STUDIES ON SCREENING OF ANTIMICROBIALS OF SOME MEDICINAL PLANTS BY BIOAUTOGRAPHY

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ABSTRACT

The methanol extracts of *Ocimum tenuiflorum , Azadirachta indica ,Mangifera indica ,Allium sativum*, were screened for their antimicrobial activity. The test organisms included bacteria (*S.aureus*), and fungi (*C. albicans*). A simple bioautographic procedure, involving spraying suspensions of the bacteria or fungi on thin layer chromatography (TLC) plates developed in solvents of varying polarities was used to detect the number of antibacterial and antifungal compounds present in the extracts. 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 salt. The bacteria tested, *S. aureus* was inhibited by the most compounds separated on the TLC plates from all the tested plants. Similarly, growth of the fungus *C. albicans* was also inhibited by many compounds present in the extracts. *Ocimum tenuiflorum , Azadirachta indica* appeared to be the plant extract with the highest number of inhibition area when compared with other plants

Qualitative phytochemical analysis demonstrated the presence of flavonoids and alkaloids in all plants tested. Thin layer chromatography bioautography assay of methanolic extracts of plants indicated flavonoids, alkaloids and tannins as major active compounds against *Staphylococcus aureus* and *Candida albicans.* Antimicrobial agar well assay revealed that all plants extract contain potent antimicrobial property against both the tested organisms with more zone of inhibition for *C.albicans than S.aureus.*

Key words: Bioautography, Medicinal Plants, Phytochemical Analysis, Agar Well Diffusion.

INTRODUCTION

Despite the existence of conventional antimicrobial agents, resistant or multi-resistant strains of pathogenic microorganisms are continuously appearing, imposing the need for a thorough search for and development of new drugs (Silver and Bostian, 1993). Fungi and bacteria cause important human and animal diseases, especially in tropical and subtropical regions, and commonly occur in immunocompromised or immunodeficient patients. 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 (Soejarto, 1996). In fact, it is also estimated that natural products are implicated in the development of 44% of all new drugs, generally as leads for the preparation of semi-synthetic derivatives (Hostettmann et al, 2001). In an effort to discover new lead compounds, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities. In this regard, several bioassays were developed for screening purposes (Hostettmann, 1991).

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. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied (Navarro et al, 1998). The method is fast, cheap, and permits a better bioassay directed fractionation of bioactive compounds (Hamburger and Cordell, 1987). Bioautography is particularly important to avoid the time-consuming isolation of inactive compounds. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Shahverdi et al, 2007). 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.

MATERIALS AND METHOD

Plant collection

Based on the available literatures, four plants (*Ocimum tenuiflorm, Azadirachta indica, Mangifera indica and Allium sativum)* were selected for the current investigation. Fresh leaves of *Ocimum tenuiflorum, Azadirachta indica, Mangifera indica* were collected and shade-dried at room temperature for one week, and then powdered. For *Allium* sp., outer skin was pealed and washed with distilled water and chopped into small pieces, shade-dried at room temperature for two weeks, and then ground into a fine paste.

Preparation of plant extracts for preliminary screening

Methnolic extracts of all the four plants were prepared by using cold extraction method where 10 g of powdered leaves/paste were mixed in 100 ml methanol (1:10) and kept for continuous shaking in orbital shaker at 250 rpm (25°C) for one week. The extracts were filtered through Whatman (No.1) filter paper. The filtrates were evaporated to dryness and stored in cold (4ºC) until use.

Bacterial and fungal cultures and growth conditions:

Clinical isolates of *S. aureus*and *Candida*albicanswere obtained from the Department of Biotechnology, Mumbai,India. All the test strains were maintained on nutrient agar and sabouraud's slants at 4°C and subcultured on to nutrient broth and sabouraud's broth for 24 h prior to testing. These bacteria served as test pathogens for antibacterial activity assay.

Antimicrobial bioassay

The antimicrobial activities were determined by agar well diffusion assay. Petri dishes (100mm) containing 20ml of Mueller Hinton Agar seeded with 0.2 ml inoculum of 10^6CFU/mL bacterial and yeast inoculum respectively were taken and a single well of 10mm diameter were cut into solidified agar media with the help of a sterile cork-borer. 50 µl of individual plant extract was poured in the agar well and the plates were kept at 4°C for pre diffusion and later incubated at 37°C overnight. Distilled water was used as negative control while Ampoxin antibiotic was used as a positive control. The experiment was performed in duplicate under strict aseptic conditions and the anti-microbial activity of each extract was expressed in terms of diameter of zone of inhibition (in mm) produced by the respective extract at the end of the incubation period**. (**Jesionek, 2015)

