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DETECTION OF ANTIMICROBIAL COMPOUNDS BY BIOAUTOGRAPHY OF SELECTED SEAWEEDS AND PLANTS EXTRACTS

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ABSTRACT

The emergence of new infectious diseases, considering the failure to acquire new molecules with antimicrobial properties from microorganisms have created the necessity for studies directed towards the development of new antimicrobials The world of plants constitutes an almost unlimited source of biologically active substances which are or could be used in the treatment of many human, animal and plant diseases[1,2] The screening methods are of great importance for the identification of antimicrobials from other natural sources. The richness of bioactive compounds in plant materials encourages continuous development of separation methods and bioassays for their isolation and identification. Thin-layer chromatography-direct bio autography links separation on the adsorbent layer with biological tests performed directly on it.[3] Therefore, the method is very convenient for searching plant and algae constituents with biological activity, such as antibiotics. Test bacteria grow directly on a plate surface excluding places where antibacterial are located. They can be detected with reagents converted by living bacteria. This paper discusses all above mentioned aspects of TLC-DB, illustrating them with literature, schemes and our own results. The objective of this study is to determine antibacterial activity of extracts by TLC-Bio autography. TLC-DB is also a very effective assay in searching for natural compounds with antifungal and antibacterial properties. The extracts from selected plants Ficus religiosa, Cynodon dactylon, Tinospora cordifolia and the sea weeds Kappaphycus alvarezii (Red Algae) and Sargassum species (Brown Algae) were screened for their antimicrobial activity.

The test organisms included bacteria Bacillus subtilis, Shigella flexneri, Escherichia Coli, Enterobacter cloacae and fungi Saccharomyces cerevisiae, Aspergillus candidus, Aspergillus Niger and Pencillium Species which were obtained from Microbial Type Culture collection, Indian Institute of Microbial Technology Chandigarh, India. A simple bioautographic procedure, involving spraying suspensions of the bacteria or fungi on thin layer chromatography (TLC) plates developed in solvent was used to detect the number of antibacterial and antifungal compounds[4] present in the extracts which showed maximum zone of inhibition in antimicrobial assay by disc diffusion method. All the extracts had antimicrobial activity against at least one of the test microorganisms.This activity was denoted by white spots against a red-purple background on the TLC plates after spraying with tetrazolium violet.[5] Of the bacteria tested, Bacillus subtilis was inhibited by the most compounds separated on the TLC plates from all the tested plants and algae. Similarly, growth of the fungus Saccharomyces cerevisiae was also inhibited by many compounds present in the extracts. The extracts of Ficus religiosa, Cynodon dactylon, Tinospora cordifolia, Kappaphycus alvarezii, and Sargassum species showed inhibition bands depicting antimicrobial activity and result was in conformity with the antimicrobial results by disc diffusion method The compilation indicates that cow urine extracts of selected algae Sargassum species and Kappaphycus alvarezii and plants Ficus religiosa, Cynodon dactylon, Tinospora cordifolia, exhibit better antimicrobial action against different clinical microbial strain. The extract showed significant antibacterial activity and this good inhibition activity can be used in control of microbial diseases by identifying novel and advanced medicines from cow urine extracts of selected plant species and algae which are future reservoir of new novel pharmaceutical agents. It was concluded that cow urine itself has antimicrobial property and inhibitory activity of plant extracts can synergistically can be used as a precursors for the synthesis of useful herbal drugs. Thus this novel extracts can be useful in solving the growing crisis and can provide antibiotic resistance for search of new antimicrobial compounds from natural resources. These products essentially help to identify newer structurally novel natural products which can be used for the treatment of bacterial and fungal infections.

KEYWORDS: Bio autography, Medicinal plants, Antifungal, Antibacterial, TLC, tetrazolium.

