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Screening of Various Plant Extracts for Antimicrobial Activity against Multi Drug Resistant *P.aeruginosa, S.aureus* and *B.subtillis*

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Abstract

A vast variety of plants in India are of medicinal importance. A large number of chemical compounds of medicinal significance have been isolated from plants. The conventional crude drug are mainly synthesized from the different plant extracts. Different parts of plants (root, leaves and flowers) produce different types of metabolites which can have different bioactivities. Plants consist of a range of different property compounds which show two different way of its effect on the microorganism; either suppress the growth of microorganism or cause the death of pathogen. Such components can be exploited as potent molecules/compounds in the search of new antibiotic drugs. In the current situation of increasing drug resistance, there is need to explore for novel antimicrobial compounds. This study was done to investigate the potential antimicrobial activity in six medicinally important plants against *P.aeruginosa*, *B.subtillis* and *S.aureus. D.innoxia, A.indica, A.marmelos* and *O.sanctum* extracts showed positive results against *S.aureus* and *B.subtillis*. Against *P.aeruginosa*, *A.marmelos D.innoxia* and *O.sanctum* seeds showed positive response. TLC bio-autographs were prepared for the extracts showing positive response. Analysis of phytochemical of the plant extracts reported the availability of various constituents.

Key words: plant extract, antimicrobial activity, phytochemical analysis, TLC bio-autography

Introduction

Plants have very significant contribution in the development of human civilization. They have provided food, clothes and medicines to humans. Today numerous natural compounds derived from plants are being used to fight with infectious microbes [1]. A vast variety of plants in India are of medicinal importance. In *Charak Samhita*, ancient literature of India, regarding medicine also describe the use of plant for medicinal purpose. The majority of chemical compounds of medicinal significance that have been isolated from plants come under the category of secondary metabolites [2]. Most of the secondary metabolites are produced as a defense mechanism against pathogen organisms such as bacteria and fungi. Therefore they can be used as antimicrobial

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agents depending on their effectiveness [3]. Failure of conventional drug against the microorganism which are acquiring multi drug resistance potential has created the situation to search the effective novel compound to fight such pathogens. Multi Drug Resistance has been shown in common pathogens [4]. The current work was designed to investigate the potential antimicrobial compounds in various plant sources which can be used to target microorganisms.

Materials and Methods

Plants to be evaluated:

Following plants were chosen for the study. The plant samples were collected from Kangra region of Himachal Pradesh:

Table No. 1: Plants and their part name used as sample for antimicrobial activity

S. No.	*Plant name*	Parts Collected
1	*Datura innoxia*	Leaves
2	*Azadirachta indica*	Leaves
3	*Aloe vera*	Leaves
4	*Ocimum sanctum*	Leaves and Seeds
5	*Terminalia arjuna*	Leaves
6	*Aegle marmelos*	Leaves

Microbes to be targeted

Following microbes were selected as test organisms:- *Bacillus subtilis* (MTCC 121), *Pseudomonas aeruginosa* (MTCC 424) and *Staphylococcus aureus* (MTCC 6908). The pure strains of test microorganisms were procured form IMTECH (Institute of Microbial Technology) Chandigarh. The strains were revived on Nutrient broth at 37^{o}C for 24hrs. The slants and glycerol stocks of the cultures were prepared for future uses.

Preparation of Plant Extracts:

Following steps were taken to prepare the plant extracts:

Drying and grinding of samples:

The plant's parts were dried in shadow and grinded in pestle and mortar to achieve powder form.

Soxhlet extraction

The powder (25g) of plant parts was placed in thimble made of Whatman filter paper. The solvents chosen for the extraction process were: Acetone, Methanol, Hexane and Chloroform. The thimble was placed in Soxhlet apparatus while solvent was placed in distilling pot. The distilling pot was then heated to the boiling temperature of solvent. This process was run for 6-8 hrs [5].

