Investigation on Antimicrobial activity of *Dioscorea Pentaphylla* from Mid-Western Ghats, India.

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ABSTRACT: Antibacterial and antifungal activity of crude extracts of medicinally important and traditionally used yam plant *Dioscorea pentaphylla* from mid Western Ghats was evaluated against 27 bacterial and 5 fungal clinical strains collected of the patients from infectious sources. The clinical strains belonging to their respective species showed concentration dependent susceptibility towards crude petroleum ether extract, chloroform extract and methanol extract at 100µg/100µL. All the extracts exhibited predominant antibacterial activity against *S. aureus* (ATCC-20852), *P. aeruginosa* (ATCC-29737) and *K. pneumoniae* (MTCC-618) respectively. and five clinically isolated pathogenic fungi, *T. rubrum, M. gypseum, T. tonsurans, M. audouini, and C. albicans* with antibacterial drug Ciprofloxacin and antifungal drug Fluconozole (50µg/100µL) as the standard drug. Out of three extracts, ethanol extracts possessed better minimum inhibition concentration against all the bacterial strains. All the three extracts showed significant result against all the five fungal pathogen strains. The results are promising and supported the traditional use of *D. Pentaphylla* for the treatment of bacterial and fungal infections.
[Prakash G., Joy Hoskeri H. and Hosetti B. B. **Investigation on Antimicrobial activity of *Dioscorea Pentaphylla* from Mid-Western Ghats, India.** *Rep Opinion* 2014;6(9):78-83]. (ISSN: 1553-9873). http://www.sciencepub.net/report. 13

Keywords : Antifungal, Antibacterial, Crude extracts, *Dioscorea pentaphylla*, MIC, Traditional medicine.

Introduction

The practice of antimicrobial chemotherapy is one of constant challenges, particularly in view of the rapid evolutionary changes and wide variety of pathogens encountered. Many investigators evaluated the bioactivity of plant extracts and their constituents against the serious infectious organisms (Parekh and Sumitra, 2006; Kausik *et al.,* 2002).

Prevalence of antibiotic-resistant strains of bacteria due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control the bacterial diseases (Cown, 1999; Bax *et al.,* 2002). Numerous studies have been carried out in different parts of the globe to extract plant products for screening antimicrobial activity (Essawi and Srour, 2000; Rajanaik *et al*., 2005).

Dioscorea yam is a member of the Yam family. The yams are vining plants with 600 known species, 71 of which are native to North America (67 species in Mexico) (Hutchens Alma, 1991). In many species of yam, the rhizome (tuber) serves as both food and medicine. Many native Americans and south Asians used a syrup of the root to relieve labour pain and later physicians gave wild yam to patients with colic pain, morning sickness, asthma, hiccough, rheumatism and gastritis related to alcoholism (Foster and Duke, 2000). Modern herbalists value wild yam to treat intestinal colic, biliary colic, and flatulence as well as menstrual cramps and rheumatoid arthritis (Tierra, 1998; Grieve, 1971; Fleming *et al.,* 1998). Herbalists combine wild yam with black cohosh (and sometimes burdock root and motherwort) (Hutchens Alma, 1991) for rheumatic complaints. Chinese herbalists use wild yam as a tonic (Hutchens Alma, 1991).

In the present investigation, *Dioscorea pentaphylla* was selected, as one of the medicinally important plant, extensively consumed by local people as food. However, there is apparently no scientific reports on the antimicrobial properties of this plant. The lack of scientific knowledge has often exerted a major constraint on the use of traditional herbal remedies as an affordable alternative to orthodox medical treatment. Thus, the different solvent extracts of the tuber were screened for its activity against three bacterial pathogens and five fungal strains.

2. Material and methods

2.1. Plant material

Tubers of *Dioscorea pentaphylla* were collected from the Lakkavalli reserve forest in and around area of Bhadra wild life sanctuary of the Mid-western Ghats region of Karnataka, India and the species was identified by comparing with the authenticated specimen deposited at the Kuvempu University herbaria (Voucher specimen KUDB/ Ang/324). The leaves were washed in running tap

water, shade dried, powdered mechanically and sieved (Sieve No. 10/44) and subjected to Soxhlet extraction using different solvents *viz.,* Petroleum ether, Chloroform and Ethanol. The extracts were concentrated under reduced pressure at 40 ± 5°C using a rotary flash evaporator (Buchi, Flawil, Switzerland). All the three extracts were subjected to phytochemical qualitative analysis.