INTRODUCTION

Bioautography is a means of target-directed isolation of active molecules on chromatogram. Organic solvents employed in chromatographic separation process can be completely removed before biological detection because these solvents cause inactivation of enzymes and/or death of living organisms.[6] This has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied.[7] Bioautography is particularly important to avoid the time-consuming isolation of inactive compounds.[8] A number of bioautographic assays have been developed, which can be divided into three groups (Rios et al., 1988). These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography (Islam et al., 2003)[9]. This review critically describes the methodologies to identify antimicrobial compounds by employing TLC -bioautography. Once the technique has been mastered, bioautography is a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, and therefore facilitates the target-directed isolation of the active constituents (Rahalison et al., 1991). Bioautography has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products.[10]

MATERIAL AND METHODS

Plant material

The three selected plants, Cynodon dactylon, Ficus religiosa, Tinosporacordifolia, were collected from forest region and wild population around Udaipur district and identity was confirmed at Maharana Pratap University of Agriculture and Technology, Udaipur. The Sea weeds, Kappaphycus alvarezii was gathered from the seacoast of Rameshwaram, Tamilnadu, India .Sargassum species was gathered from sea coast of kanya kumari district of Tamilnadu, India. Their bioactivity and identity was confirmed at Maharana Pratap University of Agriculture and Technology Udaipur. The Kappaphycus alvarezii and Sargassum species were carried in sterile bags. The selected alga 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.

Fungal and Bacterial Cultures

The test organisms Bacillus subtilis-MTCC 441,Shigella flexneri-MTCC 1457,Escherichia Coli-MTCC 739,Enterobacter cloacae, Aspergillus niger-MTCC 282,Aspergillus candidus-MTCC 1989,Saccharomyces cerevisiae-MTCC 170were obtained from Microbial Type Culture collection, Indian Institute Of Microbial Technology Chandigarh, India.

The cultures were allowed to grow on their respective selective media to check and ensure their purity and optimum growth before subjected to further analysis.

Preparation of leaf extract

The leaf samples of selected plants of Ficus religiosa, Tinosporacordiflora, Cynodon dactylon were collected, washed and dried under sunlight for 5 days. Once the leaves were crunchy dry, a fine powder was made 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 distilled cow's urine. (40ml)The samples were dissolved in a ratio of (1:10) powder to solvent, in a wide mouth test tube. The test tube mouths were then covered to prevent evaporation of the solvents. The solutions were mixed and left aside for 3 days to provide enough time for extraction. This process was repeated thrice in the same test tube for ample extraction of the samples After 3 days the supernatant of the powder and solvent solutions were pipetted out and placed in glass bottles. The extracts in the bottles were then placed without lids in the open to get powder of the extract.

Preparation of aqueous sea weed extract: The aqueous extract was prepared by dissolving 4g of powdered sample was soaked in 40 ml of the cow urine for 3 days. 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 activity of selected plants and algae against bacteria and fungi was tested by disc diffusion method. The result is displayed in the Table 1 and table 2.After antimicrobial detection by disc diffusion method the extracts with maximum inhibition zone against bacteria and fungi were selected for TLC-bio autotrophy as depicted in Table 3.

Table 1: Shows the antimicrobial activity of selected plants and algae against bacteria by disc diffusion method (Zone of inhibition including 6 mm diameter of paper disc).

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	-

Diffusion method (Zone of inhibition including 6 mm diameter of paper disc).

Table 2: Shows the antimicrobial activity of selected plants and algae against fungi by disc.

