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Molecular Characterization of Bioactive Compound from Musambi(Citrus Sinesis) Tulasi(Ocimum Sanctum) Against Selected Bacterial Pathogens

Veena. P Research Scholar, Department of Microbiology, Hindusthan College of Arts & Science, Coimbatore

Dr. J. Vimalin Hena Assistant Professor, Department of Microbiology, Hindusthan College of Arts& Science, Coimbatore

ABSTRACT

Medicinal plants are extensively used in traditional medicine to cure various infectious diseases in human. Ocimum sanctum and Citrus sinensis are two medicinal plants traditionally used in medicine which were studied.Antibacterial activity of the compound was identify against .E.coil, K.pneumoniae, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas. Pytochemical screening was done to identify the presence of antibacterial compound the presence of giycoside, tannins, and phenol were confirmed. TLC reveled the presence of carotenoid and TLC bioautography proved carotenoid to be active against the pathogens. In this regard carotenoid was purified by column chromatography and HPLC.

KEYWORDS: Medicinalplants, Antibacterialactivity, Phytochemicalscreening, Carotenoids, Column chromatography, TLC, HPLC

INTRODUCTION

Infectious diseases are the world's leading causes of premature deaths, killing almost 50000 people every day. In recent year, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Piddock and Wise,1989; Mulligen*et al*.,1993; Davis,1994; Robin *et al*.,1998). However the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. The drug-resistant bacteria pathogens have further complicated the treatment of infectious diseases in immuno compromised, AIDS and cancer patients (Rinaldi,1991; Diamond,1993). In the present scenario of emergence of multiple drug resistance to human pathogenic organism, has necessitated a search for new bioactive substances from traditional sources in the medicinal plants.

Traditionally used medicinal plants produce a variety of know therapeutic compounds (Iyengar,1985; Chopra *et al*., 1992; Harborne and Baxter,1995).These substance can either inhibit the growth of pathogens or kill them and have no or less toxicity to host cells are considered candidates for developing new antimicrobial drugs. In recent year, antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world (Grosvenor *et al*.,1995; Ratnakar and Murthy ,1995;Silva *et al*.,1996;David ,1997;Saxena ,1997; Nimri*et al*.,1999; Saxena and Sharma,1999). 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 (Hasegawa *et al* .,1995;Lee *et al*., 1998).In the present study ,we have selected two Indian medicinal plants to be screened against multi-drug resistant bacteria including *Staphylococcus aureus, Streptococcus pyogens , Pseudomonas, Klebsiella* and*, E.coil*

MATERIALS AND METHODS

Collection of plant material

Two plant samples were collected namely *Ocimum sanctum* (Tulsi), which belongs to the family *Labiatae.* The whole plant was used. *Citrus sinensis* (Musambi) which belongs to the family Rutaceae. The rind of the plant was used.

Preparation of plant extract

The plant parts were plucked, graded, cleaned, dried in shade devoied of contaminents then the dried plant material was powdered and the powdered material of *ocimum sanctum* (whole plant) and *citrus sinensis* (Rind) were extracted using soxhlet apparatus with methanol and ethanol. After extraction the extracts were kept in water bath for drying and preserved in aseptic condition. The dried extracts were used to perform various phytochemical and antibacterial screening assay.

Phytochemical screening

Phtochemical analysis of major phytoconstituents of the plant extracts was undertaken using qualitative method. The test were carried out to find the presence of the active chemical constituents such as glycoside,phenol,and Tannins by the following procedure.

Test for Glycosides

1. Keller killiani test(for deoxysugars)

To two ml of extract few drops of glacial acetic acid was added one drop of 5% of $FeCl_3$ solution and concentrate H_2So_4 was added. If Reddish brow color appears at the two liquid layer and upper layer appears bluish green it confirms the presence of deoxysugars.

2. Legal's test (test for cardenoloids)

To aqueous or alcoholic extract, 1ml of pyridine and 1ml of sodium nitroprusside solution was added.If Pink to red color appears it confirms the presence of cardenoloids.

Test for Tannins

Gelatin test

Extract was dissolved in 2% gelatine and mixed with 10% Nacl to form a white precipitate.The extract was mixed with the alcoholic vanillin solution and dilute Hcl acid added to form a pink colour.The sample was mixed with lime water to produce a brown colour. Tannins were precipitated by salts of copper,tin and lead.Tanninsshowdcolour reaction with iron salts. Ferric chloride bluish black or bluish green.These proved the presence of tannins.

Test for phenol

Two ml of ethanol was added to the extract and few drops of ferric chloride solution . Blue colouration indicates the presence of phenol .