2.2. Preparation of plant extracts for antimicrobial assay

100 µg of all the crude extracts was dissolved in 100 µl of 10% DMSO. The standard antibacterial drug Ciprofloxacin and antifungal drug Fluconozole were also tested at a concentration 50 µg/100 µl of each.

2.3. Evaluation of Minimal Inhibitory Concentrations (MIC).

The Minimal Inhibitory Concentrations (MIC) of the different solvent crude extracts was determined by micro dilution techniques in LB broth, according to Clinical and Laboratory Standards Institute (CLSI), USA guidelines. The bacterial inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard colony forming units and diluted 1:10 for the broth micro dilution procedure. The micro titre plates were incubated at 37°C and MIC was determined after 24 h of incubation. The highest activity of the isolated compounds compared to those of the crude extracts indicates that those compounds alone were solely responsible for antimicrobial activity.

2.4. Screening of Antimicrobial activity.

Twenty seven clinical strains of three of the most common bacterial pathogens, *S. aureus, P. aeruginosa and K. pneumoniae*, (*Staphylococcus aureus* – ATCC-29737; *Pseudomonas aeruginosa* - ATCC-20852 and *Klebsiella pneumoniae* - MTCC-618) strains of the corresponding bacteria and five clinically isolated pathogenic fungi, *T. rubrum, M. gypseum, T. tonsurans, M. audouini, and C. albicans* were used as test organisms. Different pathogens and their serotype were isolated from infected patients in the district health centre of Annamali nagar, and were identified in the Department of Zoology, Annamali University, India in support with National Chemical Laboratory, Pune, India. The profile of bacterial species and their strains of different clinical origin are shown in Table 1**.** All the bacterial pathogens were maintained at -30°C in Brain Heart Infusion (BHI) containing 17% (v/v) glycerol. Before testing, the suspensions were transferred to LB broth and incubated overnight at 37°C. Inocula were prepared by adjusting the turbidity of the medium to match the 0.5 McFarland standards. Dilutions of this suspension in 0.1% peptone (w/v) solution in sterile water inoculated on LB agar, to check the viability of the preparations. In case of fungal stocks cultures were stored on Brain Heart Infusion (BHI, Merck) culture media (pH 6.5).

2.5. Antimicrobial assay

The agar radial well diffusion method (Mukherjee *et al.,* 1995) was used for the assessment of antimicrobial activity of the extracts of *D. pentaphylla*. Nutrient agar medium (tryptone 10 g/l, yeast extract 5 g/l, sodium chloride 10 g/l, agar-agar 15 g/l, pH 7.2) was poured into sterilized petri dishes (90 mm diameter). LB broth containing 100µl of 24 h incubated cultures of the respective clinical isolates and the ATCC and MTCC strains were spread separately on the agar medium. Wells were created using a sterilized cork borer under aseptic conditions.

In order to identify antifungal activity of total extracts and fractions against fungal pathogens the agar diffusion assay was performed in BHI culture media (pH 6.5). Fungal spores were obtained by centrifugation at 1500 × g/4°C for 15 min and diluted in phosphate buffer saline (PBS), pH 7.2.

Spore count was performed using haemocytometer. After loading 10µl of the cell suspension in PBS and number of spores/ml was calculated, the final concentration of each strain was identified to be 106 spores/ml. Cultures were incubated for 72 h at 24°C. 100µl of fungal inoculum was spread on the BHI agar plates and wells were made using cork borer and 50µl of test compounds were loaded to each wells. The plates were refrigerated for 2 h in order to stop fungal growth and facilitate diffusion of the substances.

The reference antibacterial agent Ciprofloxacin and antifungal agent Fluconozole were loaded in the corresponding wells in the bacterial and fungal culture plates. Bacterial culture plates were then incubated at 37°C for 24 h, and fungal culture plates were incubated at 24°C for 48 h. At the end of the incubation period, inhibition zones were observed measured.

2.6. Statistical analysis

The results of these experiments are expressed as mean ± SE of three replicates in each test. The data were evaluated by one-way Analysis of Variance (ANOVA) and mean separations were carried out using Duncan's Multiple Range Test (DMRT, Gomez and Gomez 1984). Followed by Tukey's multiple comparison tests to assess the statistical significance. $P \leq 0.05$ was considered as statistically significant level.

