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Screening of Antimicrobial activities of an indigenous herb *Cassia occidentalis*

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ABSTRACT

In prospect of escalating resistance to existing antimicrobial agents, herbal drugs are being looked as an imperative source for discovery of new agents for treating various ailments related to bacterial infections. C.*occidentalis* is an annual herb in India, has been used as folk medicine and found to posses wide range of pharmacological behaviors. The objective of the current study is to evaluate the effects of C.*occidentalis* leaf extracts on the growth of various pathogenic microorganisms based on the inhibition zone using disc diffusion assay, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values. The crude aqueous extract had been fractioned in to aqueous and ethylacetate fraction using biofractionation assay. In this, the aqueous fraction of C.*occidentalis* had no antimicrobial effect against the test microorganisms where as ethyl acetate fraction had inhibitory effects on the growth of 9 strains of 9 bacterial species and one fungal isolate. Bactericidal activity of microorganism was also found using Time kill curve.

Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganism has increased [1]. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purpose has gradually increased in India. According to World Health Organization medicinal plants would be the best source to obtain variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants.[2] The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils [3] , as well as in tannin.[4]

Cassia occidentalis otherwise called as Coffee senna, belonging to the family Caesalpiniaceae, is a herb, extensively used in traditional medicine in tropical and sub-tropical countries. *C.occidentalis* commonly found in waste grounds and secondary forest. Decoctions of parts of *C.occidentalis* are uses as an anti-diabetic, anti-inflammatory, anticancerous, antibacterial, antifungal and hepatoprotective activity.[5].

It was already reported that *C. occidentalis* leaf extracts were found to be active against different microbes (*Corynebacterium diphtheriae, Mucor sp.Neisseria sp. Salmonella sp., Aspergillus niger*) [6]. The leaf extract of this plant when tested against different pathogenic bacteria was found to be active against *Salmonella enteritidis* and *Staphylococcus aureus* while a negative effect was observed against *E. coli* and *Shigella dysenteriae* [7]. It was found that the plant extract showed significant antimicrobial activities against all microorganisms and inhibition zones were comparable to that of ampicilin and gentamycin . When the ethanolic extract and metabolite rich fractions of different parts of calli of *C.occidentalis* were examined, it was observed that anthraquinones were more effective against E. coli and S. aureus (22 mm) while sennosides were more effective against *A.flavus* (28 mm).[8]

Hence, more studies pertaining to the use of plants as therapeutic agents should be emphasized, especially those related to control of antibiotic resistant microbes. The objective of this research was to evaluate the potential of plant extract using different solvents like ethyl acetate and aqueous on standard microbial strains as well as multi-drug resistant bacteria.

Materials and Methods

Plant material and extraction procedure:

The healthy plants were collected from local fields found in Chengalpattu, Kancheepuram District, TamilNadu. The taxonomic identification of plant material was confirmed by Dr.T.Sekar,Pachayappa's college,Chennai. Collected plant material was surface sterilized with tap water and drier in the shade and powdered. The dried and powered leaves of Plant (500g) were extracted successively with 1:1 of methanol by soxhlet for 72 h at a temperature not exceeding the boiling point of the solvent. Crude aqueous extract were prepared by adding 1:1 of boiling water to 500g of powdered plant material in a glass 2.5 L flask and incubated at room temperature for 2h on a rotating shaker (200rpm). The aqueous extract were filtered using Whatman. No, 1 filter paper and then concentrated in vacuum at 40°c using a rotary evaporator. The residues obtained were stored in a freezer at -80°C until use.[9]

Fractionation / Bio assay guided Extraction [10]:

Using this method the crude aqueous had fractioned into aqueous and ethyl acetate dissolved part. Based on polarity ethyl

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acetate fraction was portioned and dried in vacuum evaporator. This two portioned layers were used for antimicrobial activity.

Microorganisms:

Three strains of gram positive bacteria *Staphylococcus aureus, Streptococcus pyogenes and Bacillus subtilis.*, six strains of gram negative *Escherichia coli, Klebsiella pneumonia, Vibrio fischeri, salmonella typii, aeromonas hydrophila* and *pseudomonas aeruginosa* and one yeast *Candida albicans* were used as test microorganisms. All microorganisms were reference isolates, obtained from the division of Microbiology, Biozone research technologies, Chennai.