Antibacterial assay of crude plant extracts

1.Agar well diffusion

The antibacterial activity was tested against crude extracts. The inoculation of microorganism was prepared from bacterial culture. About 15 to 20ml of Muller Hinton Agar medium was poured in to the sterilized petridish and allowed to solidify. Wells of 6mm in diameter and about 2cm apart were punctured in the culture medium. About 40µl of the extracted sample was added to the well. Plates were incubated at 37°c for 24 hours. Antibacterial activities were evaluated by measuring inhibition zone diameters

Separation and identification of extracted compounds

Thin layer chromatography

Thin layer chromatography of the two plant extracts with strong antimicrobial activity was carried out. Different solvent system were used for different classes of compounds based on the polarity of the organic solvents. Anthrone was used as a control. Solvent system used were (1) Petroleum ether and benzene in the ratio 1:1 (2) Benzene and chloroform in the ratio1:1 (3) Benzene and ethyl acetate in the ratio 2:1 (4) Acetone and alcohol in the ratio1:1 and (5) Methanol and water in the ratio of 1:1. Individual R_f for each spot was measured. TLC is used to

support the identity of a compound in a mixture of compound by comparing the R_f of a compound with the R_f of standard.

Identification of bioactive plant compound
TLC-bioautography
TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localizationsand target-directed isolation of active constituents in a mixture. Traditionally, bioautographic technique has used for the growth inhibition of microorganisms and to detect anti-microbial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds (Shahverdi, 2007). Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (i) direct bio-autography, where the micro-organism grows directly on the thin-layer chromatographic (TLC) plate, (ii) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (iii) agar overlay bio-autography.

Contact bioautography
The sterilized petriplates were poured with mullerhinton agar medium and allowed to solidify. After solidification the bacterial cultures were swabbed onto the medium and then the spotted TLC paper was cut and placed on the MHA plate. The plates were incubated at 37^0c for 24 hours and observed for the zone formation.

Purification of bioactive compound
Column chromatography
1. Preparation of column
In a column a small amount of glass wool is pushed down to the bottom as a bed. Above this some sand is added to a height of 1.5cm above 6g adsorbent (Alumina) previously activated is taken in a beaker and a small amount of benzene is added to make a slurry. This slurry is carefully poured into the column to avoid air bubbles; the adsorbent should never be allowed to dry. Add more benzene and allow it to pass through the column.

2. Preparation of the leaf extract
About 5g of leaf was taken and adding 20ml of benzene and methanol in the ratio2:1was added. The extract was filtered through a filter paper and the filterate was transfered through the separating funnel. 10ml of water was added and shoor shake well layers were allowed to separate. The lower aqueous layer containing methanol was removed. The benzene layer was taken in a beaker and the solvent was evaporated by placing it over steam in a water bath. A pasty solid was obtained because of the presence of some water .The residue was dissolved in small volume of benzene and solid sodium sulphate was added. This removestraces of water. The clear benzene layer was decanted to another beaker and evaporated to dryness. The dried extract was dissolved in 1ml of benzene and quantitatively transfered it with a pipette to the top of the column without disturbing the adsorbent. The solution was elevated with benzene until the color pigments were well separated. Each fractionwere collected separately.

HPLC
The elute obtained from column chromatography was used for HPLC analysis. The column used was C18 and the mobile phase consisted of acetonitrile, methanol, and ethyl acetate containing 0.05% of TEA (triethylamine) used at a flow rate of 0.5 ml/min. The use of TEA was recommended by Hart and Scott (1995) to increase carotenoid recovery. Re-equilibrtion took 15 minutes.

RESULT AND DISCUSSION
Agar well diffusion method
The plant extract showed antibacterial activity against *E.coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella Pneumonia* and *Streptococcus pyogenes* in the medicinal plants of *ocimumsanctum* and *citrus sinensis*.

Ocimum sanctum
The results showed that ethanol extract has less antibacterial activity than methanol extract. The maximum inhibition zone showed was observed in *Pseudomonasaeruginosa* (1.7 cm). The minimum inhibition zone was observed in *Klebsiella pneumonia*. The methanol extract has showed antibacterial effect. *Pseudomonas acruginosa* showed the maximum inhibitory zone (2.1 cm). The minimum inhibition zone was observed in *Streptococcus pyogenes* (1.7 cm).(Table:1)

Citrus sinensis
The results showed that ethanol extract has moderate antibacterial activity. The minimum inhibition zone *Staphylococcus aureus* showed (1.5cm). The maximum inhibition zone was observed in *Pseudomonas aeruginosa*(2cm). In methanol extract, the maximum inhibition zone was observed in *Streptococcus pyogenes* (2cm). The minimum inhibition zone was observed in *Klebsiellapneumoniae* (1.4cm). (Table:2)

Among all the extracts, methanol extract was the most effective having strong antibacterial activity. The excellent antibacterial activity of methanolic extract was reported earlier by (Poonammiahra *et al*., 2001) and (Ekwenye*et al*., 2001). **Preliminary phytochemical screening**

The phytochemical screening of the plants revealed some difference in the constituents of the two plants tested.

