In vitro studies on medicinal plants used against bacterial diabetic foot ulcer (BDFU) and urinary tract infected (UTI) causing pathogens

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Abstract The pus samples from diabetic foot ulcer patients and urine samples from urinary tract infected patients were collected and inoculated in nutrient agar plates. The colonies showing different morphologies were streaked on selective agar plates. The antibacterial assay of selected commercial antibiotics was tested against the foot ulcer and urinary tract isolates. The result revealed that most of the organisms were found to be resistant against the antibiotics. Screening of antibacterial activity of selected plants, methanol extracts of plants were prepared and tested against foot ulcer pathogens. Among the plants used, the methanolic extract *Tragia involucrata* was very effective against the foot ulcer pathogens and to separate the compounds present in the methanolic extract of *T. involucrata*, when it was subjected to column chromatography. The fractions obtained were further checked for their antibacterial property and fraction 1 which inhibited the pathogens, were subjected to thin layer chromatography and the structure of the particular phytochemical compound was elucidated by NMR study. The spices were tested for their antibacterial property against the urinary tract pathogens. Among the spices tested; *Allium sativum* inhibited the growth of the pathogens isolated from urinary tract infection. It can be concluded that the plants extract can be used to discover natural products that may serve as

lead for the development of new pharmaceuticals addressing the major therapeutic needs.

Keywords Medicinal plants and spices · Antibacterial activity · Chromatography · NMR

Introduction

The use of medicinal plants in the world, and especially in south India, contributes significantly to primary health care. Many plants are used in India in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy. The Indian flora offers great possibilities for the discovery of new Compounds with important medicinal applications in combating infection and strengthening the immune system. The antimicrobial compounds found in plants may prevent bacterial infections by different mechanisms than the commercial antibiotics and therefore may have clinical value in treating resistant microorganism strains (Ellof 1998). The urinary tract infection is the widest spread microbial disease and the financial encumbering on society is substantial. Diabetes seriously burdens individuals, their families and society (Dawson et al. 2002). The chronic leg and foot ulcers are non-healing wounds that occur due to a breakdown in the underlying physiology of the leg. This breakdown is frequently associated with venous, arterial or metabolic factors (Bowler and Davies 2001). Plant constituents may be isolated and used directly as therapeutic agents or as starting materials for drug synthesis or they may serve as models for pharmacologically active compounds in drug synthesis. The wound infection always starts with local bacterial invasion and rapidly progresses often to deep abscess formation of osteomyelitis, immune system deficiency, and vascular

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insufficiency often combines to permit a rapid progression of infection (Talwalkar 2000). The medicinal plants possess immunomodulatory and antioxidant properties, leading to antibacterial activities (Kirbag et al. 2009). They are known to have versatile immunomodulatory activity by stimulating both non-specific and specific immunity (Pandey and Chowdhry 2006). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant (Nascimento et al. 2000). The alcoholic extract of *Plumbago zeylanica* (root) was tested against multi drug-resistant clinical isolates of bacteria *Salmonella paratyphi*, *Staphylococcus aureus*, *Escherichia coli* and R-plasmid harbouring activity against all test bacteria irrespective of their antibiotic resistance behaviour (Beg and Ahmad 2004). The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant (Balandrin et al. 1985). The objective of this study was to evaluate the potential of plants and spices on multi-drug resistant bacteria isolated from diabetic foot ulcer and urinary tract infection.

Materials and methods

Collection of samples

The pus and Urine samples were collected in hundred affected patients from Tuticorin Government medical

college, Tuticorin district, Tamilnadu, India. The collected samples were transported to Kamaraj college, Microbiology laboratory in an ice cold condition by adding boric acid at final bacteriostatic concentration of 1.8 % without delay (Porter and Bordie 1969). Collected pus samples were swabbed and urine samples were streaked on nutrient agar plates. These plates were incubated at 37 °C for 24 h. After incubation, the individual colonies having different colony morphologies were streaked on different selective agar plates. The isolated organisms were stored in agar slants and subjected to biochemical analysis.

Biochemical test for isolated pathogens

The bacterial pathogens identified and confirmed by using conventional microbiological and biochemical procedure were followed from Department of Microbiology, Kamaraj College, Tuticorin. Table 1 showed the various biochemical tests such as gram staining indole production test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Triple Sugar Iron test and urease test were used for the conformation of the test pathogens followed by Dubey (2007).

Isolation and identification of UTI and foot ulcer bacterial pathogens

The pus samples of diabetic foot ulcer were found to contain *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Porteus vulgaris*, *Staphylococcus aureus*, *Enterobacter aerogenes*. The urine samples of diabetic patients were found to contain *Citrobacter freundii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Salmonella typhi*, *Enterobacter aerogenes*.