Inoculum preparation:

Nutrient broth and Sabouraud dextrose agar (SDA) were used for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in nutrient broth at 37°C for 18 h and adjusted to a final density of 10^8 cfu/ml by diluting fresh cultures and comparison to McFarland density. *C. Albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled, and settled SDA medium. The petri dishes were incubated at 31°C for 48 h. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solution (normal saline), which was compared with McFarland solution. According to the manufacturer's directions, 1 ml of yeast suspension in normal saline was added to 74 ml of sterile medium and kept at 45°C to give a concentration of 2×10^7 cells/ml.

Disc-diffusion assay:

The dried plant extracts were dissolved in the same solvent (ethyl acetate and water) to a final concentration of 30mg / ml and sterilized by filtration by 0.45µm Millipore filters. Antimicrobial tests were then carried out by disc-diffusion method [11] using 100µl of suspension containing 10^8 CFU /ml of bacteria,10^6 CFU/ml of fungi spread on nutrient agar (NA) and sabourand dextrose agar(SDA) medium respectively. The well (6mm in diameter) were made by cork borer and load 25µl of the extracts (40mg/ml) at the concentration of 1000µg/25 µl and loaded on the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. Ciprofloxacin (25µg/ml) used as positive reference standard to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37°C for 24h for clinical bacterial strains, 72 h for fungi isolates. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Each assay in this experiment was repeated thrice.

Microdilution assay:

The minimal inhibition concentration (MIC) values were also studied [12] for the microorganisms which were determined as sensitive to ethyl acetate and aqueous extracts of *C.occidentalis* in disc-diffusion assay. Overnight MH broth cultures were used to prepare inocula of 10^6 CFU/ml. The MIC was defined as the lowest concentration of antimicrobial agent that prevented turbidity after 24 h of incubation at 37°C.

Minimal Bactericidal Concentration (MBC) Determination:

MBC is the smaller concentration of the drug necessary for elimination of 99.9% of the microorganisms tested[13]. The MBC was determined after the MIC assays. Tubes where the MIC results showed no bacterial growth, The MIC_{99} was considered as MBC. Bacterial growth was evaluated for the MBC determination. After 24 h, at 35°C, if MIC = MBC or if MBC is one, two or three dilutions above of MIC, the drug is considered bactericide.

Time kill study:

MICs for *C.albicans* and *Bacillus, S. aureus.* used in the time–kill and development of resistance studies were determined using the broth microdilution method.[14] Inocula were prepared from test organisms grown for 4–6 h in the appropriate broth media and diluted in saline to 0.5McFarland standard to obtain 100mL of a starting culture containing 10^6 colony-forming units (CFU)/mL, which was verified by colony counts of replicate samples. Aliquots (10 mL) of the culture were transferred to sterile plastic $25cm^2$ culture tube (Corning Inc., Corning, NY) and ethyl acetate extract was added from a sterile stock solution to give final concentrations equal to 1, 2, 4 and 8 times the MIC for *S. aureus*, *C.albicans* and *B.subtilis*. Each assay included a growth control tube with no antibiotic.

The cultures were incubated at 37 ◦C and samples were obtained at 0, 1, 2, 4, 12, 24 and 48 h following addition of extract. The samples were washed with phosphate-buffered saline and were spotted onto duplicate CAMHA (Cation adjusted muller hinton agar). Following incubation at 37 ◦C for 24 h, colonies that arose on plates with 30–300 colonies were counted.

Determination of activity index:

The activity index [15] of the crude plant extract was calculated as,

$$\text{Activity index (A.I.)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

Determination of proportion index:

The proportion index [16] was calculated as

$$\text{Proportion index (P.I.)} = \frac{\text{Number of positive results obtained for individual extract}}{\text{Total number of tests carried out for each extract}}$$

Results and Discussion

C.occidentalis is a well known herb used as ayurvedic traditional medicine for their effectiveness against wide range of diseases due to the advantage of the diversity of secondary metabolites responsible for their antibacterial activity. The antimicrobial activities of *C.occidentalis* leaf extracts against microorganism examined in the present study and their potency were quantitatively assessed by the presence or absence of inhibition zones and zone diameters (Table 1), MIC and MBC values (Table 2-3).

