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EVALUATION OF *IN VITRO* ANTIMICROBIAL ACTIVITY OF *CAESALPINIA BONDUCELLA* AND *CYCLEA PELTATA* EXTRACTS AGAINST OPPORTUNISTIC MICROBES ISOLATED FROM WOUNDS IN DIABETIC PATIENTS

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ABSTRACT

Plants are known for treating infections since ancient times, they are a good source of having phytochemicals with marked antimicrobial activity against human pathogenic microorganisms. The present study was aimed for screening of phytochemicals and to determine antimicrobial potentials of Hexane, ethyl acetate and methanol extracts of *Caesalpinia bonducella* and *Cyclea peltata* against opportunistic microbes isolated from wounds in diabetic patients. *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus mutans, Proteus mirabilis, Escherichia coli* and *Candida albicans* were identified in the diabetic patients wound infections. The plant extracts were screened for antimicrobial activity by agar disc diffusion method. Entire aerial part extracts of *Cyclea peltata* showed no zone of inhibition, however ethyl acetate leaf extract and methanolic root extract of *Caesalpinia bonducella* showed a moderate level of antimicrobial activity and their MIC were determined. Further research work is required in regarding isolation and characterization of active phytochemical compounds responsible for their antimicrobial activity.

KEYWORDS

Diabetes, Wound, Infections, Clinical isolates, Phytochemicals and MIC.

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INTRODUCTION

Wound infections are a major complication in diabetic patients and also a major healthcare burden[1]. Patients with chronic diabetic conditions are prone to slowed process of wound healing and seem to be at particularly high risk for soft tissue infections such as foot infections, urinary tract infections and surgical site infections caused by opportunistic microorganisms such as *Staphylococcus aureus, Streptococcus haemolytic us* and *Pseudomonas aeruginosa*[2-4]. Under chronic

diabetic conditions these wound infections if not treated can lead to gangrene and even amputations[5]. The long term use of existing antibiotic drugs has observed to increase drug resistance to soft tissue infection causing bacteria in diabetic patients. This has led to renewed efforts to use broad spectrum antibiotics[6]. Plants are known for treating infections since ancient times, they are found to be a good source of having phytochemicals with marked antimicrobial activity against human pathogenic microorganisms which can be used to develop new drugs for treating infections and their by usage of synthetic drugs and its associated side effects can be avoided[7]. *Caesalpinia bonducella* (Linn.) Flam and *Cyclea peltata* (Lam.) Hook. f. and Thomson. are well known in Ayurveda and folk medicine systems for their use in the treatment of various skin infections and wounds. Extensive review of literature indicated that not much work has been done on the antimicrobial properties of these plant extracts. With this background, the present study was aimed to determine antimicrobial potentials of *Caesalpinia bonducella* (leaf, bark and root) and *Cyclea peltata* (entire aerial part) extracts against opportunistic microbes isolated from wound infections in diabetic patients. *Caesalpinia bonducella* is well known as *Latakaranja* in Sanskrit, Fever nut in English, *Gejjuga* in Kannada[8]. It is a prickly shrub belongs to family Caesalpiniaceae found throughout India and tropical countries of the world[9]. Traditional herbal practioners use nuts, root, bark and leaves of this plant for treatment of various ailments[10,11]. It has been recognized for its multiple therapeutic properties such as antipyretic, antidiuretic and antihelmintic[12], antiasthmatic[13], anti-inflammatory[14] and anti-hyper glycemic[15] activities. *Cyclea peltata* belongs to family Menispermaceae is a slender twining shrub commonly known as Patha or Rajapatha in Sanskrit and Kariballi in Kannada is found throughout Southern and Eastern India. Tribal people called soliga and kadu kuruba live in tribal settlements near Nagrahole forest use this plant in several herbal preparations in combination with other herbs for treating muscular sprain and wounds. In Ayurveda system of medicine, it is used to treat wounds, snake bite, skin disorders, cardiac pains and urinary related disorders[16,17]. Tuberous roots of this plant are used in the treatment of jaundice, stomach ache, diabetes, fever and asthma[18, 19].

