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Antibiogram of *Candida albicans* using *Tabernaemontana divaricata* leaf extracts by AWD assay.

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Abstract

A total of about 25 samples of diarrheal infection samples were collected from the patients by using sterile screw capped test tubes from Government Hospital, Kanchipuram. Samples were inoculated in sterile peptone water in screw – capped tubes and transported to the laboratory within an hour. The culture was inoculated in the Nutrient agar plates and the culture obtained was further identified by classical cultural methods. The identified isolate was subjected to the antimicrobial suspectibility using standard antibiotic discs. The resistant strains were subjected to screening for Extended Spectrum Beta Lactamase production by Double disk synergy test, Disk replacement test and E-Strip test. The leaf extracts were prepared for different solvents such as Acetone, Butanol, Chloroform and Ethanol. Each extract was tested against ESBL producing *Escherichia coli by* AWD assay. Zone of inhibition of each extract were recorded. All the extracts were found to be effective. The different extract were also subjected to antibacterial assay at different concentrations such as 25 µg, 50µg, 75 µg and a standard streptomycin of 75 µg As a global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, development of modern drugs from these seeds should be emphasized for the control of ESBL producing *Escherichia coli*.

Keywords: *Tabernaemontana divaricata*, *Candida albicans*, ESBL producing *Escherichia coli*., AWD assay.

Introduction

Plant use in treating diseases is as old as human civilization and traditional medicines are still a major part of regular treatments of different maladies (Alviano and Alviano, 2009). Plants are no doubt one of the main sources of biologically active materials. According to a recent report medicinal herbs are used by 80% of world population living in rural areas for their primary healthcare (Sakarkar and Deshmukh, 2011). In Europe and USA 50% of the prescriptions are prescribing natural products including plants or their derivatives for different maladies (Cordell, 2002; Newman *et al.*, 2007). Interestingly out of the 250,000-500,000 plant species on earth, only 1-10% has been studied chemically and pharmacologically for their innumerable medicinal values (Verpoorte, 2000). Recently the synthetic chemistry has dominated the market of drug discovery and production still there is enormous scope for bioactive principles or extracts derived from plants (Raskin *et al*., 2002). If we compare them with synthetic drugs than plant derived natural products are surely good source of biologically active agents since they are natural and cost effective (Ghosh *et al*., 2008). Again plant molecules may have different mode of action than conventional drugs derived agents may have different mechanisms than conventional drugs so as they may be better for health care improvement (Eloff *et al*., 1998). Plant derived natural products are generally bioactive secondary metabolites with better potential to treat different diseases. Some examples of these compounds include terpenoids, alkaloids, phenols, unsaturated lactones, phenolic glycosides, saponins, sulphur compounds,

cyanogenic glycosides and glucosinolates (Mukherjee *et al*., 2001; Quiroga *et al*., 2001). These plant based natural products are recently of interest among many scientists to design new remedies for different afflictions like microbial infections.

During the last two decades scientists have tried to develop antimicrobial drugs with low side effects from the natural sources and it may be due to the increasing development of drug resistance to human pathogenic organisms (Savita *et al., 2013).*

Tabernaemontana divaricata

Tabernaemontana divaricata (Apocynaceae), commonly called Pinwheel Flower, Crape Jasmine, East India Rosebay and Nero's Crown is an evergreen shrub probably native to India and now cultivated throughout South East Asia and the warmer regions of continental Asia. In zones where it is not hardy it is grown as a house/glasshouse plant for its attractive flowers and foliage. The stem exudes a milky latex when broken, whence the name Milk Flower in the Bengali language. Known as Wathusudda (meaning "garden white") in the Sinhalese language. In telugu language it's called as nandivardhanam. Other Indian names include Chandni and Taggar. The plant contains several alkaloids and, like many other Apocynaceae, is toxic and medicinal.