Concentration of Plant Extracts

The solution containing the plant components after the extraction operation was heated to evaporate the solvents. The plant extracts were dried and again dissolved in the same solvent to give final concentration of 100mg/ml Concentrated plant extracts were stored in test tubes at 4^oC for further use [5].

Screening of plant extracts for the antimicrobial activity

Testing selected bacteria for Antibiotic sensitivity

To find the antibiotic sensitivities of the bacteria chosen, the bacterial cultures were inoculated on the Muller Hinton Agar using swabbing method. The antibiotic discs were applied on the surface of plate. Next step was incubation at 37^oC for 24 hrs. Presence of Zone of inhibition indicated the sensitivity of bacteria towards the antibiotic.

Testing plant extracts for antimicrobial activity

Agar Gel Diffusion assay was utilised to test the effectiveness of plant extracts against the microorganism. Muller Hinton Agar was prepared, autoclaved and poured into petridishes. Small wells (3mm diameter) were cut in the gel through the sterile puncher. The bacterial culture was spread on the nutrient agar using swabbing method. 50µl of Plant extract was added into the wells. Incubation of the dishes at 37^oC for 24 hours was given. In this experiment, Streptomycin (2mg/ml) and autoclaved distilled water were selected for positive control and negative control respectively. Next step was to look for the zone of inhibition and measure the diameter of it. The effectiveness of plant extract was proportional to the diameter of clear zone [6].

Thin layer chromatography of plant extracts

Thin Layer Chromatography of the plants was performed by the methods described by Sasidharan *et al*., [7]. TLC plates of silica gel were prepared; 10µl of plant extract was applied on the bottom of plate 2 cm above the edge. Plates were placed in the mobile phase- Chloroform: Methanol: Water (65:30:5) in a running chamber. When solvent front reached near the top of plate, the plate was removed and the solvent was air dried. Methanol: H_2SO_4 (95:5) was used as spraying agent to visualize the spots or bands.

TLC Bioautography

TLC Bioautography was performed by the method described by Selowa *et al.*, [8]. The developed plates were sprayed with growing culture of test microorganisms. The plates were placed in a humid chamber and incubation at 37^{o}C was given for 24 hours. TTC solution (1mg/ml) was then sprayed on the plate and again incubation of 12 hrs was given. The appearance of pinkish red colour indicated the presence of viable organism while the areas where the colour was not changed were considered as ones where the growth was inhibited [9].

Phytochemical analysis

Phytochemical analysis was done for the 10 selected plant extracts which gave best results as per standard methods.

Detection of Alkaloids

For the Alkaloids detection, diluted HCl was taken as solvent and after dissolution, extract was filtered [10].

Mayer's Test

Mayer's Reagent (Potassium Mercuric Iodide) was added in the filtrate. Presence of yellowish precipitates indicated the availability of alkaloids [10].

Wagner's Test

Wagner's Reagent (Iodine in Potassium Iodide) was put down in the filtrate. Presence of brownish red precipitates indicated the availability of alkaloids [10].

Hager's Test

Hager's Reagent (Saturated Picric acid solution) was added in the filtrate. Yellow colored precipitates showed the presence of alkaloids [11].

Detection of Carbohydrates

For the Carbohydrates detection, distilled water was taken as solvent and after dissolution, extract was filtered [12].

Molisch's Test

Few drops of alcoholic α-napthol solution were added in test tube containing filtrate. Presence of blue violet ring at junction confirmed the availability of carbohydrates.

Benedict's test

Benedict's reagent was added in the filtrate and were heated in boiling water bath. Orange red precipitates indicated the presence of reducing sugars.

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Detection of glycosides

Extracts were hydrolyzed with dilute HCl and were then tested for the presence of glycosides [13].

Modified Borntrager's Test

To 5ml of extract 5ml Ferric Chloride (5%) was added. The mixture was kept in boiling water for 5 minutes. The solution was cooled and Chloroform was added. After shaking the mixture the chloroform layer was separated and equal volume of dilute ammonia was added. Appearance of pinkish red colour in ammonia layer indicated the presence of glycosides.