MATERIALS AND METHOD

Collection of Plant Material

Fresh plant materials of *Caesalpinia bonducella* (leaf, bark and root) and *Cyclea peltata* (entire aerial part) were collected from Nagapura haadi and Haemmegae haadi tribal settlements of Hunsur taluk, Mysore district, Karnataka, India. The plants were photographed, taxonomically identified and their herbarium specimens were deposited in the Department of Pharmacognosy, Herbarium Collection Centre, JSS College of Pharmacy, Mysore, India. The fresh plant materials were washed under running tap water, wiped with clean dry cloth, shade dried for 7 days and ground to a coarse powder and stored in air tight containers separately.

Extraction of Plant Materials

500 gm of *Caesalpinia bonducella* (leaf, bark and root) and *Cyclea peltata* (entire aerial part) powder was subjected to soxhlet extraction using hexane, ethyl acetate, and methanol respectively for eight hours at 50^{o}C. They were first filtered separately using double layered muslin cloth and then filtered through Whatmann No.1 filter paper to get clear filtrate. Solvents were evaporated in rotary evaporator and then extracts were dried separately in desiccators to get dried solvent free extracts and were stored at 4^{0}C in air tight brown bottles until further use[20, 21].

Phytochemical screening of plant extracts[22, 23]

The test plant extracts were subjected to preliminary qualitative phytochemical screening as per the standard procedures of Evans and Harborne to determine the presence of various phytoconstituents such as Tannins, Alkaloids, Saponins, Flavonoids, Phytosterols, Triterpenoids and Cardiac glycosides in the extracts.

Isolation and identification of microorganisms from wound swab samples

Pus swab samples were collected by standard method based on Levin technique of swab sample collection using sterile High Media swabs[24] from

surgical site infections and infected wounds on foot, hands and ankles of 50 diabetic patients in JSS Ayurveda Hospital, Mysore, Karnataka, India. The swabs samples were taken immediately to Microbiology laboratory for isolation of aerobic bacteria using Blood agar and MacConkeys agar media (High-Media). After 24 hours of incubation at 37^0C the bacterial isolates were subjected to Gram's staining for identification. The identification of bacterial pathogens were made based on microscopic examination, morphological and cultural characters, staining characteristics and biochemical properties using standard laboratory procedures[25]. The fungal pathogens were identified based on its morphology and cultural characteristics. *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Streptococcus mutans, Proteus mirabilis* and *Candida albicans* were isolated from swabs. Pure cultures of these clinical isolates were prepared using Mueller Hinton agar medium (High-Media) for bacterial culture and for fungal culture, Sabouraud Dextrose Agar media was used for culturing. The clinical isolates along with their MTCC Quality control strains *Staphylococcus aureus* MTCC 7443, *Pseudomonas aeruginosa* MTCC 7903, *Escherichia coli* MTCC 7410, *Streptococcus mutans* MTCC 890, *Proteus mirabilis* MTCC 425 *and Candida albicans* MTCC 183 were used for antibiotic susceptibility tests and antimicrobial activity studies.

Antimicrobial susceptibility test against microbial isolates

Antimicrobial susceptibility tests were conducted for the clinical isolates and its respective MTCC control strains by the standard disk diffusion technique against High-Media standard antimicrobial discs Gentamicin (10mcg), Cefotaxime (30mcg), Erythromycin (15mcg), Amikacin (30mcg), Nitrofurantoin (300mcg), Ciprofloxacin (5mcg), and Fluconazole (25mcg). The diameter of the zones of inhibition produced by each antimicrobial discs were measured using a High-Media antibiotic zone scale and the results were recorded in millimetres. Antimicrobial susceptibility results of each isolates were interpreted as either susceptible (S) or resistance (R) to the antibiotic agent, depending on the diameter of inhibition zone produced in comparison with High-Media zone size interpretative chart[26, 27].