Table 1. list of the clinical strains used for antimicrobial activity.

Clinical strains	Clinical condition	Source
P. aeruginosa		
Ps-1	Bronchitis	Wounds
Ps-2	Otitis media	Pus
Ps-3	Burns	Sputum
Ps-4 and Ps-5	Upper UTI	Stool
Ps-6	Food poisoning	Hospital effluent
Ps-7	Cross infections in UTI	Hospital effluent
Ps-8	Septicemia	Old wounds
Ps-9	Unknown	Ear swab
K. pneumoniae		
Kp-1	Pneumonia	Mucus
Kp-2	Gram negative	Follicullitis stipules
Kp-3	Burns	Pus
Kp-4	UTI	Urine
Kp-5	Septicemia	Sputum
Kp-6	Cross infections in UTI	Urine
Kp-7	Abscess in immunodeficiency	Wounds
Kp-8	Upper UTI	Urine
Kp-9	Unknown	Hospital effluent
S. aureus		
Sa-1	Abscess in immunodeficiency	Wounds
Sa-2	Burns	Pus
Sa-3	Septicemia	Old wounds
Sa-4	Food poisoning	Stool
Sa-5	Burns	Pus
Sa-6 and Sa-7	Unknown	Hospital effluent
Sa-8	Abscess in immunodeficiency	Sputum
Sa-9	Otitis media	Ear swab
Fungal strains		
T. rubrum	Cutaneous mycoses	Skin
T. tonsurans	Scaring of the scalp	Scalp ringworm
M. gypseum	Ringworm	Infections Skin
M. audouini	Cutaneous mycoses	Skin and hairs
C. albicans	Opportunistic mycoses candidosis	Lungs

Ps = clinical strains of *Pseudomonas aeruginosa,* Kp = clinical strains of *Klebsiella pneumoniae,* Sa = clinical strains of *Staphylococcus aureus*.

3. Results

Results of phytochemical analysis of *Dioscorea pentaphylla* tuber extracts are printed in Table 2. All the three extracts were tested for the presence of phenols/polyphenols, flavonoids, terpenoids, tannins, alkaloids and saponins. Phenols and Saponins were invariably present in all the solvent extracts. The analysis of different tuber extracts (*viz.,* petroleum ether, chloroform and ethanol) also showed presence of combinations of other phytochemical constituents as well.

The results of antimicrobial investigation revealed that the Minimal Inhibitory Concentrations (MIC) of the petroleum ether, Chloroform and ethanol extracts was 100µl/100µl for each. The results of antibacterial activity of all crude extracts a synchronizing effect on clinical strains of pathogenic bacteria and dermatitis fungi was detected. The zones of inhibition of the microbial colony are depicted in Tables 1. The pet ether extract demonstrated antibacterial activity against all the clinical strains of bacteria. It showed maximum activity against *S. aureus* (16.13 mm) followed by *P. aeruginosa* (12.30 mm), *K. pneumoniae* (12.23 mm) and among fungal strains *Microsporum audouini* (14.42 mm) when compared to standard. Chloroform extract showed

least inhibition activity against all the strains of bacteria and fungi. It was 19.40 mm against *S. aureus*, *P aeruginosa* (16.00 mm), *K pneumoniae* (14.33 mm) and in fungal strain *Tricophyton tonsurans* (16.23 mm) respectively. Whereas, ethanol extract showed significant inhibition zone as similar to standard. Ethanol extract illustrated inhibition zone against S. aureus (20.63 mm), *P aeruginosa* (20.50 mm), *K pneumoniae* (19.26 mm) and in fungal strain *Microsporum gypseum* (20.37 mm) and *Candida albicans* (18.13 mm) respectively (Table 2).

Among all the tested extracts ethanol proved to be most potent bactericidal agent against all the strains as compared to other extracts, but it is not up to the standard drug Ciprofloxacin. Among the five dermatitis fungi cultured for antifungal assay, all the crude extracts showed the zone of inhibition of the colony was found to be very good. The ethanol extracts showed significant inhibition against in *C. albicans, T. rubrum, M. gypseum* and *T. tonsurans* at par with standard drug Fluconozole.