Qualitative analysis of the phytochemicals

Phytochemical tests were performed using the standard protocols for extracts that responded significantly to antimicrobial assay (Harborne 1973).

a. Test for carbohydrates: Benedict's test

Equal volumes of Benedict's reagent and crude extract were mixed in a test tube. The mixture was heated in boiling water bath for 5 minutes. Solution appeared reddish brown showing the presence of reducing sugar.

b. Tests for Proteins:

To 2ml of extract, add 4 drops of 0.2% of ninhydrin reagent. This resulted in the formation of blue color.

c. Test for glycosides

To the 2ml of plant extract, add 1ml of glacial acetic acid and 5% ferric chloride and drops of concentrated sulphuric acid. The presence of greenish blue color showing the presence of glycosides.

d. Test for Steroids: Salkowski Test:

To 2ml of extract, 2ml of chloroform and 2ml of conc.H_2SO_4 was added. The solution was shaken well. As a result, chloroform layer turned red and acid layer showed greenish yellow fluorescence.

e. Tests for alkaloids: The plant extract is mixed with dilute HCl was added, shaken well and filtered. To the filtrate add Mayer's reagent. Green or white precipitate shows the presence of alkaloids.

f. Tests for flavonoids- To small quantity of extract add 1ml of 1 n NaOH. Formation of yellow precipitate showed the presence of alkaloids.

g. Test for Tannins- For 2ml of extract adds few drops of 5% ferric chloride. A dark blue or greenish black precipitate showed the presence of tannins.

h. Test for saponins- 2ml of extract was added to 10 ml of distilled water, foam formation indicated the presence of saponins.

i.Test for phenols- To 1ml of plant extract add distilled water and 5 drops of 10% Ferric chloride. Blue or green color formation indicates the formation of phenols.

Direct TLC bioautography

The phytocompounds of the methanol extracts showing significant antimicrobial activity were analysed using direct TLC bioautography. About 10 µl of each extract was applied on TLC silica gel plates. Developing solvent systems used was ethyl acetate: methanol: water and Chloroform: ethyl acetate: formic acid. The TLC plates were run in duplicate. The plates were exposed to UV light (365 or 254 nm) to observe the bands of secondary metabolites. In direct TLC bioautography, the developed TLC plate is sprayed with fungal or bacterial suspension. A suspension of 10^6CFU/mL is employed for both bacteria and fungi. The bioautogram is then incubated at 25'C for 48 h under humid condition. For visualization of microbial growth, tetrazolium salts are used. These salts are converted by the dehydrogenases of living microorganisms to intensely colored formazen. These salts are sprayed onto the bioautogram and are reincubated at 25'C for 24 h, clear white zones against a purple background on the TLC plate indicate antimicrobial activity of the sample. (Suleimana, 2010).

RESULT AND DISCUSSION

Phytochemical screening of the crude extract of medicinal plants was performed and the presence of secondary metabolites was detected. According to the results, the tests for carbohydrates, alkaloids and flavonoids were positive in all plant extracts. (Table 1). It is the believed that the presence of these secondary metabolites is responsible for the antimicrobial property of plants. Phytochemical screening revealed the presence of varied chemical components in the different extracts of the plants. This is notable from the different colour changes depicted by individual compounds due to their reaction with the spray reagent used (vanillin/sulphuric acid) .For example, flavonids exhibit red or blue colouration on the chromatograms when sprayed withvanillin/sulphuric acid (Gibbons 1998).

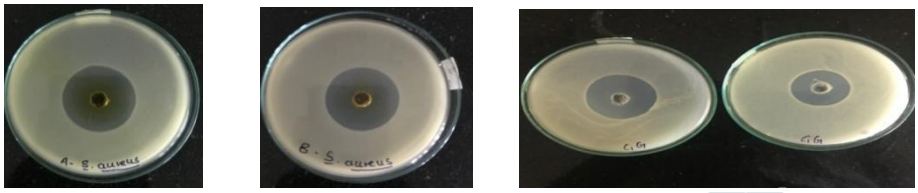
Table 1- Phytochemical tests shows the following secondary metabolites in crude extract of medicinal plants.