Selected Plants and algae	FUNGI	Cow Urine	Benzene	Ethanol	Acetone	Positive Control	Negative Control
Ficus religiosa	Aspergillus niger	6	6	6	6	13.5	6
	Aspergillus candidus	8.5	6	6	6	-	-
	Penicillium Species	6	9.5	8	6	-	-
	Saccharomyces cerevisiae	6	6	8.5	6	10	6
Cynodon dactylon	Aspergillus niger	6	6	6	6	13.5	-
	Aspergillus candidus	9.5	6	12	6	-	-
	Penicillium species	6	6	12	6	-	-
	Saccharomyces cerevisiae	6	6	8	6	10	6
Kappaphycus alvarezii	Aspergillus niger	10	11	7	9	-	
	Aspergillus candidus	10	6	6	6	-	-
	Penicillium Species	9.5	11	6	6	-	-
	Saccharomyces cerevisiae	6.5	11.5	6	6	-	-
Sargassum species	Aspergillus niger	9	9.5	8	7	20	-
	Aspergillus candidus	6	6	6	6	11	-
	Penicillium corylophilum	10	10	6	6	10	-
	Saccharomyces cerevisiae	6.5	11.5	6	6	12	-
Tinospora cordifolia	Aspergillus niger	6	6	6	6	6	13
	Aspergillus candidus	6	6	8	9.5	6	11
	Penicillium Species	6	6	6	6	6	9.5
	Saccharomyces cerevisiae	13	10	6	6	25.5	27

Table 3: Bioautotrophy of extracts with maximum inhibition zone in disc diffusion method against bacteria and fungi.

Bacteria	Extracts	Fungi	Extracts
Bacillus subtils		**Aspergillus niger**	
Ficus religiosa	Ethanol	Ficus religiosa	Ethanol
Cynodon dactylon	Ethanol	Cynodon dactylon	Ethanol
Tinospora cordifolia	Ethanol	Tinospora cordifolia	Ethanol
Sargassum species	Cow urine	Sargassum species	Benzene
Kappaphycus alvarezii	Acetone	Kappaphycus alvarezii	Benzene
Shigella flexneri		**Saccharomyces cerevisiae**	
Ficus religiosa	Ethanol	Ficus religiosa	Ethanol
Cynodon dactylon	Ethanol	Cynodon dactylon	Ethanol
Tinospora cordifolia	Benzene	Tinospora cordifolia	Cow urine
Sargassum species	Cow urine	Sargassum species	Benzene
Kappaphycus alvarezii	Cow urine	Kappaphycus alvarezii	Benzene
E.coli		**Aspergillus candidus**	
Ficus religiosa	Ethanol	Ficus religiosa	Cow urine
Cynodon dactylon	Ethanol	Cynodon dactylon	Ethanol
Tinospora cordifolia	Ethanol	Tinospora cordifolia	Acetone
Sargassum species	Acetone	Sargassum species	Ethanol
Kappaphycus alvarezii	Acetone	Kappaphycus alvarezii	Cow urine
Enterobacter cloacae		**Penicillium Species**	
Ficus religiosa	Ethanol	Ficus religiosa	Benzene
Cynodon dactylon	Ethanol	Cynodon dactylon	Ethanol
Tinospora cordifolia	Ethanol	Tinospora cordifolia	Ethanol
Sargassum species	Cow urine	Sargassum species	Benzene
Kappaphycus alvarezii	Ethanol	Kappaphycus alvarezii	Cow urine

Thin Layer Chromatography (TLC)

Chemical constituents of the selected plant and algae extracts were separated on aluminium-backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F_{254}). The TLC plates were developed under saturated conditions with one the eluent systems Chloroform + Methanol (7:3 v/v). Kieselgel GF_{254} plates (Merck), 20 X 20 cm^2 and 0.2 mm thick, were used for TLC. Lightly a spot was put with a pencil above 1cm from the end of the plate. While putting the spot the surface of the plate should not be scratched deeply. Using a micropipette, 5 µL of the sample was taken and each sample spot as small as possible was made (less than about 3mm diameter).The sample was simply tabbed and released.

Development of chamber

The prepared plate was developed in a closed, pre-saturated chamber using an ascending mobile solvent Chloroform + Methanol (7:3 v/v).The solvent was then evaporated before development by placing the plate in the mobile phase at a level below the applied sample.