Detection of Phenols

Ferric Chloride test: Treated Extracts with 3-4 drops of Ferric Chloride (5%) solution. Presence of bluish black precipitates indicated availability of phenols [14].

Detection of Tannins

Gelatin test: Added extracts to gelatin solution (1%) containing sodium chloride (10%). Presence of tannins was indicated by formation of white precipitates [11].

Detection of Flavonoids

Alkaline Reagent Test:

Plant extract were treated with few drops of Sodium Hydroxide. Appearance of yellow colour which disappeared with addition of dilute HCl indicated the availability of flavonoids **[**15].

Results and Discussions

Dry Weight of plant extracts

The weight of dried plant extracts was measured in grams mentioned in fig. no.1. Different quantities of extracts were obtained from different solvents and different plants as shown in the graph.

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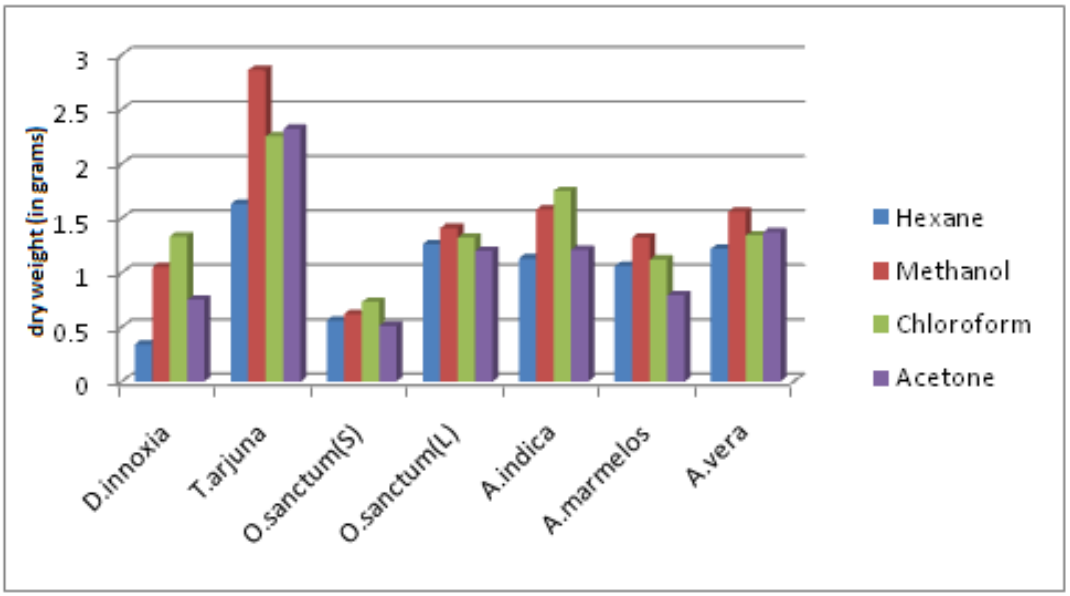
Fig. No. 1: Amount of dry extract obtained per 25 g of plant sample; x axis: Plant samples; Y axis: Dry weight in grams



Antimicrobial Testing of Plant Extracts

Antimicrobial testing of plant extracts was done on test microorganisms. *A.indica* (M, C, A), *A.marmelos* (H, C, A) and *D.innoxia* (H, C, M, A) were giving positive results against *S.aureus*. Against *B.subtilis*, *Aloe vera* (M), *A.indica* (M, C, A), *A.marmelos* (M, C, A) and *D.innoxia* (H, M, C, A) were showing positive results. Against *P.aeruginosa* only *A.marmelos* (M, C, A) and *O.sanctum* extracts were giving positive results.