Candida albicans

Candida is a genus of yeasts and is the most common cause of fungal infections worldwide. Many species are harmless commensals or endosymbionts of hosts including humans; however, when mucosal barriers are disrupted or the immune system is compromised they can invade and cause disease.

Among Candida species, *Candida albicans* which is a normal constituent of the human flora, a commensal of the skin and the gastrointestinal and genitourinary tracts, is responsible for the majority of Candida bloodstream infections (candidemia).

Many species are found in gut flora, including *Candida albicans* in mammalian hosts, whereas others live as endosymbionts in insect hosts.

Systemic infections of the bloodstream and major organs (candidemia or invasive candidiasis), particularly in immunocompromised patients, affect over 90,000 people a year in the U.S.

Aim and objectives

Collection of oral thrush sample from cancer patients
Isolation and identification of *Candida albicans*
Antifungal assay against standard antifungal agents
Preparation of leaf extract using solvents such as acetone, butanol, chloroform and ethanol.
Phytochemical analysis of leaf extracts
Antibiogram of *Candida albicans* using *Tabernaemontana divaricata* leaf extracts by AWD assay.

Materials and Methods

Collection of samples

A total of about 25 oral thrush samples were collected from the cancer patients by using appropriate sterile swabs from Aringar Anna Memorial Cancer Hospital, Kanchipuram.

Transportation of sample

Samples were inoculated in sterile saline in screw – capped tubes and transported to the laboratory within an hour.

Processing of sample

Microscopic Examination

Gram’s Staining Method

The collected specimen was subjected to differential staining by Gram's Staining techniques and observed for the presence of Gram negative rod under oil - immersion lens of light Microscope.

Lacto Phenol Cotton Blue Mount

Take a clean grease free slide. Add a drop of mounting fluid that is lactophenol cotton blue solution on a slide. Sterilize the needle and cool it then transfer a small portion of the sample and mix with the stain. Take a clean cover slip and with the help of a forcep place the cover slip over the stain. Take a blotting paper and wipe the excess stain .Observe under low to high power objectives of microscope.

Identification of isolate

After incubation the isolate is subjected to the following tests for the identification of the yeast.

Gram's Staining Method

The collected specimen was subjected to differential staining by Gram's Staining techniques and observed for the presence of Gram positive yeast under oil - immersion lens of light Microscope.

Lacto Phenol Cotton Blue Mount

Take a clean grease free slide. Add a drop of mounting fluid that is lactophenol cotton blue solution on a slide. Sterilize the needle and cool it then transfer a small portion of the sample and mix with the stain. Take a clean cover slip and with the help of a forcep place the cover slip over the stain. Take a blotting paper and wipe the excess stain .Observe under low to high power objectives of microscope

Germ Tube Test

Emulsify a small amount of yeast culture in a tube of fresh human serum. Incubate the tube for 1 hour at 35ºC. Examine the culture by placing a loopful of the serum culture on a clean glass slide. Place a coverslip and observe microscopically for germ tube formation. If germ tubes are not seen, reincubate as long as an additional 2 hours and repeat proceeding step every 15 minutes.

Carbohydrate fermentation test

The 5 ml of carbohydrate (pH, 7.4) containing 1 % peptone, 1 % sugar, 0.3 % beef extract and 0.5 % NaCl, 0.2 % Bromothymol blue in distilled water medium was dispensed in sterilized test tubes with Durham's tube and 0.2 ml of saline suspension of the test organism was added and incubated at 37^0 C for 10 days.

Sugar Assimilation Test

For sugar assimilation test, discs with 4% sugars were prepared and placed on inoculated yeast nitrogen base agar and incubated at 30°C for 48 hours. Presence of growth around the disc indicates assimilation of that carbohydrate. The sugars used for assimilation testing were glucose, maltose, lactose and sucrose.

Urease Test

Inoculate two tubes of urea agar with fragments of mold or yeast culture being tested. Incubate one tube at 25 ºC and the other at 35º C. Observe daily for one week for change in color to red.