When the solvent front near the top edge of the stationary phase, after 15 minutes up to several hours, the plate was removed from the solvent reservoir. The sample ascended the plate by capillary action of the mobile phase and the various components of the sample were retarded in proportion to their interaction. Separations in TLC involve disturbing a mixture of two or more substances between a stationary phase and a mobile phase Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.

Calculate

The Rf value

Rf Value = Distance from Baseline travelled by Solute.

Distance from Baseline travelled by Solvent (Solvent Front).

Direct TLC bioautographic detection

Ten µl (10 mg/ml) of each extract were loaded onto TLC plates in a narrow band and eluted using the chloroform methanol (7:3) solvent system The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates. One week old cultures of fungal organisms grown on SD agar were each transferred into 250 ml of freshly prepared SD broth using a sterile swab., In the case of bacteria, overnight cultures grown on MH broth were used .1 % of inoculams of different bacteria and fungi was poured on the TLC plates until wet They were incubated for 24 hours at 37ºC.Subsequently, the bioautogram was sprayed with a 1 % aqueous of 3 (4,5- dimethyl-2-thiazolyl) -2,5- diphenyl-2H-tetrazolium bromide (MTT), based on the reduction of MTT by mitochondrial dehydrogenase of viable cells resulting in a blue formazan product after incubation. It was then incubated at 30ºC for few hours. Zones in which the bacterial growth was inhibited failed to take the bluish purplish stain and indicated the presence of active compounds on that area of TLC plate. White bands indicate the presence of compounds that inhibited the growth of tested organisms.[11]

RESULTS

The Rf value of different extracts of selected plants and algae separated by TLC are shown in table 4.The appearance of white areas against a purple-bluish background on the chromatograms denotes inhibition of growth of the bacteria (Figure 2,3) or fungi (Figure 3,4) due to presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce MNT to a purple-bluish colour (Begue and Klein, 1972). In the

presence of active plant compounds on the chromatograms, the growth of the organism is inhibited. An important factor in quantifying the movement of a compound on a stationary phase e.g. silica with a certain solvent system is the Rf (retardation factor) value and is the ratio of the distance moved by the compound from its origin to the movement of the solvent from the origin. R_f If a development solvent of too high a polarity is used, all components in the mixture will move along with the solvent and no separation will be observed (RF's will be too large). If the solvent is of too low a polarity the components will not move enough, and again separation will not occur (RF's will be too small). In practice, different solvents or mixtures of solvents are tried until a good separation is observed. Typically an effective solvent is one that gives Rf's in the range of 0.3 - 0.7.12. By using these compounds with a wide range of polarities can be separated. Because the R_f value is constant for the same compound under defined conditions, the presence of clear bands with the same[12] Rf value may mean that the same compounds are probably responsible for the antimicrobial activity in the same extract tested against different microorganisms. This would suggest non-selective antimicrobial activity. In some cases, no inhibition of microbial growth was observed The absence of activity could be due to evaporation of the active compounds, photo-oxidation or due to very little amount of the active compound. The antimicrobial agents detected in this study were present in extracts of relatively non-polar solvent. These findings agreed with previously published results (Masoko and Eloff, 2005, 2006) that the substances responsible for the antimicrobial activity were mainly non-polar in nature. Bacillus subtillis was most inhibited bacteria.

Table 4: Rf value of different extracts of selected plants and algae separated by TLC.

Plant	Solvent Extracts system	R_f values	Active bands
Ficus religiosa	Chloroform + Methanol (7:3 v/v)	0.987	7
Cynodon dactylon	Chloroform + Methanol (7:3 v/v)	0.98	5
Tinospora cordifolia	Chloroform + Methanol (7:3 v/v)	0.9	7
Kappaphycus alvarezii	Chloroform + Methanol (7:3 v/v)	0.25	2
Sargassum species	Chloroform + Methanol (7:3 v/v)	0.18	2

P= Peepal, D= Doob Grass, T=*Tinospora cordifolia*, B= Brown Algae, R=Red Algae

Silica GF 254 plates (Merck), 20 X 20 cm^2 and 0.2 mm thick, were used for analytical TLC. TLC run length = 8 cm, Running solvent =Chloroform + Methanol (7:3 v/v).