Table No. 2: Antimicrobial testing for Plant Extracts

S. No	Plant Extract	Solvent	*S.aureus*	*B.subtilis*	*P.aeruginosa*
1	***Aloe vera***	Hexane	-	-	-
2	***Aloe vera***	Methanol	-	+	-
3	***Aloe vera***	Chloroform	-	-	-
4	***Aloe vera***	Acetone	-	-	-
5	***Azadirachta indica***	Hexane	-	-	-

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6	***Azadirachta indica***	Methanol	+	+	-
7	***Azadirachta indica***	Chloroform	+	+	-
8	***Azadirachta indica***	Acetone	+	+	-
9	***Aegle marmelos***	Hexane	+	-	-
10	***Aegle marmelos***	Methanol	-	+	+
11	***Aegle marmelos***	Chloroform	+	+	+
12	***Aegle marmelos***	Acetone	+	+	+
13	***Datura innoxia***	Hexane	+	+	-
14	***Datura innoxia***	Methanol	+	+	-
15	***Datura innoxia***	Chloroform	+	+	-
16	***Datura innoxia***	Acetone	+	+	-
17	***Ocimum sanctum***	Hexane	-	-	-
18	***Ocimum sanctum***	Methanol	-	-	-
19	***Ocimum sanctum***	Chloroform	-	-	+
20	***Ocimum sanctum***	Acetone	-	-	+
21	***Terminalia arjuna***	Hexane	-	-	-
22	***Terminalia arjuna***	Methanol	-	-	-
23	***Terminalia arjuna***	Chloroform	-	-	-
24	***Terminalia arjuna***	Acetone	-	-	-

Where (+) sensitive, (-) resistant

Antimicrobial activity of plant extracts against *P.aeruginosa*

The diameter of clear zone was measured. Seven extracts were showing positive results against *P.aeruginosa*, in Fig. No. 2, out of all the extracts tested. Maximum clear zone (13mm) was shown by Chloroform extract of *O.sanctum* leaves. Other effective extracts were *O.sanctum* seeds(A, C) and *A.marmelos* (M, C, A).

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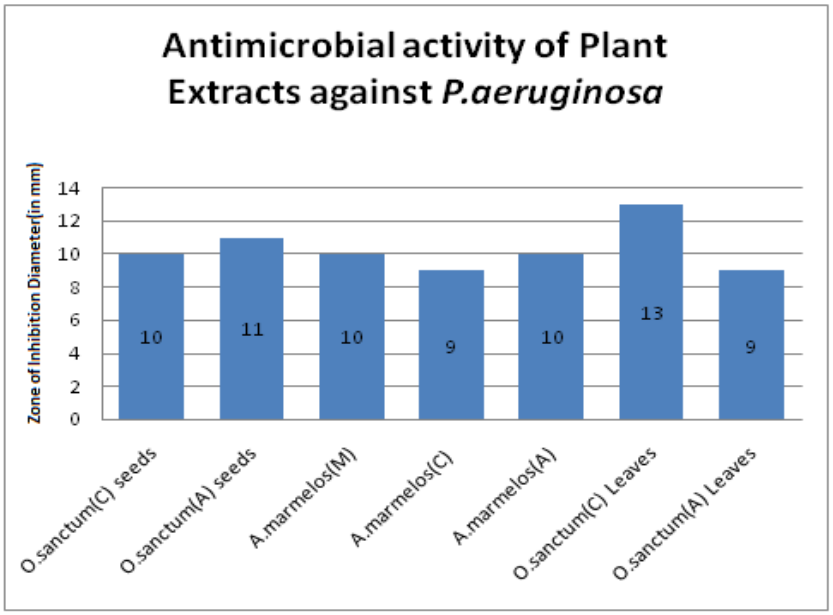
Fig. No. 2: Antimicrobial activity of Plant Extracts against *P.aeruginosa.* X axis: Plant Extracts, Y axis: clear zone diameter (in mm)



Antimicrobial activity of Plant Extracts against *S.aureus*

Out of total extracts tested, ten extracts were showing positive results against *S.aureus* in Fig. No. 3. Extracts of *D.innoxia* were most effective against the test organism. All four extracts of *D.innoxia* showed positive results. Methanol extract of *D.innoxia* was giving maximum zone of inhibition with a diameter of 24 mm followed by hexane extract (20 mm), acetone extract (19 mm) and chloroform extract (17 mm) [16]. Three extracts of *A.indica* (M, C, A) and *A.marmelos* (H, C, A) were showing positive results[17].