Chlamydospore Formation

This test is used for differentiating *Candida albicans* from other species of Candida on the basis of Chlamydospore Formation.
The pH of the medium was adjusted to 5.1 $\pm$ 0.2.
Then the medium was sterilized by autoclaving at 121^0C for 15 minutes.
20ml of the sterilized medium was poured into the Petri plates.
After, the sample was inoculated and incubated at 20-25 °C for 24-48 hours.
After incubation, the culture was observed under the microscope by placing it in a drop of lactophenol cotton blue mount.

Antimicrobial susceptibility of *Candida albicans* against standard antifungal agents

The sterilized Mueller Hinton Agar medium was poured into a sterile Petri plate.
After solidification, a lawn culture of the organism was made and it is allowed to dry for 5 minutes.
The standard antifungal discs were placed on to the surface of the inoculated plates (Amphotericin B, Fluconazole, Voriconazole and Itraconazole) and gently pressed in order to adhere the discs.
Then the plates were incubated at 37^0C for 18 - 24 hours.

Collection of plant leaves

The fresh leaves of the *Tabernaemontana divaricata* plant were collected.
The leaves were washed with running tap water to remove the surface dust particles and blotted with clean white muslin cloth.
The leaves were shade dried completely and were grind into a fine powder.

Preparation of Extracts

A known quantity of leaf powder (25gm each) was taken in a
separate 250 ml beaker and 100 ml of each solvent Acetone, Butanol, Chloroform and Ethanol was added.
The preparation was kept at room temperature for 48 hrs and rapidly stirred using glass rod every 4 hrs.
After 48 hrs, the leaf extracts were filtered through Whatmann No. 1 fitter paper to exclude the leaf powder.

Then the extract was taken in separate beaker and kept in a water bath at 40 – 50 ^{0}C until the solvent gets evaporated.
A greasy final material (ethanolic extract) obtained from the plant was transferred to sterile screw capped bottle and stored under refrigerated condition till use.

Phytochemical Analysis

Qualitative analysis of phytonutrients of leaf extracts

Qualitative analysis of phytonutrients was done for the leaf extract of *Tabernaemontana divaricata.*

Benedict's test

To 0.5 ml of different leaf extract, 5 ml of Benedict's reagent was added. The mixture is then boiled for 5 minutes. Presence of a bluish green precipitate indicated the presence of carbohydrates.

Test for Glycosides

To 2ml of different leaf extract 1ml of aqueous NaOH solution was added. The appearance of a yellow color indicated the presence of glycosides.

Test for Proteins and Amino acids

Ninhydrin test

Lactophenol Cotton Blue
A small quantity extract solution was boiled with 0.2% solution of ninhydrin. Purple color indicated the presence of free amino acids.

Test for Phytosterols and Triterpenoids

Sabouraud' Dextrose Agar -

Salkowski test

To 2 ml of the different leaf extract, 1 ml of concentrated sulfuric acid added.
Chloroform was added along the sides of the test tube.
A red color produced in the chloroform layer indicated the presence of Phytosterols or if it is yellow in color at the lower layer indicated the presence of triterpenoids.

Antibiogram of *Candida albicans* using *Tabernaemontana divaricata* leaf extracts

The sterilized Muller Hinton Agar medium was poured into a sterile Petri plate.
After solidification, a lawn culture of the organism was made and it is allowed to dry for 5 minutes.

Using gel punch four wells were made and a known quantity of 20µl of each extract was added to the wells.
Then the plates were incubated at 37^{0}C for 18 - 24 hours.
After incubation the zone of inhibition around the disc were measured.

Results

Out of 25 samples collected all were positive for the presence of yeast *Candida albicans*.

Identification of isolates

Morphology -
Gram positive yeast cells

- Blue stained budding yeast cells.

Colony morphology

White to cream colored, smooth, convex opaque, grey white colonies

Biochemical characterization

The biochemical characterization of the isolate was given in the Table - 1.