From the result obtained (Table 1) the aqueous extract has got maximum activity when compared with other solvents like ethanol and methanol. The mechanistic aspects of antimicrobial nature of *Cassia occidentalis* was also observed [17].The antimicrobial efficacy of *C.occidentalis* may result from damages and inactivation of enzymes due to their ability to induce leakage of sodium and potassium ions.[18] Ethanolic and hot water extract of *C. occidentalis* was investigated for their capacity to release sodium and potassium ions for some selected pathogenic bacteria in the genera *Bacillus subtillus, Staphylococcus, Echerichia, Streptococcus, Klebsiella, Pseudomonas* and *Salmonella* using flame photometer. It was found that the aqueous extract was most effective in the leakage of Na and K ions than the ethanolic extract of all organisms except *Salmonella* .It is very much correlated with the obtained results that aqueous alone has got maximum activity.(5-15mm) The proportion index for the antimicrobial activity of aqueous extract is 0.7. Similarly, activity index of aqueous plant extract varies from maximum 0.63 for *C.albicans* and minimum 0.19 for *Aeromonas*.

Using Biofractionation assay, this aqueous has further fractioned in to aqueous and ethyl acetate fraction in order to assess the potential compound. It was observed that the ethylacetate extract has inhibition effect on the growth of 9 of 9 bacterial species and 1 fungi isolates which were *Aeromonas, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Methicillin resistant Staphylococcus aureus, Vancomycin resistant Pseudomonas , Salmonella paratyphi, Staphylococcus pyrogens,Vibrio fischeri and Candida albicans*. However, the fractioned aqueous extract had no antimicrobial activity against any of the bacterial and fungal isolates tested in this study which may be due to the presence of active components in ethylacetate fraction. Maximum inhibition zones, MIC and MBC values for the microorganism sensitive to the ethylacetate extract were in the range of 12-24mm, 62.5-500µg/ml, 250-1000 µg/ml respectively (Tables 2-3).

This is the first study to demonstrate that ethylacetate extract of *C.occidentalis* contains antimicrobial substances with antibacterial and anticandidal effect. The MIC and MBC of the ethylacetate extract were showing lower concentration and higher zone of inhibition, *Aeromonas* (20mm,MIC 250µg/mL,MBC 1000 µg/mL), *B.Subtilis* (18mm,MIC 125µg/mL,MBC 250 µg/mL) , *MRSA* (20mm,MIC 62.5µg/mL,MBC 500 µg/mL), *V.fischeri* (24mm,MIC 250µg/mL,MBC 1000 µg/mL). Most of the MIC values were lower than the MBC values indicating that the extracts could be bactericidal in action. Low MIC and MBC values are also an indication of high efficiency as well as inhibit 99% of microbes. Lower MIC and MBC values and higher zones of inhibition for ethylacetate extract depicts higher solubility of phytoconstituents in the ethylacetate compared with the aqueous fraction. [19]

Saganuvan and Gulumbe [20] observed that the *E. coli* was sensitive to methanol, hexane, chloroform and aqueous extract of leaves of *C. occidentalis* at a concentration range 900-1000 mg/mL ,but in the present study it is observed that *E.coli* has inhibited at the concentration of 500µg/mL (MIC µg/mL ,MBC µg/mL). Similarly there was no antimicrobial activity exhibited against other tested microorganisms (*Salmonella typhi*, *S. pyogenes*). In contrast, our study revealed that the extract of leaves of *C. occidentalis* was effective against *S.paratyphi and S.pyrogens* at concentration of 500µg/ml with 13 and 14 mm inhibition zone respectively. These differences in the plant extracts activities may be due to spatial and temporal variations of the plants. Infections caused by *MRSA*, especially those with multidrug resistance, are among the most difficult to treat conventional antibiotics. In our study, the growth of *MRSA* was remarkably inhibited by the ethylacetate extract of the leaves of *C. occidentalis*.

Although MIC determination is still the gold standard for characterizing the potency of an antimicrobial agent, it does not provide information about the time course of the antibiotic's action.This limitation is overcome by the use of time–kill studies [21],which were performed using strains of high effective range in MIC studies. The results of time–kill investigations showed that it exhibits rapid initial killing against C.*albicans and B.subtilis*.

The time kill curve characterized by a rapid and significant decline (>3 log drop). Re-growth was observed at lower concentrations of ethyl acetate extract (1× and 2× MIC for *C.albicans* and 2× and 4× MIC for *B.subtilis*) but not at concentrations >8× MIC for all strains.(Figure 1-2) At 4X and 8X MIC inhibit *MRSA* (Figure 3) and *B.subtilis* within 1 hour. Confined inhibition was developed in C.albicans after 4 hour incubation. The CLSI defines a bactericidal agent as one for which a given concentration reduces the original inoculum by 99.9% (>3 log10 CFU/mL) for each time period, and bacteriostatic if the inoculum was reduced by 0–3 log10 CFU/mL. [22] According to that definition, time–kill studies reveal that it is bactericidal against *C.albicans*, *B.subtili* and,*MRSA* at concentrations 1× and 2× MIC.