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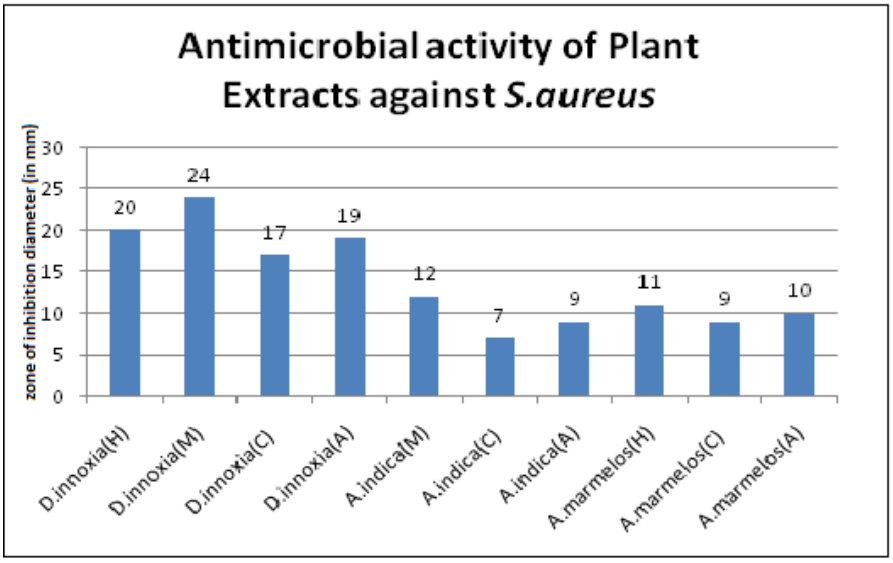
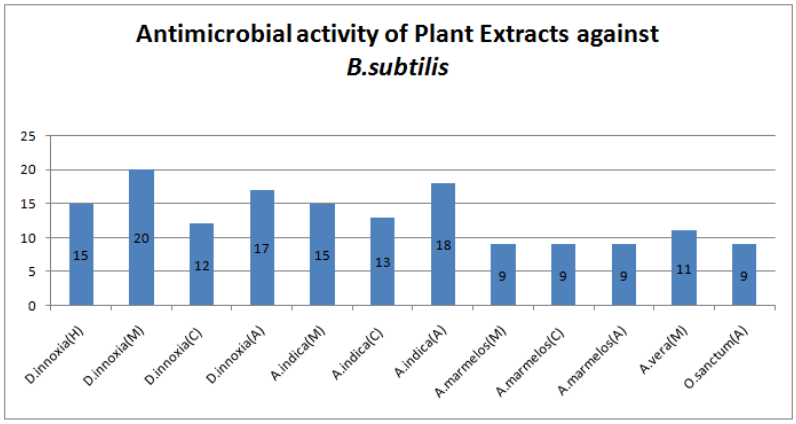
Fig. No. 3: Antimicrobial activity of Plant Extracts against *S.aureus.* X axis: Plant Extracts, Y axis: clear zone diameter (in mm)



Antimicrobial activity of Plant Extracts against *B.subtilis*

Against *B. subtilis*, 12 extracts were giving positive results in Fig. No. 4. Methanol extract of *D.innoxia* was most effective with zone of inhibition of 20 mm. All four extracts (H, M, C, A) of *D.innoxia* showed positive results. While for *A.indica* only M, C and A extracts showed positive results. For *A.marmelos* the extracts showing positive results were H, C and A. Other samples showing positive results were *A. vera* (M extract)[18] and *O.sanctum* (A extract) [19].