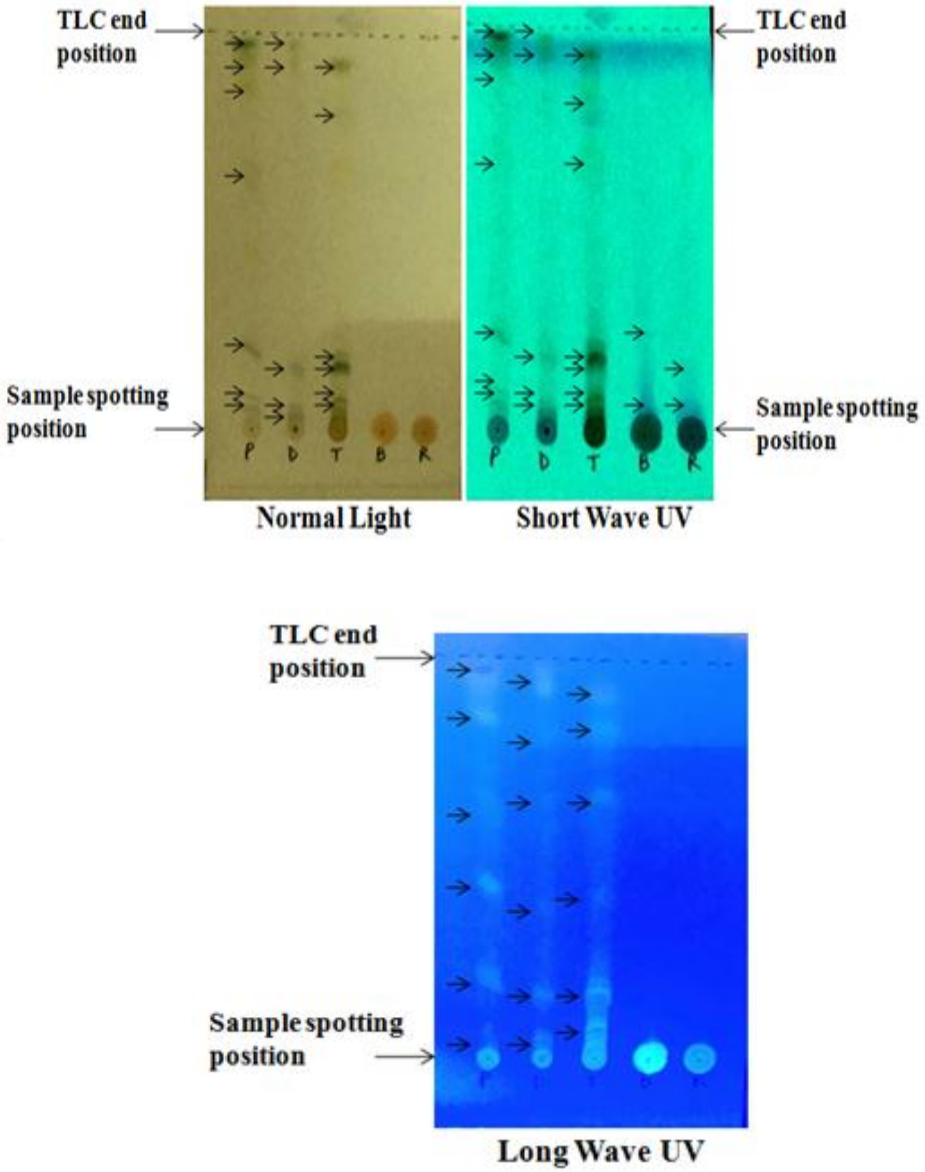

Fig 1: TLC Bio autography result of extracts from selected plants Ficus religiosa (Peepal), Cynodon dactylon (Doob Grass), Tinospora cordifolia and the sea weeds Kappaphycus alvarezii (Red Algae) and Sargasso species (Brown Algae).

