6
Kappaphycus alvarezii	6	11.5	6	6	13	
Sargassum species	8	12	11.5	6	14	

Table2d.Shows the antimicrobial activity of Enterobacter cloacaeagainst Ficusreligiosa, Cynodondactylon, Tinosporacordifolia, Kappaphycus alvarezii Sargassum species of, ethanol, acetone, cow urine and benzene extracts. In these four extracts Tinosporacordifolia showed highest antimicrobial activity against Bacillus subtilis (13) mm in ethanol extract. acetone, cow urine and benzene extracts. In these four extracts Ficus religiosa and Cynodondactylon showed highest antimicrobial activity against Enterobacter cloacae (23) mm in ethanol extract

Enterobacter cloacae	Ethanol	Acetone	Benzene	Cow Urine	+ Control	- Control
Ficus religiosa	23*	12.5	15	6	15.5	6
Cynodon dactylon	23*	17.5	18.5	6	18.5	6
Tinospora cordifolia	8.5	6	6	8	26.5	6
Kappaphycus alvarezii	6.5	6.5	6.5	6	16	-
Sargassum species	8.5	6	10	11	10	-

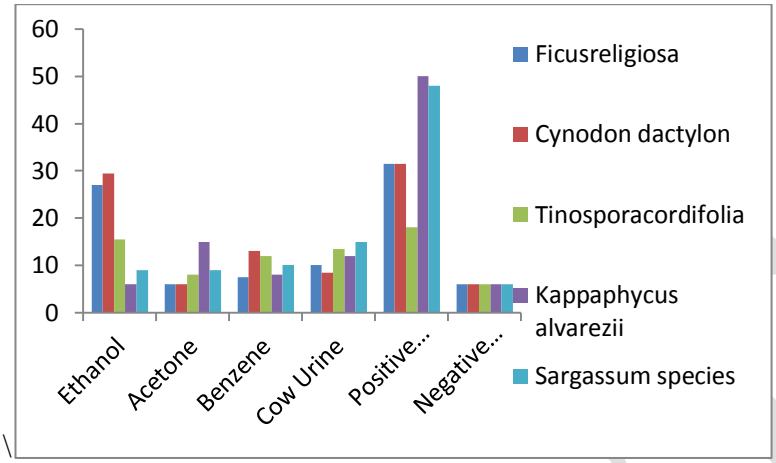

Figure 1: Cynodondactylon showed highest antimicrobial activity against Bacillus subtilis (29.5) mm in ethanol extract Zone of inhibition including 6 mm diameter of paper disc.

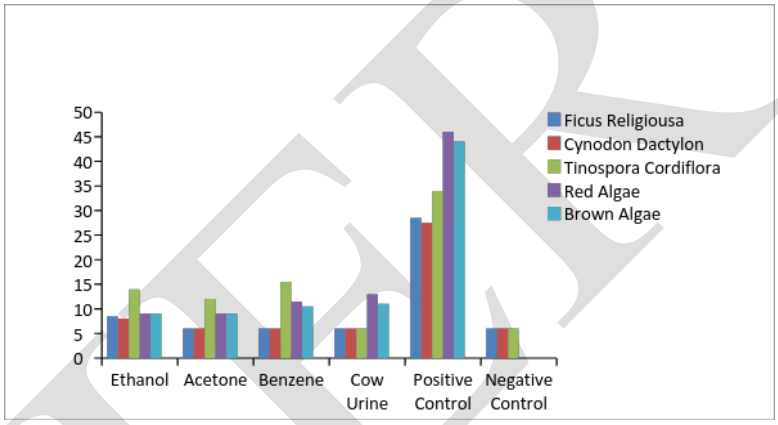

Figure 2: Tinosporacordifolia showed highest antimicrobial activity against bacteria Shigella flexneri (15.5) mm in benzene extract Zone of inhibition including 6 mm diameter of paper disc.

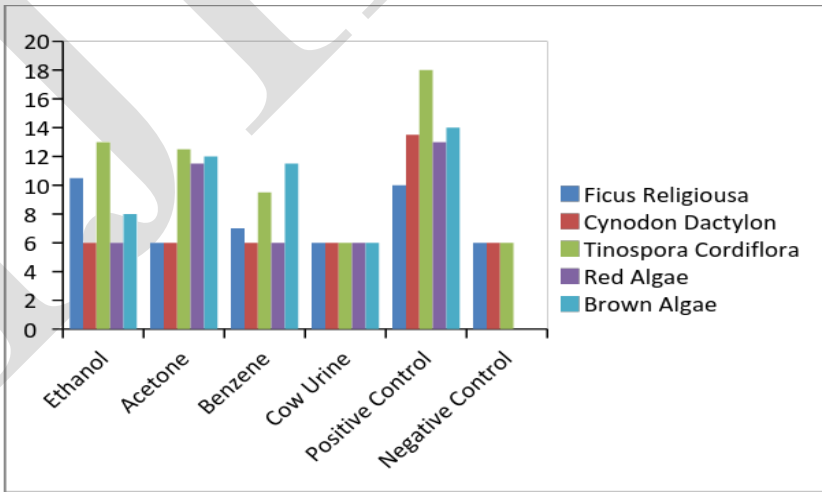

Figure 3: Tinosporacordiflora showed highest antimicrobial activity against (13) mm in ethanol extract. Zone of inhibition including 6 mm diameter of paper disc.

