Antimicrobial Activities in Crude Extracts of the Flowers of *Plumeriaobtusa*L. and Localization of Active Compounds by Applying Bioautography

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Abstract

Plumeria is a genus of laticiferous tree or shrub grown in lower as well as upper Myanmar. *Plumeria obtusa* Linn belongs to Family Apocynaceae and not only well known as ornamental plant but also as useful medicinal plant for our people in the country. In the research of microbiology and Plant phytochemistry, the study of antimicrobial activities of medicinal plants have clearly been routine or essential works and progressive performance to provide concrete results of antibiotic potentials in the crude drugs of Myanmar Traditional medicinal plants. In the present work, the crude drug obtained by aqueous extract, ethanolic extracts and fresh juice of the flowers of *Plumeria obtuse* L. were used in the antimicrobial activity tests against eleven test organisms. The paper disc diffusion technique revealed that the crude extracts showed high antimicrobial activity against the eight test organisms. The second part of present work is to localize the active constituents in the flower crude extracts by applying "bioautography". The flower extracts were chromatographed onto the filter paper using ethyl acetate saturated with water as solvent and then bioautography was done on the agar plates containing respective test organisms. By reviewing the respective R_f values of clear zones, the localization of compounds with high antimicrobial activity were recorded. It was observed that the flower extract with ethyl acetate showed highest activity on *Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas fluorescens* and *Mallasezia furfur.* It was also found that clear sport of ethyl acetate extracts were recorded with the R_f value of 0.92 on *Bacillus subtilis,* 0.58 on *Candida albicans,* 0.7 on *Escherichia coli,* 0.67 on *Pseudomonas fluorescens* and 0.83 on *Mallasezia furfur* indicated that spot localization of bioactive compound involved in flower extracts *Plumeria obtuse* L.

Keyword: *Plumeria obtuse* L., antimicrobial activity, Bioautography

Introduction

Myanmar is sitting on a gold mine of well-recorded and traditionally well-practiced knowledge of herbal medicines which are clinically important. The major merits of herbal medicines seem to be their efficacy, low incidence of side effects and low cost. One of the ornamental plants in Myanmar, namely Akyaw, is scientifically known as *Plumeria obtuse* L. and belongs to the Apocynaceae family. The plants from this genus are widely cultivated in the tropical and subtropical regions throughout Myanmar. *Plumeria* plants are famous for their attractiveness and fragrant flowers. The essential oils from the flowers are used for perfumery and aromatherapy purposes (Shaida *et al*., 2008).

According to the World Health Organization, 2003, about 80% of the population of developing countries being unable to afford pharmaceutical drugs rely on traditional medicines, mainly plant based, to sustain their primary health care needs. Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological and medicinal activities, higher safety margins and lesser costs. The flowers of *Plumeria obtuse* L.

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are borne in inflorescences (clusters) that form at the ends of the branches on a long thick stalk. Each inflorescence contains many white flowers with a small yellow center creating splashes of color throughout the tree. The flowers are aromatic and use as cough suppressant and widely used in pectoral syrups. The flowers decoction of *Plumeria* was reported to use in Mexico for control of diabetes.

After a literature survey of some Ayurvedic texts and published research articles, for present study, was shortlisted in the aspect of antimicrobial potential. Therefore, the screening of antimicrobial properties of crude extract from *Plumeria obtusa* flower against 11 pathogens has been conducted localization of active components are detected by applying Bioautography.

Materials and Methods

Morphological study was done in Botany Department by using available literature of Lawrence 1969; Dassanayake 1980 and Kress 2003 in library.

Sample collection of Akyaw (*Plumeria obtusa* L.)

The plant samples (the flowers) of Akyaw (*Plumeria obtusa* L.) were collected from the campus of Yangon University.

Preparation of aqueous extract from *Plumeriaobtusa* L.

The flowers were cut into small pieces and dried in the air for two weeks. Then, they were powdered. About 100 g of air dried samples of the flowers were put into the two of 500 ml conical flasks (each putting 100g of samples) and distilled water (1L) was added. The samples were extracted with distilled water by using water-bath (70^{o}C) for six hrs and shaking culture (room temperature) for three days. The solvents with powdered samples were filtered by using filter paper. The remaining residues were discarded. The extracted filtrate was evaporated to dry on the water bath (70^{o}C). The extracts obtained were stored in the refrigerator.