Fig. No. 4: Antimicrobial activity of Plant Extracts against *B.subtilis.* X axis: Plant Extracts, Y axis: clear zone diameter (in mm)



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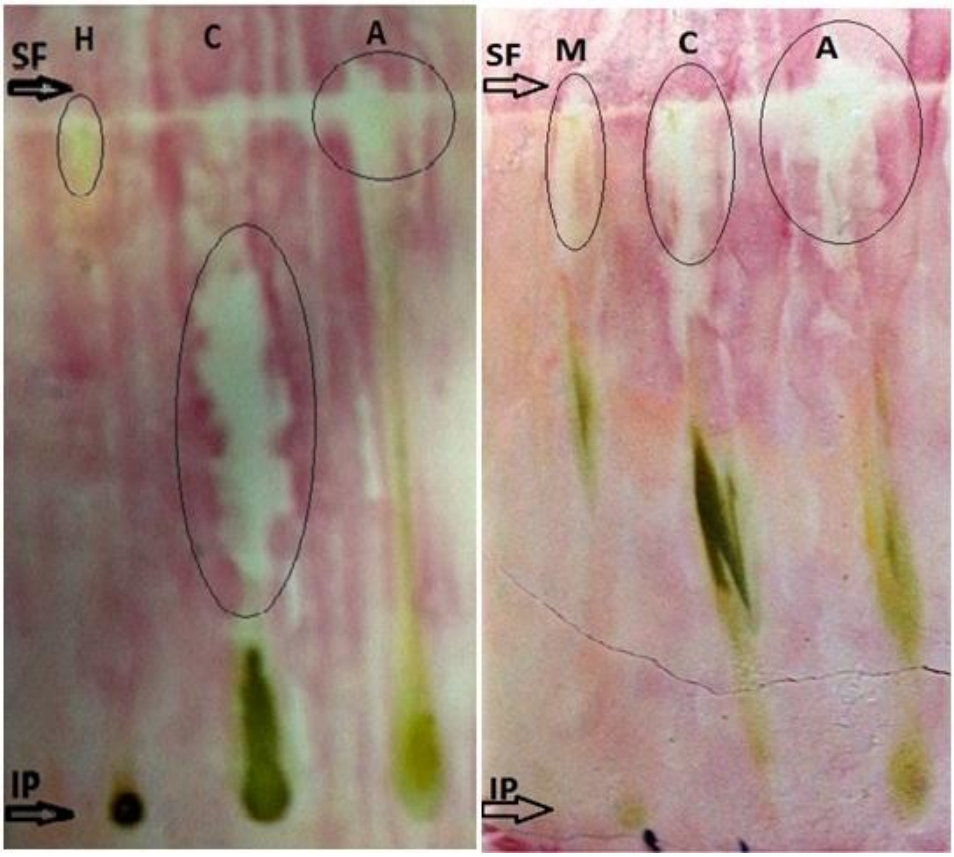
TLC Bioautography

To screen the different antibiotic compound available in various plant extract used Bioautography. Inhibition zones were identified as white bands on pink background in Fig. No. 5. White areas indicated the regions where the reduction of TTC to formazan could not happened due to availability of bioactive compounds that inhibited the growth of the test organism.

Fig. No. 5: Bioautograms of extracts (H: Hexane, M: Methanol, C: Chloroform, A: Acetone) of *A. marmelos* against *S.aureus* (Left) and *B.subtilis* (Right). Zone of inhibitions are indicated by the encircled areas along the running track of extracts in plate. SF indicates Solvent Front; IP indicates Initial Point where extracts were applied



Phytochemical Analysis

Phytochemical analysis was performed on the 10 plant extracts of *D. innoxia, A. indica* and *A. marmelos* which gave best results for antimicrobial activity.