P= Peepal, D= Doob Grass, T=*Tinospora cordifolia*, B= Brown Algae, R=Red Algae

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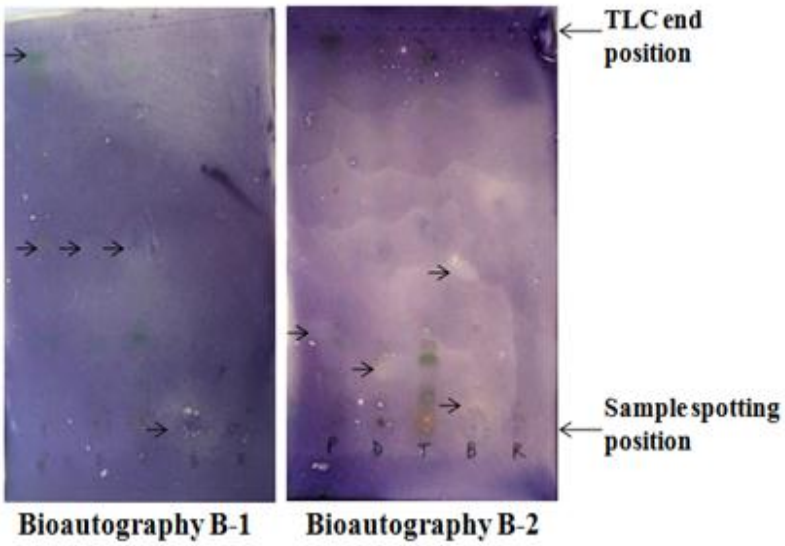

Fig 2: TLC-Bioautography of extracts of Ficus religiosa(Peepal), Cynodon dactylon (Doob Grass) ,Tinospora cordifolia and the sea weeds Kappaphycus alvarezii (Red Algae) and Sargassum species (Brown Algae)against Bacillus subtilis (B1) and Shigella flexneri(B2).

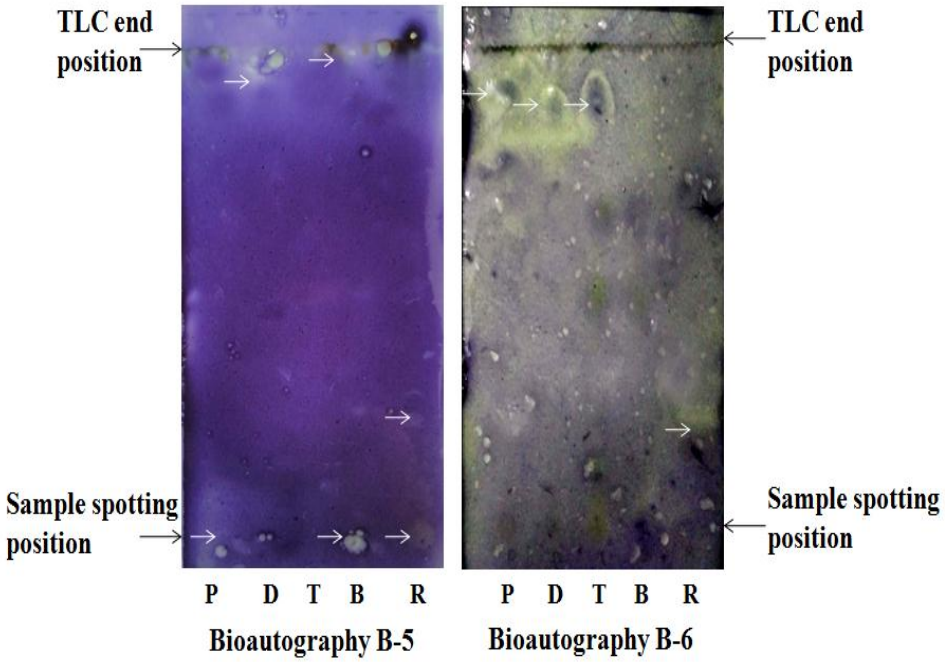

Fig 3: TLC-Bio autography of extracts of Ficus religiosa (Peepal), Cynodon dactylon (Doob Grass), Tinospora cordifolia and the sea weeds Kappaphycus alvarezii (Red Algae) and Sargassum species (Brown Algae) against Escherichia Coli (B-5) Enterobacter cloacae (B-6).