Preparation of ethanolic extract of *Plumeriaobtusa* L.

The flowers of Akyaw (*Plumeria obtusa* L.) were cut into small pieces and dried in the air for two weeks. Then, they were powdered. About 100 g of air dried samples of the flowers were put into the six conical flasks (500 mL) and ethyl alcohol was added. The samples were extracted with 90%, 70% and 50% ethanol by using water-bath (70^{o}C) for six hrs and shaking culture (room temperature) for three days. The extracts with powdered samples were filtered by using filter paper. The remaining residues were discarded. The extracted filtrates were evaporated to dry on the water bath at 70^{o}C. The extracts obtained were stored in the refrigerator.

Preparation of (fresh juice) extract from Akyaw (*Plumeria obtusa* Linn.)

First, the collected flowers were washed in water two to three times. Then, the flowers are sterilized with 70% ethanol for 2 min. After that, they were washed with distilled water. The flowers are done by the use of mortar and pestel. The extract of the flowers was centrifuged at 3000 rpm for 15 min. Finally, the supernatant was collected.

Antimicrobial activity of extracts by paper disc diffusion assay

Screening of antimicrobial activity was done by paper disc diffusion assay according to modified Kirby and Bauer method (WHO, 2003). The size of the paper disc is 6 mm in diameter. The modified Kirby and Bauer method was currently used as a reference method in clinical laboratory. The eleven test organisms (four fungal strains & seven bacterial strains) were utilized for antimicrobial activity at Microbiology Lab. from Department of Botany, University of Yangon.

Preparation of test organisms suspension

A few colonies of the organisms to be tested were picked with a wire loop from the original test tube and introduced into a test tube containing 5 ml of nutrient broth. To obtain a bacterial suspension of moderate cloudiness it was incubated previous day before the actual testing of the sample.

Table (1) Test Organism and Diseases

	Test organisms	Diseases	Code No.
I	*Agrobacterium tumefaciens*	Plant tumor cell	-
II	*Aspergillus flavous*	Bronchitics	-
III	*Bacillus subtilis*	Fever	JAP-0225025
IV	*Candida albicans*	Skin infection, vaginal candidasis, alimentary tract infection	IFO-1060
V	*Escherichia coli*	Cholera, diarrhea and vomiting, urinary tract infections	ATCC-25922
VI	*Micrococcus luteus*	Skin disease	ATCC-23840
VII	*Pseudomonas fluorescens*	Bacteria for leaf blight	-
VIII	*Salmonella typhi*	Typhoid	3/ Sep. 69
IX	*Staphylococcus aureus*	Skin disease, food poison, boils, wound infection	ATCC-12877
X	*Xanthomonas oryzae*	Bacteria for leaf blight	-
XI	*Mallasazia furfur*	Dundraff	

Preparation of plant extract

Extract of Akyaw (*Plumeria obtusa* L.) 0.1 g was dissolved with 1 ml of respective solvent to form 100 mg/ml plant extract solution. From the stock solution, 20µl of solution were impregnated to discs resulting in (2 mg/disc).

Preparation of disc

The disc, six millimeter in diameter were punched from No. 4 Whatmen filter paper and were sterilized by autoclaving followed by dry heat at 60ºC for one hour. They were then impregnated with extracts (2 mg/disc) and then allowed to be dried at 37ºC.

Antimicrobial susceptibility testing

Nutrient agar is usually prepared. After autoclaving, 30 ml of media was poured into petridishes. Before pour, 0.5 ml of culture media is added into petridishes. After the inoculums have dried for a few minutes, the dried discs impregnated with plant extracts were placed on the agar. A control disc, impregnated with solvent only. Plates were incubated at 37ºC in an incubator within 30 minutes after inoculation. After overnight incubation, the zone diameters (including 6 mm disc) were measured with the thin plastic transparent ruler.

Extraction (Paper Chromatography)

The filter paper (Toyo Advance, Japan) and ethyl acetate saturated with water was used for the characterization of antimicrobial agent. The ethanolic extracted samples (50µl) were applied on the paper strip and allowed to dry. The papers were chromatographed in ethyl acctate solvent. Then bioautography was done to check the antimicrobial activity. Each paper was placed on assay agar plates. In the same method as paper disc diffusion assay. After one hour the paper was taken out, and then the plates were incubated for 24-36 hours. Then, the inhibitory zone was measured yielding an R_f value for the corresponding bioactive compound. The R_f value was calculated in the following formula.

$$R_f \text{ value} = \frac{\text{Distance of compound from the origin}}{\text{Distance of solvent from the origin}}$$

Results

Morphological characters of Akyaw (*Plumeria obtuse* L.)