Table -1 Biochemical characterization

Biochemical Test	Result
Germ tube Test	Positive
Carbohydrate fermentation test	Ferments Glucose and Maltose
Sugar Assimilation test	Assimilates Glucose, Maltose and Lactose
Urease Test	Negative
Chlamydospore formation	Positive

Based on the Gram's staining and Lactophenol cotton blue mount, colony morphology, Germ tube test and biochemical characterization the fungal isolate is identified as *Candida albicans* yeast.

Antimicrobial susceptibility of *Candida albicans* against standard antifungal agents

The antimicrobial susceptibility of the isolates against the standard antifungal agent was given in Table – 2.

Table - 2 Antimicrobial susceptibility of *Candida albicans* against standard antifungal agents

S. No	Standard antifungal agent	Zone of inhibition (diameter in cm)
1.	Amphotericin B	-
2.	Fluconazole	-
3.	Itraconazole	2.5
4.	Voriconazole	-

Phytochemical Analysis

Qualitative analysis of phytonutrients of leaf extracts

The results for the qualitative analysis of phytonutrients of leaf extracts were given in Table – 3

Table – 3 Qualitative analysis of phytonutrients of leaf extracts

S.No	Phytoconstituent	Acetone	Butanol	Chloroform	Ethanol
1.	Carbohydrates	+	+	+	+
2.	Glycosides	+	+	+	+
3.	Free aminoacids	+	+	+	+
4.	Phytosterols	+	+	+	+
5.	Triterpenoids	+	+	+	+

Antibiogram of *Candida albicans* using *Tabernaemontana divaricata leaf* extracts

The antimicrobial susceptibility of the isolates against the leaf extracts was given in Table -4

Table -4 Antibiogram of *Candida albicans* using *Tabernaemontana divaricata leaf* extracts

S. no	Leaf extract used	Zone of inhibition (diameter in mm)
1.	Acetone	10
2.	Butanol	12
3.	Chloroform	13
4.	Ethanol	16

Discussion

ESBLs constitute a growing class of plasmid-mediated β-lactamases which confer resistance to broad spectrum beta-lactam antibiotic. They are commonly expressed by Enterobacteriaceae but the species of organisms producing these enzymes are increasing and this is a cause for great concern.

The prevalence of ESBL-producing organisms is increasing worldwide and several outbreaks have been reported. Serious infections with these organisms are associated with high mortality rates as therapeutic options are limited.

The emergence of ESBLs creates a real challenge for both clinical microbiology laboratories and clinicians because of their dynamic evolution and epidemiology, wide substrate specificity with its therapeutic implications, their significant diagnostic challenges and their prevention and infection control issues.

In this present study an attempt is made to isolate and identify the ESBL producing *Escherichia coli* in patients with diarrheal infection by Double disk synergy test, Disk replacement test and E-Strip test (Kenneth *et al*., 1992).

Tabernaemontana divaricata leaves are large, shiny and deep green in colour and the size is about 6-inches in length and 2-inches in width. The flowers are commonly known as Crape jasmine. It blooms in spring but flowers appear sporatically all year. The waxy blossoms are white five-petaled pinwheels that are borne in small clusters on the stem tips.

The leaf extracts were prepared for different solvents such as Acetone, Butanol, Chloroform and Ethanol.

In addition the ESBL producing *Escherichia coli* was subjected to antibiogram using *Tabernaemontana divaricata* leaf extracts (Sumitha *et al.*, 2015).

They showed reasonable zone of inhibition against the ESBL producing *Escherichia coli*. The zone of inhibition for Acetone extract is 10mm, butanol extract is 12 mm, chloroform extract is 13 mm and for ethanol extract is 16 mm. Among the four, the ethanolic leaf extract was found to be very effective as it shows maximum zone of inhibition.

The different extract were also subjected to antibacterial assay at different concentrations such as 25 µg, 50µg, 75 µg and a standard streptomycin of 75 µg. Among the different concentrations 75 µg is more effective for all the extracts.

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