***In vitro* antimicrobial activity studies of the plant extracts**

The antimicrobial activity of hexane, ethyl acetate and methanol extracts of *Caesalpinia bonducella* (Leaf, root and bark) and *Cyclea peltata* (entire aerial part) extracts were determined by agar disc diffusion method[27]. The test bacteria were inoculated into sterile Mueller Hinton broth and incubated at 37^0C for 24 hours, fungal isolate *Candida albicans* was inoculated into sterile Sabouraud dextrose broth and kept under room temperature for 48 hours and used as inoculums. 0.1 ml [i.e. 100 µl (1×10^5 cfu/ml)] of the inoculums of *test bacteria* and *Candida albicans* were spread aseptically on sterile Mueller Hinton agar and Sabouraud dextrose agar plates respectively. 100mg/ml concentration of the plant extracts stock was prepared using the respective solvents (Ethyl acetate/ hexane/ methanol) from which they have been extracted. 10 µl of plant extracts were impregnated on 6mm sterile Whatmann No.1 discs using sterile forcipes and allowed to dry in laminar air flow system under aseptic condition and were placed on the inoculated plate. 10 µl of Ciprofloxacin (0.5 mg/ml) for bacterial cultures plates and 10 µl of Fluconazole (2.5 mg/ml) for fungal cultures were impregnated on 6mm sterile Whatmann No.1 discs and were used as positive control. The bacterial plates were incubated at 37^oC for 16-18 hours, for *Candida albicans* the plates were kept under room temperature for 4-5 days. After incubation, the plates were examined for zone of inhibition around the disk. The antimicrobial activity was evaluated by measuring the diameter of inhibition zones. The mean value of the diameter of the inhibition zone of the triplicates was taken as the final value.

Determination of MIC by micro broth dilution technique[28]

Minimum inhibitory concentration of *Ceasalpinia bonducella* Ethyl acetate Leaf extract (LE) and *Ceasalpinia bonducella* Methanolic Root Extract (RM) were determined by micro broth dilution

technique as per NCCLS method against clinical isolates *Pseudomonas aeruginosa, Staphylococcus aureus* and *Proteus mirabilis* isolated from swab samples of wounds infections in diabetic patients. MIC of test extracts against the respective MTCC strains were also determined and used as a standard reference for comparison to the MIC of extracts against clinical isolates.

Preparation of inoculums

Cell suspensions were prepared from bacterial cultures (MTCC cultures and clinical isolates) grown on Trypticose soya broth was adjusted to 1 to 2 x 10^5 cells/mL according to the Macferland's standards and used as inoculum.

Micro broth dilution technique procedures as per NCCLS method

1 to 64 µg/ml (Two fold dilutions) Concentrations of the standard drug Ciprofloxacin was prepared in Muller Hinton broth. 90 µl of this each individual drug concentrations were mixed with 10 µl Inoculum in 96 well plates in triplicates. 16 to 1024 µg/ml (Two fold dilutions) Concentrations of *Ceasalpinia bonducella* Ethyl acetate Leaf extract (LE) and *Ceasalpinia bonducella* Methanolic Root Extract (RM) were prepared in Muller Hinton broth for bacterial test cultures. 90 µl of this each test plant extracts concentrations (LE / RM separately) were mixed with 10 µl Inoculum in 96 well plate in triplicates. 90 µl Muller Hinton broths without drug or plant extract was mixed with 10 µl Inoculum in 96 well plates in triplicates and used as Positive control. All these above treated bacterial culture test well plates were incubated at 37^0C and were observed for after 24-48 hrs and O.D was measured at 600 nm in Tecan plate reader. OD of different concentrations of test plant extracts (LE and RM) and standard drug Ciproflaxacin were observed.

Minimum inhibitory concentration of drug and Test plant extracts

MIC of standard drug was determined as Minimum concentration of drug giving 50% inhibition of OD as compared with control, similarly MIC of test extracts were determined as Minimum concentration of test extract giving 50% inhibition of OD as compared with control. MIC of test plant extracts were compared with the MIC of standard drug Ciproflaxacin. MIC of standard drug Ciproflaxacin and test plant extracts against MTCC control strains and microbial isolates are all listed in Table No.4.