Habit	-	Small tree.
Stem	-	Aerial, erect, cylindrical, branched, wood solid, green; latex present.
Leaves	-	Simple, petiolate, exstipulate, large, entire, oblong shape and the tip of the leaf is obtuse.
Inflorescence	-	Umbellate cyme.
Flower	-	Ebracteate; pedicellate, complete actinomorphic, pentamerous, large, fragrant, white, perigynous.
Calyx	-	sepals 5, synsepalous, quincuncial aestivation.
Corolla	-	Petal 5, synepetalous, tubular, twisted, the lobe obvate, the inner white with a yellow centre.
Androecium	-	Stamens 5, apostemanous, free, alternate to petals, epipetalous, anther dithecous, basifixed and introrse.

Gynaecium - Bicarpellary, syncarpous, ovary half-inferior, many ovules in each loculus, axile placentation; stigma bifid.

Extraction of dried crude powder of Akyaw (*Plumeria obtusa* L.)

The powder of dried flower was extracted with polar solvents (watery, ethanol). Some of the active constituents from the flower by the aid of successive extraction. After removing each solvent by evaporation, crude extract were dried and kept in refrigerator. Then, the residues were weight again. The watery extract and ethanolic extract of 100 g of dried crude powder data were tabulated in Table (2).

Table (2) Extraction value of dried crude powder

Solvent	Extraction Value (%) g	
	Heating (H)	Shaking (S)
Water	5.6	5.6
90% ethanol	6.4	6.0
70% ethanol	5.8	5
50% ethanol	6.1	4.6

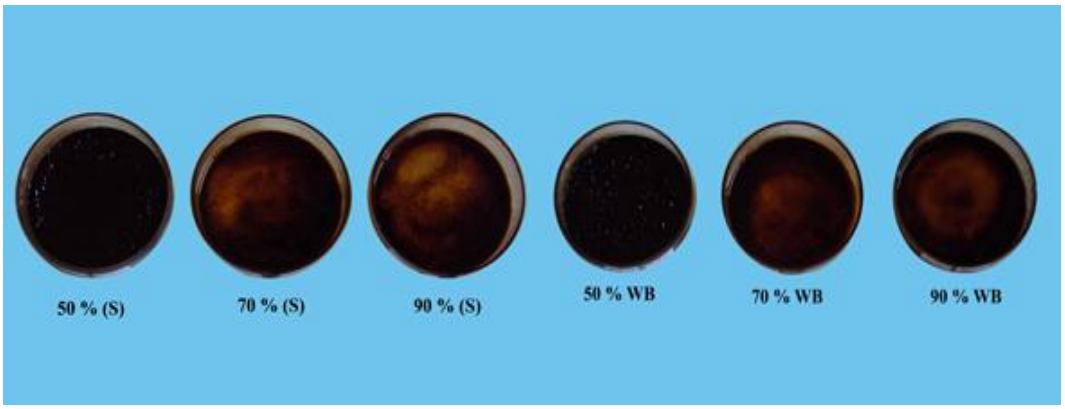
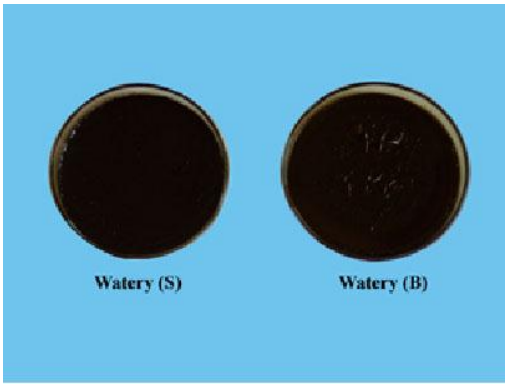
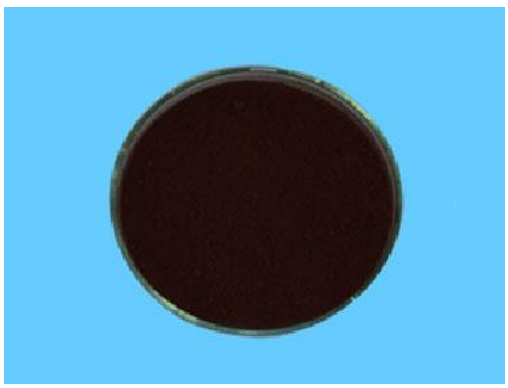
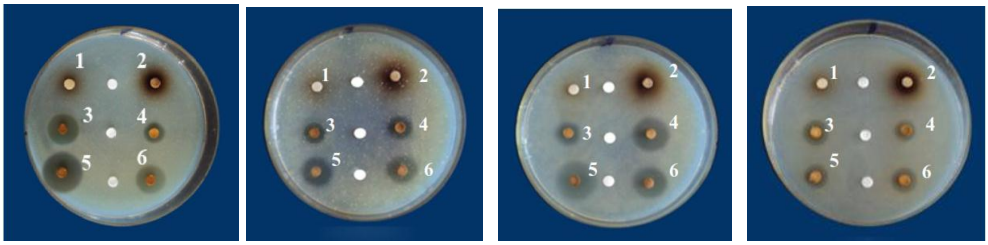