The phytochemical screening was done in the ethanolic extracts of *Ocimum sanctum* and *Citrus sinensis*. The presence of common phyto constituents like phemo l0.22%,Tannin 0.19%,and glycosides 3.13 in *citrus sinensis* and *ocimum sanctum* had acontent of phenol 0.12%, tannin 0.10% and glycosides 0.94%.

After the addition of glacial acetic acid FcI_3 and H_2SO_4 ,to the extract reddish brown colour was observed which indicated the presence of glycosides.

After the addition of 2 ml of ethanol, and ferric chloride solution,to the extract a blue colour was observed which indicates the presence of phenol.

The preliminary phytochemicals investigation revealed the presence of various constituents of *Ocimum sanctum* and *Citrus sinensis*. The results are shown in the table 3.

Thin layer chromatography
The presence of phenols, Tannins, glycoside components was also detected by thin layer chromatography(TLC). In addition of qualitative results TLC also provides a chromatographic measurement known as an R_f values. The R_f value is the retardation factor or the ratio- to- front value expressed as a decimal fraction.

The R_f value was calculated and was found to be0.489 and it is the retardation factor for carotenoid.

TLC bioautography
TLC bioautography was performed against two highly active plant extracts . In the majority of the plants tested by TLC bioautography, carotenoid was observed as the most common active constituents

Column chromatography
The mobile phase slowly eluted down through the alumina column. The different components in the leaf extract passed through the column at different rates due to differences in the portioning behaviour between the mobile liquid phase and the stationary phase. The various colour pigments which eluted out were collected and stored separately. Table 4 show the results of column chromatography.

HPLC
The elute from column chromatography was used for HPLC analysis. When subjected to HPLC. A single peak was observed, whose R_f value was 0.42 which confirm the presence of carotenoid.

TABLE-1
ANTIBACTERIAL EFFECT OF *OCIMUM SANCTUM* EXTRACT ON DIFFERENT MICROORGANISMS

Ocimum sanctumExtract	Methanol	Ethanol
Concentration of extract	40µl	40µl
Microorganisms	Zone of inhibition in cm	
Escherichia coli	2.0	1.5
Klebsiella pneumonia	1.8	1.4
Streptococcus pyogenes	1.7	1.5
Pseudomonas aeruginosa	2.1	1.7
Staphylococcus aureus	2.0	1.6

TABLE-2ANTIBACTERIAL EFFECTS OF *CITRUS SINESIS* ON DIF-

FERENT MICROORGANISMS

Citrus sinensisExtract	Methanol	Ethanol
Concentration of extract	40µl	40µl
Microorganisms	Zone of inhibition in cm	
Escherichia coli	1.5	1.6
Klebsiella pneumonia	1.5	1.8
Streptococcus pyogenes	2.0	1.5
Pseudomonas aeruginosa	2.1	1.7
Staphylococcus aureus	1.7	2.0

TABLE-3 PHYTOCHEMICAL SCREENING OF CITRUS SINESIS AND OCIMUM SANTUM

S.NO	ACID REAGENT	OBSERVATION	RESULT
1	Extract+Glacial acetic acid+$FeCl_2$+H_2SO_4	Reddish brown colour	Presence of glycosides
2	Extract+Pyridine+Sodium nitro prusside	Pink to red colour	Presence of Tannins
3	Extract+lime+H_2O	Brown colour	
4	Extract+Alcoholic vanillin solution+dilute HCL	Pink colour	
5	Extract+Ferric chloride solution	Bluish green colour or Bluish black	
6	Extract+2ml of Ethanol+Ferric chloride	Blue colour	Presence of phenol

TABLE-4
COLUMN CHROMATOGRAPHY

PIGMENTS	COLOUR
Carotenes	Golden yellow
Pheophytin	Olivegreen
Lutein	Yellow colour
Xanthophylls	Deep yellow
Chlorophyll a	Blue green
Chlorophyll b	Green

SUMMARY AND CONCLUSION

Bioactive compounds isolated from the plants are used in herbal medicine. Based on their mode of action they are divided into different categories like chemotherapeutic, bactericidal and antimicrobial agent. Several medicinal plants have been screened for their antibacterial activity so that be used as alternative medicine.

In this study *Ocimum sanctum and Citrussinesis* were screened for their antibacterial activity against gram positive organisms like*Staphylococcus aureus* and *Streptococcus pyogenes* and gram negative organisms like*E.coli, Klebsiellapneumonial,* and *Pseudomonas aeruginosa.*

From our study it is concluded that *Ocimumsanctum* is the plant with maximum antibacterial activity towards *E.coli* and *pseudomonas*. Among all the solvents used, methanol extract has strong antibacterial activity. Phytochemical analysis showed that tulsi and musambi were rich in glycoside, phenol and tannin.

In thin layer chromatography, methanol and water at 1:1ratio when used as solvent system .The spot was observed .Tulsi when observed in the bioautography, produced zone of inhibition in solvent system .

The bioactive compound purified by coloum chromatography and HPLC and the bioactive compound was found to be carotenoid.

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