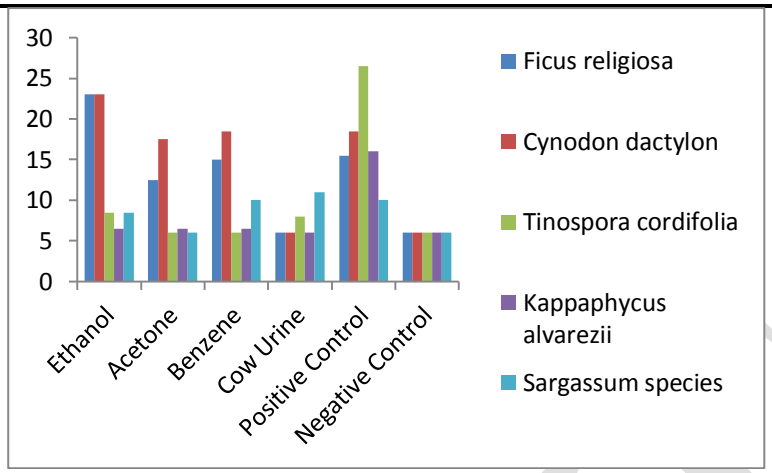

Figure 4: Ficus religiosa and Cynodon dactylon showed highest antimicrobial activity against Enterobacter cloacae(23)mm in ethanol extract Zone of inhibition including 6 mm diameter of paper disc.

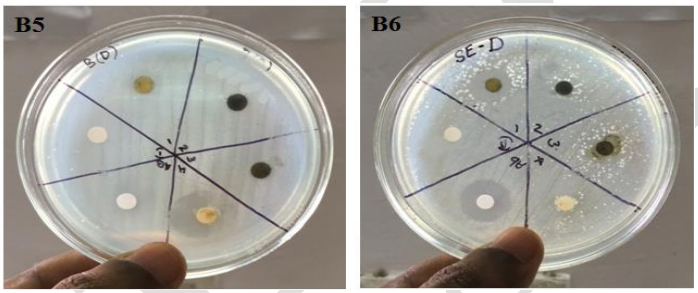

Figure 5: Disc diffusion results of Cynodondactylon against four Bacteria.1: Ethanol, 2: Acetone, 3: Benzene, 4: Cow Urine, Ab: Amoxicillin, (-): Negative control.B1: Bacillus subtilis, B2: Shigella flexneri, B5: E.coli, B6: Enterobacter cloacae

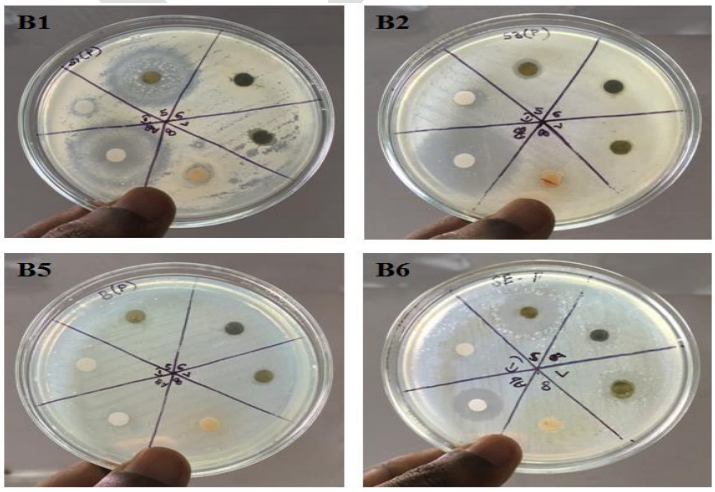

Figure 6: Disc diffusion results of Ficus religiosa against four Bacteria. 5: Ethanol, 6: Acetone, 7: Benzene, 8: Cow Urine, Ab: Amoxicilin, (-): Negative control. B1: Bacillus subtilis, B2: Shigella flexneri, B5: E.coli, B6: Enterobacter cloacae

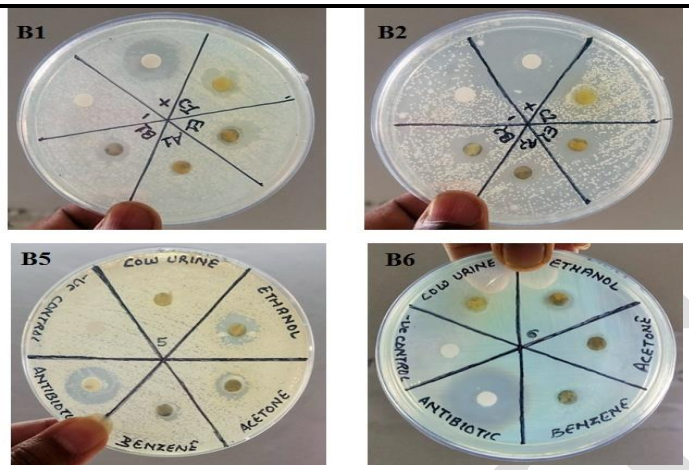

Figure 7: Disc diffusion results of Tinosporacordifolia against four isolated Bacteria. C1: Cow Urine, E1: Ethanol, A1: Acetone, B1: Benzene, +: Amoxicillin, - : Negative control. B1: Bacillus subtilis, B2: Shigella flexneri, B5: E.coli, B6: Enterobacter cloacae

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