Fig (2) Ethanolic extract of dried crude (Heating condition)



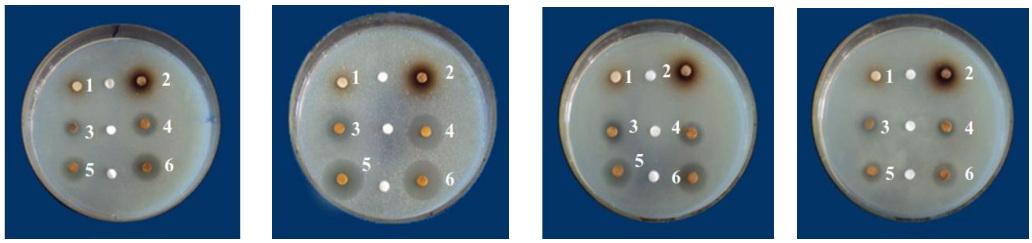




Aspergillus flavous *Bacillus subtilis* *Candida albicans* *Escherichia coli*

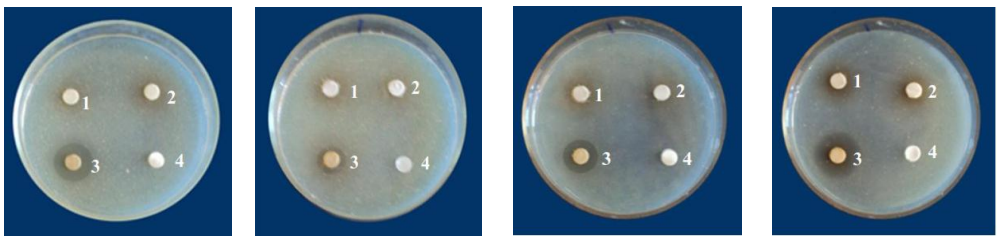
1,2 = 50% ethanolic extract (H&S) 3,4 = 70% ethanolic extract (H&S)

5,6. 90% ethanolic extract (H&S)



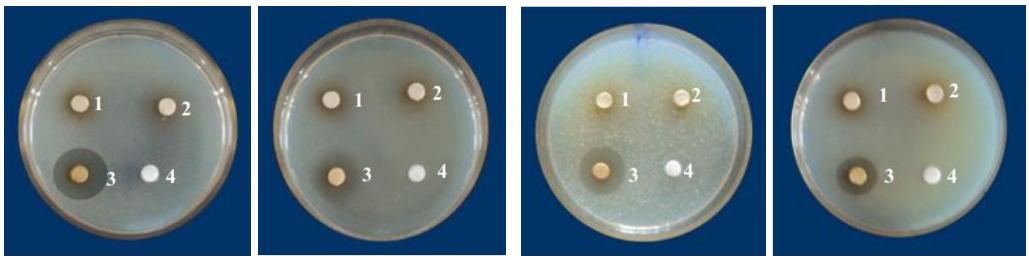
Pseudomonas fluorescens *Salmonella typhi* *Staphylococcus aureus* *Mallasezia furfur*