4. Discussion

The results obtained in the hitherto study showed wide spectrum of antimicrobial properties for the petroleum ether, chloroform and ethanolic extracts of *D. pentaphylla*. The organic solvent extracts of *D. Pentaphylla* tubers studied in the current work showed remarkable antibacterial activities against *Pseudomonas aeruginosa* , *Klebsiella pneumoniae* and *Staphylococcus aureus* responsible for causing diseases in animals and humans. These microorganisms pose an important public health and economic concerns for human society. However, the solvent extracts proved to be significant in their activity against the above bacterial strains.

There are reports showing that alkaloids and flavonoids are the responsible for the antifungal activities in higher plants (Cordell *et al.,* 2001). Moreover, secondary metabolites such as tannins and other compounds of phenolic nature are also classified as active antimicrobial compounds. Phenols, the aromatic compounds with hydroxyl groups are widespread in plant kingdom. They occur in all parts of plants. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amount of polyphenols are resistant to bird attack (Sadasivam and Manickam, 2006).

Interestingly, phytochemical screening of the current investigation revealed that extracts from both the plant parts and the tuber possess at least three to four of the following classes of secondary metabolites: phenols, flavonoids, terpenoids, tannins, alkaloids and saponins. Therefore, the presence of these phytochemicals, could justify the observed antifungal activities in the current study. These results are in agreement with earlier studies realized on other plant species belonging to the euphorbiaceae (Mahomoodally *et al.,* 2005) and asteraceae (Boussaada *et al.,* 2008).

Dioscorea (Sautor *et al.,* 2003) attributing antimicrobial activities to the presence of secondary metabolites. Diosgenyl saponins are one of the most abundant steroid saponins, with diosgenin as the steroidal sapogenin, are reported to exert a large variety of biological functions, such as anti-fungal, anti-bacterial, and anticancer (Li *et al.,* 2001).

Earlier chemical investigation of yam tubers afforded two norclerodane diterpenoids (Murray *et al.,* 1984). Clerodane class of diterpenes is a group of compounds that has attracted considerable interest because of problems associated with their stereochemistry and because of the diverse biological activities shown by some members (Roengsunran *et al.,* 2002). They are known to possess anti-tumor, anti-bacterial, anti-feedant, and anti-fungal activities (Biswanath *et al.,* 2005; Harding *et al.,* 2006).

The studies of Quan *et al* (2006) reported efficient antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* of diosgenin deratives like 2,6-iodopseudogiosgenin and 2,6-iodopsuedogiosgenone. Sautour *et al* (2004) showed Steroidal Saponin from *Dioscorea cayenensis* showed a positive results aganist *Candida albicans* (IP 1180-79), *C. glabrata* and *C. tropicalis* (clinical isolates). The CH_2Cl_2-soluble portion of the crude extract and the two clerodanes were showed significant activities against *P. aeruginosa, S. typhi, S. paratyphi A and S. paratyphi B,* was reported by Teponno *et al* (2006).

In the heitherto the traditional use of tubers of *Dioscorea pentaphylla* for the treatment of bacterial and fungal infections is realised. For follow-up research, it is needed to determine the active components in each extract and confirm their mechanism of action.

Table 2. Antibacterial activity of the crude extracts of *Dioscorea pentaphylla* against clinical bacterial and fungal isolates.