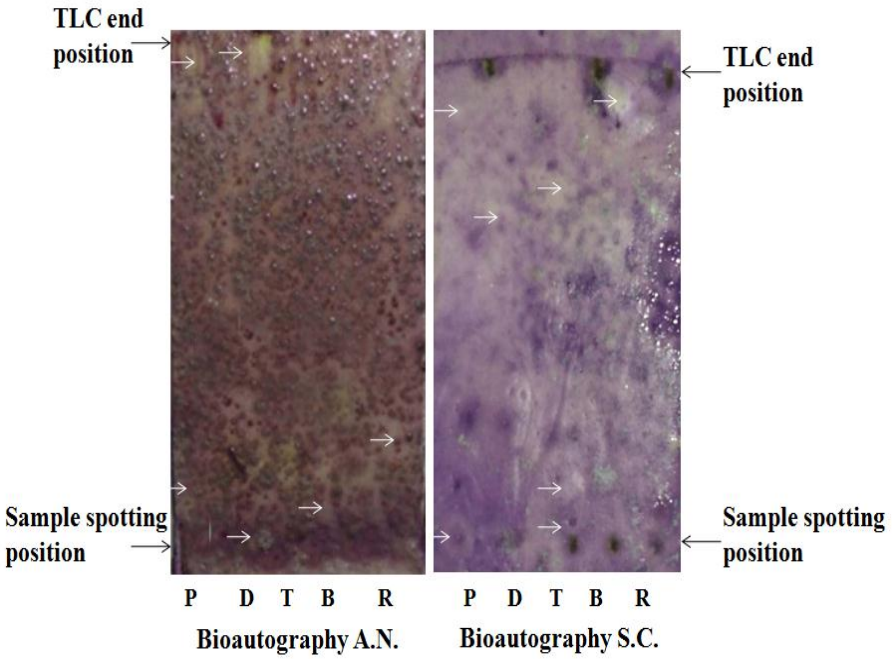

Fig 4: TLC-Bioautography of extracts of Ficus religiosa(Peepal), Cynodon dactylon (Doob Grass) ,Tinospora cordifolia and the sea weeds Kappaphycus alvarezii (Red Algae) and Sargassum species (Brown Algae) against Aspergillus Niger and Saccharomyces cerevisiae.