Figure (5) Zone of inhibition of crude extract of flower on test organisms



Aspergillus flavous *Bacillus subtilis* *Candida albicans* *Escherichia coli*

1,2 = watery extract (S&H) 3.fresh juice 4.control



Pseudomonas fluorescens *Salmonella typhi* *Staphylococcus aureus* *Mallasezia furfur*

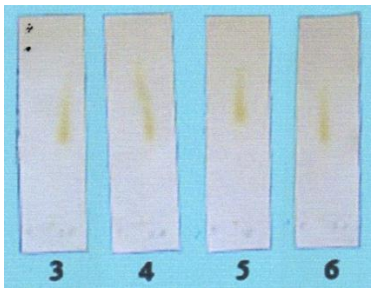
Figure (6) Zone of inhibition of watery and fresh juice extract of flower on test organisms

Table (3) Determination of zone of inhibition on ethanolic extract of flower on different microorganisms

Test Organisms	1 H (50%)	2 S (50%)	3 H (70%)	4 S (70%)	5 H (90%)	6 S (90%)
I	-	-	-	-	-	-
II	-	-	17	10	21	15
III	-	-	15	17	22	19
IV	-	-	15	19	23	20
V	-	-	14	11	15	15
VI	-	-	-	-	-	-
VII	-	-	10	14	18	16
VIII	-	-	15	13	18	18
IX	-	-	15	20	22	22
X	-	-	-	-	-	-
XI	-	-	11	12	14	16

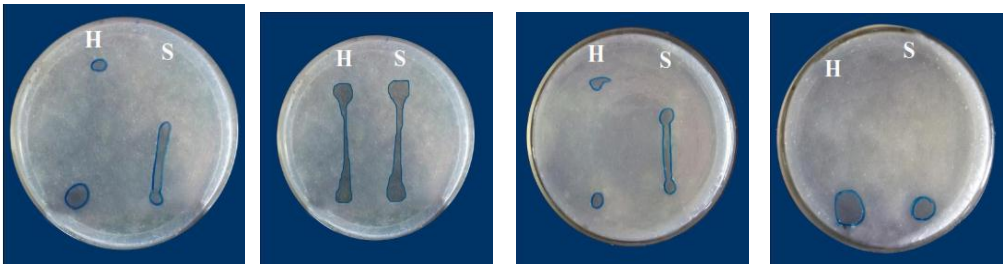
Table (4) Determination of zone of inhibition on watery extract of flower on different microorganisms

Test Organisms	Diameter Zone of inhibition (mm)		
	Watery extract		
	1Shaking	2Heating	3Fresh Juice
I	-	-	-
II	-	-	15
III	-	-	10
IV	-	-	13
V	-	-	18
VI	-	-	-
VII	-	-	20
VIII	-	-	14
IX	-	-	17
X	-	-	-
XI	-	-	16

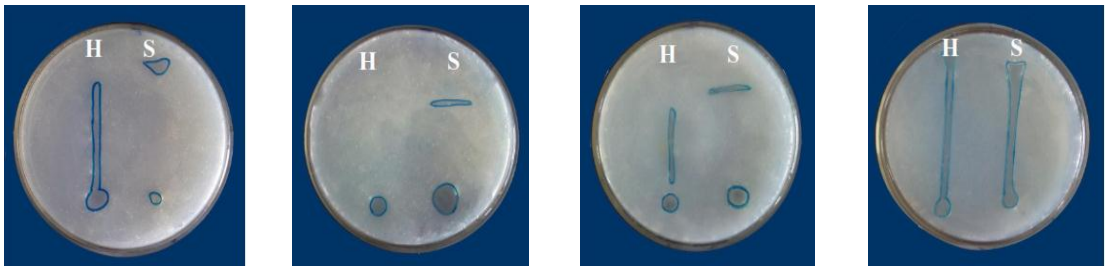


3, 4 = 70 % ethanolic extract 5, 6 = 90 % ethanolic extract

Figure (7) Paper chromatography test by filter paper

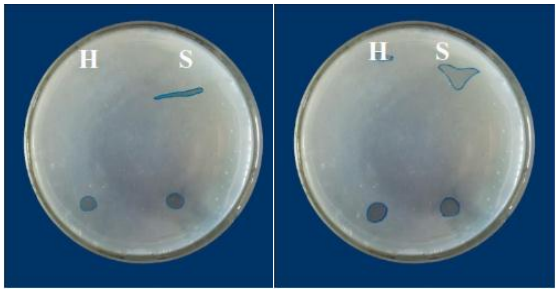


Aspergillus flavous *Bacillus subtilis*



Candida albicans *Escherichia coli*

Pseudomonas fluorescens *Staphylococcus aureus*



Mallasezia furfur

Figure (8) Bioautography of ethanolic flower extract on test organisms

Table (5) The Results of Bioautography of extract *Plumeriaobtusa*L.flower by using Ethyl acetate