COMPOUNDS	*Azadirachta indica*	*Ocimum tenuiflorum*	*Mangifera indica*
1.Carbohydrate	+	++	+++
2.Tannins	-	-	++
3. Saponins	-	-	-
4. Flavonoids	++	++	+++
5. Alkaloids	++	++	+
6. Glycosides	-	-	-
7. Steroids	+	+	-
8. Phenols	-	-	+
KEY: +: Present; +: Low ; ++: moderate ; +++: High ; -: Absent			

The findings of the present study for antimicrobial assay revealed that all plant extract contain potent antimicrobial property against both the tested organisms. It was observed that the zone of inhibition of *C.albicans* was found to be more than *S.aureus* against all plant extract(Table 2, Figure 1) while *S.aureus* showed almost similar zone of inhibition in both *Azadirachta indica and Mangifera indica plant* extracts and a higher zone of inhibition against *Ocimum tenuiflorum and Allium sativum.*

Table 2- Shows zone of inhibition of *S.aureus and C.albicans for* methanolic plants extract

Crude plant extract	***Ocimum tenuiflorum***		***Azadirachta indica***		***Mangifera indica***		***Allium sativum***	
Test organism	***S.auerus***	***C.albicans***	***S.auerus***	***C.albicans***	***S.auerus***	***C.albicans***	***S.auerus***	***C.albicans***
Zone of inhibition (mm)	18	20	16	22	16	22	20	24



(A) **(B)** **(C)**

Figure 1- MH agar plate shows zone of inhibition of *S.aureus and C.albicans* in methanolic extract of (A- *Azadirachta indica.*,**B-** *Ocimum tenuiflorum and* ,**C-** *Allium sativum)* plant respectively.

In direct TLC bioautography, methanolic extracts of *Azadirachta indica and Ocimum tenuiflorum* had the highest area of inhibition against S.*aureus* and *Candida albicans* , while *Allium sativum and magnifera indica* had the lowest area of inhibition against S.*aureus* and *Candida* albicans . The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the S.*aureus* (Figure 2) or *Candida* (Figure 2) due to presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce INT to a purple-red colour (Ayoola, 2008). Creamy spots appearing against a purple background, so-called inhibition zones, point to the presence of antimicrobial agents. In the presence of active plant compounds on the chromatograms, the growth of the organism is inhibited. The bioautography results revealed the different compounds present in the extracts that were responsible for the antibacterial activity.

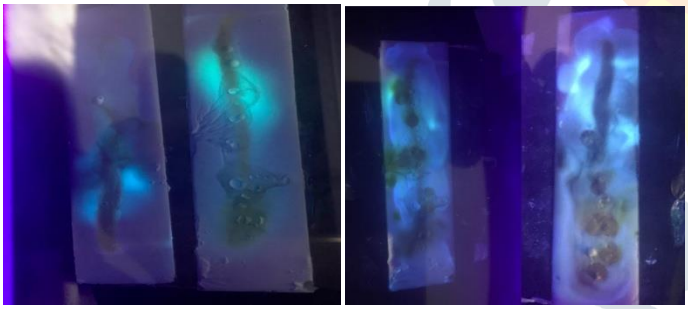


Fig 2. TLC bioautragraphy showing appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the S.*aureus* and *Candida albicans* by *Azadirachta indica and Ocimum tenuiflorum*

This study showed that *Azadirachta indica and Ocimum tenuiflorum were* active against *C. albicans*. This agrees with report by Rios et al.1998 who tested the activity of leaves and stems of this plant using several organic solvents and found that they were active. In another study, the methanol extract of the stem bark of *Khaya anthotheca* was reported to be very active against the fungus *Candida krusei* (Hamza et al., 2006). Many compounds isolated from *Combretum* and *Terminalia spp.* (Combretaceae) have antimicrobial actvity against S.*aureus* and *Candida albicans*. (Eloff et al 2008).

It is important to realize that bioautography is not a quantitative measure of antimicrobial activity. It only indicates the number of compounds that were separated with 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 (Eloff, 2008). Most of the antimicrobial agents detected in this study were present in extracts of relatively non-polar solvents. These findings agreed with previously published results (Eloff 2008) that the substances responsible for the antimicrobial activity were mainly non-polar in nature.

Conclusion

The results indicate that agar well diffusion method is more sensitive than normal disc diffusion method. Like wise, according to the presented results, direct bioautographic method showed to be more sensitive than the indirect variant. Also, preparation of the bacterial inoculum from 24 h grown culture, rather than 48 h, is suitable for performing the tests by bioautographic method and a concentration of 0.2 mg/mL of *p*-iodonitrotetrazolium violet indicator solution is eligible to allow visualization of results. The bioautography results revealed the different compounds present in the extracts that were responsible for the antibacterial activity. These two procedures take relatively little time to perform and save financial resources.

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