Clinical strains	Clinical condition	Pet. Ether extract	Chloroform Extract	Ethanol Extract	Ciprofloxacin
P. aeruginosa					
Ps-1	Bronchitis	07.40 ± 0.10	12.83 ± 0.20	17.23 ± 0.14	23.30 ± 0.15
Ps-2	Otitis media	09.00 ± 0.12	10.16 ± 0.16	16.33 ± 0.24	20.50 ± 0.28
Ps-3	Burns	11.23 ± 0.15	11.73 ± 0.33	19.23 ± 0.14	22.23 ± 0.14
Ps-4	Upper UTI	10.23 ± 0.15	13.50 ± 0.17	18.43 ± 0.23	20.20 ± 0.26
Ps-5	Upper UTI	12.30 ± 0.10	13.23 ± 0.14	18.06 ± 0.18	23.30 ± 0.15
Ps-6	Food poisoning	7.40 ± 0.10	17.33 ± 0.20	20.50 ± 0.28	24.33 ± 0.20
Ps-7	Cross infections in UTI	10.60 ± 0.12	16.00 ± 0.28	17.00 ± 0.28	20.00 ± 0.28
Ps-8	Septicemia	08.53 ± 0.29	12.13 ± 0.13	19.23 ± 0.17	23.16 ± 0.16
Ps-9	Unknown	11.37 ± 0.19	13.33 ± 0.16	19.12 ± 0.00	24.50 ± 0.28
K. pneumoniae					
Kp-1	Pneumonia	09.23 ± 0.14	13.23 ± 0.15	16.23 ± 0.14	25.00 ± 0.12
Kp-2	Gram negative	08.56 ± 0.12	14.33 ± 0.17	15.40 ± 0.10	20.23 ± 0.15
Kp-3	Burns	09.23 ± 0.14	12.30 ± 0.15	14.23 ± 0.14	21.37 ± 0.09
Kp-4	UTI	10.23 ± 0.14	12.77 ± 0.09	14.50 ± 0.28	20.20 ± 0.26
Kp-5	Septicemia	09.40 ± 0.10	14.27 ± 0.18	17.73 ± 0.12	23.37 ± 0.09
Kp-6	Cross infections in UTI	12.23 ± 0.14	10.43 ± 0.23	18.30 ± 0.15	22.53 ± 0.18
Kp-7	Abscess in immunodeficiency	12.30 ± 0.10	12.33 ± 0.17	19.26 ± 0.14	24.37 ± 0.19
Kp-8	Upper UTI	11.16 ± 0.16	14.30 ±0.17	16.17 ±0.17	23.43 ± 0.12
Kp-9	Unknown	14.06 ± 0.06	13.30 ± 0.15	18.43 ± 0.03	24.43 ± 0.12
S. aureus					
Sa-1	Abscess in immunodeficiency	19.40 ± 0.23	13.33 ± 0.17	18.37 ± 0.19	28.33 ± 0.17
Sa-2	Burns	18.43 ± 0.23	14.23 ± 0.15	20.60 ± 0.10	26.90 ± 0.21
Sa-3	Septicemia	14.27 ± 0.18	14.87 ± 0.37	16.70 ± 0.10	21.50 ± 0.29
Sa-4	Food poisoning	12.40 ± 0.21	12.17 ± 0.17	20.63 ± 0.09	24.50 ± 0.29
Sa-5	Burns	13.47 ± 0.24	14.33 ± 0.17	17.27 ± 0.12	20.43 ± 0.23
Sa-6	Unknown	16.30 ± 0.15	12.17 ± 0.17	19.33 ± 0.22	27.10 ± 0.21
Sa-7	Unknown	17.60 ± 0.15	13.17 ± 0.17	20.30 ± 0.25	25.50 ± 0.29
Sa-8	Abscess in immunodeficiency	12.57 ± 0.07	16.13 ± 0.12	19.57 ± 0.12	23.50 ± 0.29
Sa-9	Otitis media	11.43 ± 0.23	13.33 ± 0.17	18.60 ± 0.10	23.83 ± 0.44
Clinical strains	**Clinical condition**	**Pet. Ether extract**	**Chloroform Extract**	**Ethanol Extract**	**Fluconozole**
T. rubrum	Cutaneous mycoses	10.63 ± 0.09	13.30 ± 0.15	14.37 ± 0.19	15.43 ± 0.23
T. tonsurans	Scaring of the scalp	12.37 ± 0.09	15.37 ± 0.09	20.37 ± 0.09	21.37 ± 0.19
M. gypseum	Ringworm	11.60 ± 0.10	16.23 ± 0.15	16.15 ± 0.09	16.57 ± 0.12
M. audouini	Cutaneous mycoses	14.42 ± 0.09	12.77 ± 0.15	10.37 ± 0.19	16.40 ± 0.10
C. albicans	Opportunisticmycoses candidosis	10.06 ± 0.13	14.37 ± 0.19	18.13 ± 0.09	18.23 ± 0.15

Ps = clinical strains of *Pseudomonas aeruginosa*, Kp = clinical strains of *Klebsiella pneumoniae*, Sa = clinical strains of *Staphylococcus aureus*. The values are the mean of three experiments ± S.E.

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4/22/2013