Organisms	Concentration of ethanol	R_f values obtained	
		Heating	Shaking
Aspergillusflavous	70% 90%	**0.98** 0.95	0.67 0.95
Bacillus subtilis	70% 90%	**0.92** 0.03	0.67 0.03
Candida albicans	70% 90%	0.67 0.10	**0.98** **0.58**
Escherichia coli	70% 90%	0.63 0.95	**0.70** 0.95
Pseudomonas fluorescens	70% 90%	0.50 0.00	0.45 **0.67**
Staphylococcsaureus	70% 90%	0.91 0.95	0.98 0.50
Mallasezia furfur	70% 90%	0.10 **0.95**	**0.83** **0.93**

Discussion and Conclusion

Plumeria obtusa L. (Akyaw) is an ornamental tree with latex. In rural area the plant parts are used to treat the disease such as asthma, diarrhea, dysentery, toothache, earache and wound healing etc. Most of these diseases are caused by microbes. In the present work, the main constituents of *Plumera obtuse* L. flowers may be essential oil so that they provided fragrance. According to Talpade *et al.* (2015) also reported that *Plumeria* flowers also contain resin, quercetain and traces of kaemfeol. In the present work, the flower powdered was extracted with water, 50%, 70%, 90% ethanol and then fresh juice was also made by mortar and pestel. These extracts are subjected in the antimicrobial activity tests. The results are shown in Table (2) and Figure (1, 2, 3) that the higest antimicrobial activity was provided by 90% ethanol extract against on *Candida albicans, Staphylococcus aures, Bacillus subtilis* and moderate activity on *Aspergillusflavous, Pseudomonoassubtilis*and *Salmonella typhi*.

There was no antimicrobial activity on *Agrobacterium tumefaciens* and *Xanthomonas oryzae*. Moreover, the fresh juice obtained by pounding the flower also gave high antimicrobial activity in *Escheria. Coli* and *Pseudomonas fluorescens*. The moderate activity in *Aspergillus* flavous, *Staphylococcus aureus* and *Mallasezia furfur*. Fresh juice was no activity on *Agrobacterium tumefaciens*, *Micrococcus luteus* and *Xanthomonas oryzae*. In the beginning of present work, altogether eleven test organisms but the activity of extracts were giving by eight test organisms as describe the Table (3, 4), Figure (5, 6).The discovery of antimicrobial agent are undeniably one of the most important factors in the study of plants phytochemistry in the 20th century. In, 2014, Shinde, was reported that *Plumeria bark* extract able to show antiulcer effects and heal Indomethacin included stomach ulceration by triterpenoids action.

These researches also review that antimicrobial activity on test organisms. The localization of antimicrobial compound in the different extracts were supported by biooutgraphy technique. The respective results of R_f value are tabulated in Table (5) and Figure (8). It is observed that R_f value obtained by two condition (Heating and Shaking) provided the better data of localization of active constituents against the some test organism. The result from the present study may be helpful in the further study concerning, extraction and purification of bioactive compound in the crude extract of *Plumeria obtuse* flower. An important conclusion made during the present study was that the plant extracts are highly effective against *Pseudomonas fluorescens,* an important gram negative pathogen as well as *Streptococus aureus* an important gram positive pathogen. In 2014, Ali *et al*., also provided the similar result.

One of the approaches is to screen local medicinal plants for possible antimicrobial properties. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, very little information is available on such activity of medicinal plants. This can be considered as an important observation as *Pseudomonas fluorescens,* an opportunistic pathogen and *Staphylococcus aureus* have been found to be resistant to a variety of known antibiotics. On the basic of the results obtained, it can be deduced that the crude extract of *Plumeria obtusa* used in the present study exhibit good antimicrobial activity against both Gram positive as well as Gram negative organisms and also on some fungi.

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