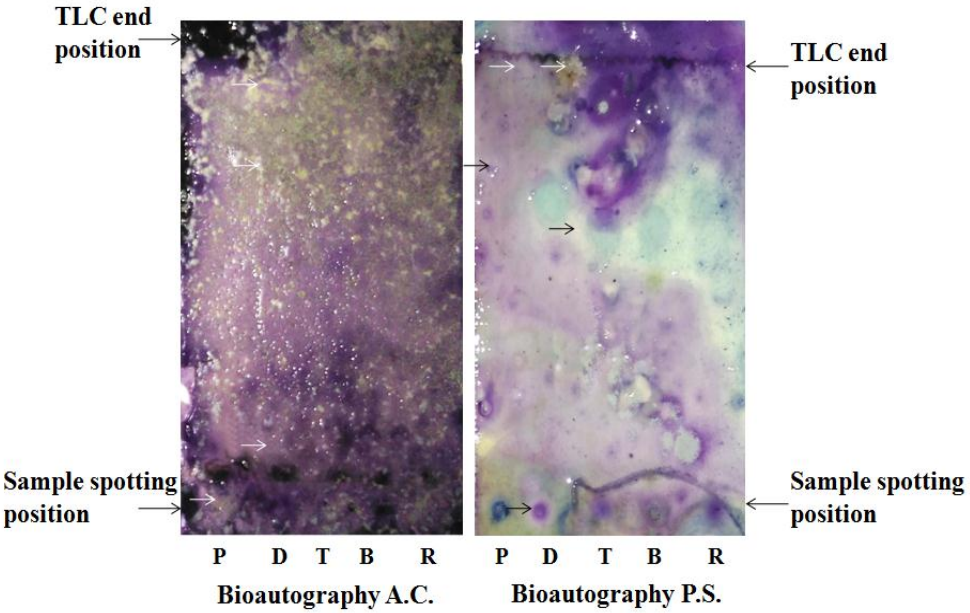

Fig 5: TLC-Bio autography of extracts of Ficus religiosa (Peepal), Cynodon dactylon (Doob Grass), Tinospora cordifolia and the sea weeds Kappaphycus alvarezii (Red Algae) and Sargassum species (Brown Algae) against Aspergillus candidus and Pencillum species.

DISCUSSION

Phytochemical screening revealed the presence of varied chemical components in the different extracts of the plants. It is important to realize that bioautography is not a quantitative measure of antimicrobial activity.[13] It only indicates the number of compounds that were separated with antimicrobial activity. The fact that of the bacteria tested, *Bacillus subtilis* had the highest number of inhibition bands does not mean that this was the most susceptible organism. Similarly, Saccharomyces cerevisiae with the most inhibition bands against fungi does not necessarily have the highest susceptibility among fungal organisms. Some of the compounds are active against both bacteria and fungi, while others are selective in their activity. It is possible that compounds that have activity against all the tested organisms possess a broad antimicrobial action or they may even be general metabolic toxins that could be toxic to animals as well. The absence of bioactivity in some of the plant extracts with this screening method does not preclude that they may be active, as synergistic or additive interactions of plant extracts or phytocompounds is the basis of activity of several herbal formulations (Ahmad and Aqil, 2007). Although some experience is required to obtain good bioautograms especially with fungi, the method is very useful in isolating compounds with antimicrobial activity because the R_f of the active compound can be used in bioassay guided fractionation instead of requiring labour intensive determination of activity of fractions. In an effort to discover new lead compounds from marine algae the inhibitions bands in Sargassum species and Kappaphycus alvarezii do attribute to antimicrobial compounds in these algae extracts, many research groups screen algae extracts to detect secondary metabolites with relevant biological activities. However, more research has to be done on isolation, purification and identification of the active ingredients in algae species in order to understand their bio prospects. The compilation indicates that cow urine extracts of selected algae Sargassum species and Kappaphycus alvarezii and plants Ficus religiosa, Cynodon dactylon, Tinospora cordifolia, exhibit better antimicrobial action against different clinical microbial strain. Over the last decade, there has been a renewed interest in plants; and the pharmaceutical industry considers plants as a viable option for the discovery of new leads One should however keep the possibility that volatile compounds may have evaporated from the chromatogram in mind when using this approach. The extract showed significant antibacterial activity and this good inhibition activity can be used in control of microbial diseases by identifying novel and advanced medicines from cow urine extracts of selected plant species and algae which are future reservoir of new novel pharmaceutical agents. If an extract had a high antimicrobial activity and this was caused by an interaction of compounds

that were not active individually attempts to isolate single active compounds would be futile, this is an important procedure to select species for further work. It was concluded that cow urine itself has antimicrobial property and inhibitory activity of plant extracts can synergistically can be used as a precursors for the synthesis of useful herbal drugs. Thus this novel extracts can be useful in solving the growing crisis and can provide antibiotic resistance for search of new antimicrobial compounds from natural resources.. These products essentially help to identify newer structurally novel natural products which can be used for the treatment of bacterial and fungal infections.

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