RESULTS AND DISCUSSION

Folk medicinal plants *Caesalpinia bonducella* (Leaf, root and bark) and *Cyclea peltata* (entire aerial part) were selected for the present study aimed for screening of phytochemicals to know the presence of various groups of secondary metabolites and to determine antimicrobial potentials of these plant extracts against opportunistic microbes isolated from infected wounds in diabetic patients. Hexane, ethyl acetate and methanol extracts of *Caesalpinia bonducella* (Leaf, root and bark) and *Cyclea peltata* (entire aerial part) were tested for the presence of phytochemical constituents such as sterols, triterpenes, alkaloids, glycosides, saponins, flavonoids, phenolics, tannins, proteins, amino acids, carbohydrates, fixed oils and fats were all listed in the Table No.1. For the present study pus swab samples of infected wounds were taken from 50 diabetic patients in JSS Ayurveda Hospital, Mysore, Karnataka, India**.** In the study diabetic foot infections were common among diabetic patients, however, there were cases where these wounds were also found on hands, ankles and also surgical site infections were observed in chronic diabetic patients. The wound infections were of polymicrobial nature, *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Streptococcus mutans, Proteus mirabilis* and *Candida albicans* were predominantly found in the swab samples among these patients. Antimicrobial susceptibility test was conducted for these microbial isolates against standard antibiotics by disc diffusion method indicated that the opportunistic microbial isolates were resistant to most of the antibiotics i.e. they were of highly virulent strains, the results of susceptibility tests are listed in Table No.2. Hexane, ethyl acetate and methanol extracts of *Caesalpinia bonducella* (leaf, bark and root) and *Cyclea peltata* (entire aerial part) were tested for their antimicrobial activity potentials against these clinical microbial isolates and their respective MTCC control strains. Among all these test extracts only ethyl acetate leaf

extract and methanolic root extract of *Caesalpinia bonducella* showed a moderate level of antimicrobial activity and rest of the extracts of *Caesalpinia bonducella* and entire aerial part extracts of *Cyclea peltata* did not show any zone of inhibition against the test microbial clinical isolates and their MTCC control strains. The antimicrobial activity results are all listed in the Table No.3. MIC of ethyl acetate leaf extract and methanolic root extract of *Caesalpinia bonducella* against the test microbial clinical isolates and their MTCC control strains were determined. MIC of ethyl acetate leaf extract of *Caesalpinia bonducella* against *Pseudomonas aeruginosa* clinical isolates and *Pseudomonas aeruginosa* MTCC 7903 was 1024 µg/ml. MIC of ethyl acetate leaf extract of *Caesalpinia bonducella* against *Staphylococcus aureus* clinical isolates and *Staphylococcus aureus* MTCC 7443 were >1024 and 1024 µg/ml respectively. Similarly MIC of methanolic root extract of *Caesalpinia bonducella* against *Proteus mirabilis* clinical isolates and *Proteus mirabilis* MTCC 425 were >1024 and 1024 µg/ml respectively. MIC results are all listed in the Table No.4.

Table No.1: Phytochemical Components of *Caesalpinia bonducella* (leaf, bark and root) and *Cyclea peltata* (aerial part) extracts

S.No	Phytochemicals	Plant extracts											
		LH	**LE**	**LM**	**BH**	**BE**	**BM**	**RH**	**RE**	**RM**	**AH**	**AE**	**AM**
1	Sterols	+	+	+	+	+	+	+	+	+	-	-	-
2	Triterpenes	-	-	-	-	-	-	-	-	-	-	-	+
3	Alkaloids	-	-	-	-	-	-	-	-	-	-	+	+
4	Glycosides	-	-	+	-	-	+	-	-	+	-	-	+
5	Saponins	-	-	+	-	-	+	-	-	+	-	+	+
6	Flavonoids	-	+	+	-	+	+	-	+	+	-	+	+
7	Phenolics And Tannins	-	+	+	-	+	+	-	+	+	-	+	+
8	Proteins And Amino acids	-	-	+	-	-	-	-	-	-	-	-	+
9	Carbohydrates	-	+	+	-	-	+	-	-	+	-	+	+
10	Fixed oils And Fats	-	-	-	+	+	+	-	+	+	-	-	-