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Table No. 3: Phytochemical Analysis results of plant extracts

S.No.	Plant Extract	Mayer's Test	Wagner's Test	Hager's Test	Gelatin Test	Modified Borntrager's test	Alkaline Reagent Test	$FeCl_3$ test	Benedict's test	Molisch's test
1	*D.innoxia(H)*	-	-	-	-	+	-	+	-	+
2	*D.innoxia(M)*	+	+	+	-	+	-	-	-	+
3	*D.innoxia(C)*	-	+	-	-	+	+	+	+	+
4	*D.innoxia(A)*	+	+	+	-	+	+	-	+	+
5	*A.indica(M)*	+	+	+	-	+	+	+	+	+
6	*A.indica(C)*	-	+	+	-	+	+	+	+	+
7	*A.indica(A)*	+	+	+	-	+	-	-	+	+
8	*A.marmelos(H)*	-	-	-	-	+	-	-	-	+
9	*A.marmelos(C)*	-	-	-	-	+	-	+	+	+
10	*A.marmelos(A)*	+	+	+	-	+	+	-	+	+

Discussion:

Total twenty eight extracts were tested on each test microorganism. Extracts of *D. innoxia* were showing antimicrobial activity against *S. aureus* and *B. subtilis.* Methanol extract was found to be most active against both of the microorganisms with zone of inhibition diameter of 24 mm and 20 mm respectively. There was no activity indentified against *P. aeruginosa* by extracts of *D. innoxia.*

Antimicrobial activity of *A. indica* was observed against targeted *S. aureus* and *B. subtilis*. The best results were shown by Methanol extract against *S. aureus* (12 mm) and by Acetone extract against *B. subtilis* (18 mm). There was no activity observed against *P. aeruginosa*.

T. arjuna did not show antimicrobial activity against any of microorganisms used in the study. The extracts of *O. sanctum* were shown its activity against *P. aeruginosa* and *B. subtilis.* Only Chloroform and Acetone extracts of both seeds and leaves showed positive results against *P. aeruginosa.* The maximum clear zone diameter for targeted *P. aeruginosa* was 13 mm in case of seeds extract in chloroform solvent.

A. marmelos exhibited antimicrobial activity against all three test microorganisms. Against *S. aureus* Hexane, Chloroform and Acetone extracts were active. The best result against *S. aureus*

was shown by Hexane extract. Against *B. subtilis* the extracts displaying positive results were acetone, chloroform and methanol. All three extracts were having same activity against *B. subtilis* with zone of inhibition diameter of 9 mm. Only Chloroform and Acetone extracts showed positive results against *P. aeruginosa* with zone of inhibition of 9 mm and 10 mm respectively.

In case of *A. vera,* only methanol extract showed antimicrobial activity against *B. subtilis.* The diameter of zone of inhibition was 11mm.

Extracts giving best results were selected for further studies. Thin Layer Chromatography was done on the selected extracts using Chloroform: Methanol: Distilled Water (65:30:5) as solvent system. The components of extracts were separated on TLC plate and were visualized as separate bands.

To check further bioactivity on TLC plates, bioautography was performed. Clear zones were identified as white bands on pink background. White areas indicated the regions where the reduction of TTC to formazan could not occurred due to presence of bioactive compounds that inhibited the growth of the test organism.

Phytochemical studies of the extracts revealed the presence of various components. Glycosides were present in all the extracts. Alkaloids were present in extracts of *D.innoxia* (Methanol, Chloroform and Acetone), *A.indica* (Methanol, Chloroform and Acetone) and *A.marmelos* (Acetone).

Tannins were absent in all extracts as gelatin test was negative for all. Flavonoids were present in *D.innoxia* (Chloroform, Acetone), *A.indica* (Methanol, Chloroform) and *A.marmelos* (Acetone).

Presence of phenols was detected by Ferric Chloride Test. Phenols were present in *D.innoxia* (Hexane, Chloroform), *A. marmelos* (Chloroform) and *A.indica* (Methanol, Chloroform). Carbohydrates were found to be present in all the extracts. Further studies are required to make the relation of present phytochemical with antimicrobial activity. *A. marmelos* can be used as source for broad spectrum antimicrobial agent so further exploration are required. *D. innoxia* with the combination of Methanol may be effective source to fight with pathogen.

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