Figure-1: Time Kill curve of ethyl acetate against Candida albicans

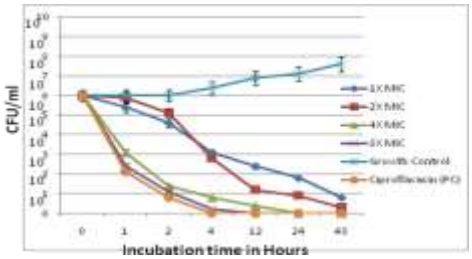


Figure-2- Time kill curve of ethyl acetate against Vancomycin resistant Bacillus subtilis

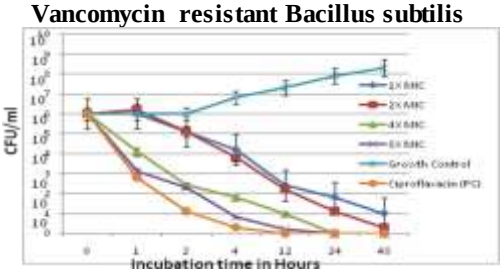
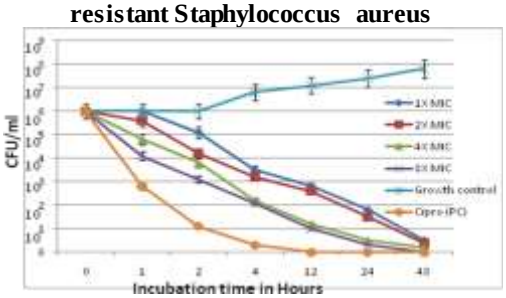


Figure-3 Time kill curve of ethyl acetate against Methicillin resistant Staphylococcus aureus



The broad spectrum antibacterial activities of the plant extract possibly due to the identified alkaloids, further confirm its use as a health remedy in folklore medicine. The result may suggest that ethylacetate extract of the *C.occidentalis* posses compounds with antibacterial and anticandidal properties which can be used as antimicrobial agents in new drugs for therapy of infectious diseases.

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Table 1 Antimicrobial activity of C.occidentalis leaf extracts on different solvents based on disc-diffusion method

Microorganisms	Inhibition zone diameter around test disc			
	Ethanol extract	Methanol extract	Aqueous extract	Standard antibiotic disc
Aeromonas	-	-	5mm	26mm
B.Subtilis	-	-	13mm	22mm
E.coli	-	-	-	18mm
Klebsiella	-	-	14mm	26mm
MRSA	-	-	14mm	26mm
VRPA	-	-	10mm	20mm
S.paratyphi	-	-	15mm	26mm
S.pyogenes	-	-	8mm	20mm
V.fischeri	-	-	9mm	20mm
C.albicans	-	-	15mm	24 mm

*Ciproflaxin (500µg/disc) used as a standard antibiotic, *Each value recorded here was taken as triplets

Table 2 Antimicrobial activity of C.occidentalis leaf extracts (500µg/disc) against the bacterial strains tested based on disc-diffusion method

Microorganisms	Inhibition zone diameter around test disc		
	Ethyl acetate extract	Aqueous extract	Standard antibiotic disc
Aeromonas	20mm	3mm	28mm
B.Subtilis	18mm	-	28mm
E.coli	14mm	-	18mm
Klebsiella	15mm	-	14mm
MRSA	20mm	4mm	28mm
VRPA	12mm	3mm	28mm
S.paratyphi	13mm	3mm	24mm
S.pyrogens	14mm	3mm	28mm
V.fischeri	24mm	-	26mm
C.albicans	22mm	4mm	28mm

*Ciproflaxin (500µg/disc) used as a standard antibiotic, *Each value recorded here was taken as triplets

Table 3 The MIC and MBC values of C.occidentalis leaf extracts against the microorganism tested based in microdilution assay

Microorganisms	Ethyl acetate extract MIC	Ethyl acetate extract MBC
Aeromonas	250µg	1000 µg
B.Subtilis	125 µg	250 µg
E.coli	62.5 µg	500 µg
Klebsiella	62.5 µg	250 µg
MRSA	62.5 µg	500 µg
VRPA	250 µg	1000 µg
S.paratyphi	500µg	1000 µg
S.pyrogens	125 µg	1000 µg
V.fischeri	250 µg	2000 µg
C.albicans	62.5 µg	250 µg

*Each value recorded here was taken as triplets