Note

Caesalpinia bonducella leaf- Hexane, Ethyl acetate and Methanol extracts- (LH, LE and LM)
Caesalpinia bonducella bark - Hexane, Ethyl acetate and Methanol extracts- (BH, BE and BM)
Caesalpinia bonducella root - Hexane, Ethyl acetate and Methanol extracts- (RH, RE and RM)
Cyclea peltata aerial part- Hexane, Ethyl acetate and Methanol extracts- (AH, AE and AM)
+ = present, - = absent

Table No.2: Antimicrobial susceptibility test for the clinical isolates and it's respective MTCC control strains by standard disc diffusion method

S.No	Micro organisms (Clinical isolates)	Zone of Inhibition (mm) and Sensitivity						
		GEN (10 mcg)	**CTX (30mcg)**	**E (15mcg)**	**AK (30mcg)**	**NIT (300mcg)**	**CIP (5mcg)**	**FLC (25mcg)**
1	*Pseudomonas aeroginosa*	11mm (R)	16 mm (R)	6mm (R)	10mm (R)	6mm (R)	14 mm (R)	-
2	*Escherichia coli*	18mm (R)	24mm (R)	6mm (R)	12mm (R)	10mm (R)	16mm (R)	-
3	*Staphylococcus aureus*	14mm (S)	18mm (S)	14mm (R)	20mm (S)	11mm (R)	21mm (S)	-
4	*Streptococcus mutans*	20mm (S)	6mm (R)	19mm (S)	20mm (S)	10mm (R)	19mm (I)	-
5	*Proteus mirabilis*	10mm (R)	20mm (S)	12mm (R)	10mm (R)	6mm (R)	12mm (R)	-
6	*Candida albicans*	-	-	-	-	-	-	13mm (R)
S.No	Micro organisms (MTCC samples)	GEN (10 mcg)	CTX (30mcg)	E (15mcg)	AK (30mcg)	NIT (300mcg)	CIP (5mcg)	FLC (25mcg)
1	*Pseudomonas aeroginosa* MTCC 7903	22 mm (S)	22mm (S)	10 mm (R)	19mm (S)	11mm (R)	28mm (S)	-
2	*Escherichia coli* MTCC 7410	21 mm (S)	30mm (S)	6mm (R)	20mm (S)	21mm (S)	31mm (S)	-
3	*Staphylococcus aureus* MTCC 7443	20 mm (S)	25mm (S)	23mm (S)	22mm (S)	19mm (S)	25mm (S)	-
4	*Streptococcus mutans* MTCC 890	26 mm (S)	11mm (R)	26mm (S)	29mm (S)	16mm (S)	25mm (S)	-
5	*Proteus mirabilis* MTCC 425	20mm (S)	25mm (S)	17mm (S)	16mm (S)	10mm (R)	27mm (S)	-
6	*Candida albicans* MTCC 183	-	-	-	-	-	-	22 mm (S)

Note

Antimicrobial agents (HIMEDIA) -

Gentamicin (SD016) - GEN, Cefotaxime (SD040) - CTX, Erythromycin (SD013) - E, Amikacin (SD035) - AK, Nitrofurantoin (SD023) - NIT, Ciprofloxacin (SD060) - CIP, Fluconazole (SD232) - FLC.

MTCC- Microbial Type Culture Collection.

(S) - Sensitive, (R) - Resistant, (I) - Intermediate.

Table No.3: *In vitro* Antimicrobial Activity of Plant Extracts Against Clinical Isolates and MTCC Control strains

Micro organisms (Clinical isolates)	Mean Diameter Inhibition zone (mm)																
	Caesalpinia bonducella extracts 10 µl/disc (Conc 100mg/ml)									*Cyclea peltata* extracts 10 µl/disc (Conc 100mg/ml)			Antimicrobial agents (+ve control) 10 µl/disc		Solvents (-ve control) 10 µl/disc		
	LH	LE	LM	BH	BE	BM	RH	RE	RM	AH	AE	AM	Cip 0.5 mg/ml	Flu 2.5 mg/ml	H	E	M
Pseudomonas aeroginosa	-	8.00 ± 0.25	-	-	-	-	-	-	-	-	-	-	14.0 ± 0.00	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	16.0 ± 0.50	-	-	-	-
Staphylococcus aureus	-	9.00 ± 0.25	-	-	-	-	-	-	-	-	-	-	21.0 ± 0.50	-	-	-	-
Streptococcus mutans	-	-	-	-	-	-	-	-	-	-	-	-	19.00 ± 1.00	-	-	-	-
Proteus mirabilis	-	-	-	-	-	-	-	-	7.00 ± 0.25	-	-	-	12.00 ± 1.00	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	13.00 ±0.25	-	-	-
Microorganisms (MTCC samples)	LH	LE	LM	BH	BE	BM	RH	RE	RM	AH	AE	AM	Cip 0.5 mg/ml	Flu 2.5 mg/ml	H	E	M
Pseudomonas aeroginosa MTCC 7903	-	10.00 ± 0.25	-	-	-	-	-	-	-	-	-	-	28.0 ± 0.00	-	-	-	-
Escherichia coli MTCC 7410	-	-	-	-	-	-	-	-	-	-	-	-	31.0 ± 0.50	-	-	-	-
Staphylococcus aureus MTCC 7443	-	12.00 ± 0.25	-	-	-	-	-	-	-	-	-	-	25.5 ± 2.50	-	-	-	-
Streptococcus mutans MTCC 890	-	-	-	-	-	-	-	-	-	-	-	-	25.00 ± 1.00	-	-	-	-
Proteus mirabilis MTCC 425	-	-	-	-	-	-	-	-	9.00 ± 0.25	-	-	-	27.00 ± 1.00	-	-	-	-
Candida albicans MTCC 183	-	-	-	-	-	-	-	-	-	-	-	-	-	22.00 ± 0.25	-	-	-

Note:

Caesalpinia bonducella leaf- Hexane, Ethyl acetate and Methanol extracts- (LH, LE and LM)
Caesalpinia bonducella bark- Hexane, Ethyl acetate and Methanol extracts- (BH, BE and BM)
Caesalpinia bonducella root- Hexane, Ethyl acetate and Methanol extracts- (RH, RE and RM)
Cyclea peltata aerial part- Hexane, Ethyl acetate and Methanol extracts- (AH, AE and AM)
Cip- Ciproflaxacin, Flu- Flucanazole, E- Ethyl acetate, H-Hexane and M- Methanol - = no activity.

Table No.4: Minimum Inhibitory Concentration of plant extracts against microbial isolates

S.No	Test organisms			Standard Antibiotic MIC (µg/ml)
		Ceasalpinia bonducella Extracts MIC (µg/ml)		
		Root methanolic extract	Leaf Ethyl acetate extract	Ciproflaxacin
1	*Pseudomonas aeruginosa* MTCC 7903	-	1024	<1
2	*Pseudomonas aeruginosa* clinical isolate	-	1024	8
3	*Staphylococcus aureus* MTCC 7443	-	1024	<1
4	*Staphylococcus aureus* clinical isolate	-	>1024	8
5	*Proteus mirabilis* MTCC 425	1024	-	<1
6	*Proteus mirabilis* clinical isolate	>1024	-	8

CONCLUSION

Results reveal that ethyl acetate leaf extract and methanolic root extract of *Caesalpinia bonducella* has showed a moderate level of antimicrobial activities against virulent strains of microbial isolates from wound infections in chronic diabetic patients. Further research work is required in regarding isolation and characterization of active phytochemical constituents from these plant extracts which are responsible for their antimicrobial activity. Entire aerial part extracts of *Cyclea peltata* did not showed any antimicrobial activity against the test microbial clinical isolates and their MTCC control strains. However in folk system of medicine herbal medicinal practioners use these plants for curing skin infections and wounds, where these plants are used as one of the ingredients in their polyherbal formulations along with other herbs. The synergetic effect of different plants used in polyherbal formulations may be responsible for curing infections and wounds.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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