Table 1 Biochemical characters of the isolated pathogens

Tests	<i>Staphylococcus aureus</i>	<i>Enterobacter aerogenes</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>	<i>Proteus vulgaris</i>	<i>Citrobacter freundii</i>
Gram's stain	+	–	–	–	–	–	–
Motility	NM	M	M	M	M	M	M
Indole production	–	–	–	–	+	+	–
Voges-proskauer	–	+	–	–	–	–	–
Methyl red	+	–	–	–	+	+	+
Citrate	–	+	+	+	–	–	+
Urease	–	–	–	–	–	+	+
Catalase	+	+	+	+	+	+	+
TSI	A/A	A/A	K/K	A/A	A/A	K/A	A/A

+ positive, – negative, A acid, K alkali, M motile, NM non motile

Activity of selected commercial antibiotics against the isolated pathogens

Assay of antibiotic activity was performed by Kirby-Bauer disc diffusion method. The Mueller–Hinton agar plates were prepared and the organism was swabbed over it using a sterile cotton swab. The antibiotic discs were placed on the surface of the agar plates and then, the plates were incubated at 37 °C for 24 h. After incubation, the zone of inhibition was measured.

Collection of plants and spices

Medicinal plants such as *Acalypha indica*, *Azadirachta indica*, *Calotropis gigantea*, *Centella asiatica*, *Cyanodon dactylon*, *Leucas aspera*, *Ocimum sanctum*, *Psidium guajava*, *Thymus vulgaris*, *Tragia involucrata* and *Tridax procumbens* were collected from Tuticorin district and were further confirmed in the Department of Botany, Kamaraj College, Tuticorin. The collection of Fresh spices such as *Allium cepa* (Onion), *Allium sativum* (Garlic), *Myristica fragrans* (Nutmeg) *Zingiber officinale* (Ginger) and *Piper nigrum* (pepper) were obtained from the local market of Tuticorin district.

Extraction of plant and spices

The plant and spices were washed with distilled water and air dried in the laboratory for two weeks and grounded. The 50 g of each ground material was soaked in 500 ml of methanol for 72 h with frequent shakings. The samples were then filtered through Whatman No: 1 filter paper (Grade 595: 4–7 µm). The filtrate was evaporated to dryness under reduced pressure using oven at 40 °C. The stock solution of 0.2 g/ml in DMSO was made for each extract. The extract was kept at 40 °C until used (Samie et al. 2005).

Antibacterial activity of selected plants and spices against the isolated pathogens

The pathogens isolated from diabetic foot ulcer sample and urinary tract infected sample were inoculated into 10 ml of sterile nutrient broth and incubated at 37 °C for 24 h. Using a sterile cotton swab, the nutrient broth cultures were aseptically swabbed on sterile Mueller Hinton agar plates. Wells of 5 mm in diameter were made aseptically using well cutter, and 50 µl of methanolic extract of plant and spices were inoculated. The stock solutions were prepared at a concentration of 20 mg/ml. The result was calculated by measuring the zone of inhibition in millimeters. For each concentration tested, triplicates were maintained for the confirmation of activity.

Column chromatography

The active plant extracts were submitted to column chromatography on silica gel melted with hexane:ethyl acetate (50:50), hexane:petroleum ether (50:50). Proper packing of the column is one of the important factors in achieving good separation of samples.

Thin layer chromatography

TLC has become an important method for rapid analysis of bio molecules, the active plant extracts were submitted to Silica gel plates, 20 × 20 cm, 1 mm thick was used. A line was drawn with a pencil about 1.5–2.0 cm from the bottom in the plate. The samples were spotted using capillary tube at 1.5 cm distance between them.

Nuclear magnetic resonance (NMR)

¹H NMR spectra of 10 mg of the dried powder taken from TLC were subjected to proton nuclear magnetic resonance (NMR) study and the structure of the active compound is elucidated. The result graph was compared with the reference chart and possible functional group present in the plant were determined.

Results

Antibacterial activity of selected commercial antibiotics

The commercially available antibiotics such as Streptomycin, Gentamycin, Chloramphenicol, Oxacillin, Vancomycin, Ciprofloxacin and Methicillin were tested against the foot ulcer isolates and urinary tract pathogens (Tables 2, 3). The antibiotics used did not show any inhibitory activity against the foot ulcer isolates except chloramphenicol which inhibited *E. aerogenes* and *P. aeruginosa*. *P. vulgaris* was inhibited only by the antibiotic methicillin and urinary tract infection was found to be highly resistant against all the selected commercial antibiotics.

Antibacterial activity of medicinal plants against foot ulcer microflora pathogens

The methanolic extract of *O. sanctum* showed inhibitory activity against *E. aerogenes*, *P. aeruginosa* and *P. vulgaris*. The extract of *A. indica* highly inhibited *E. aerogenes*, *P. aeruginosa* and *E. coli*. Table 4 showed that the extract of *T. involucrata* (–, –, –) was found to inhibit the growth of all organisms isolated from foot ulcer. It was

Table 2 Selected commercial drugs for foot ulcer microorganisms

Organisms	Antibiotics						
	Streptomycin	Gentamycin	Chloramphenicol	Oxacillin	Vancomycin	Ciprofloxacin	Methicillin
<i>Staphylococcus aureus</i>	—	—	—	—	—	—	—
<i>Enterobacter aerogenes</i>	—	—	++	—	—	—	+
<i>Proteus vulgaris</i>	—	+	—	—	+	—	++
<i>Pseudomonas aeruginosa</i>	—	—	+	—	—	+	+
<i>Escherichia coli</i>	—	—	—	—	—	+	—

— above 8.5 mm resistant, + below 2.4 mm intermediate, ++ below 1.5 mm sensitive

Table 3 Selected commercial drugs for foot ulcer microorganisms

Organisms	Antibiotics						
	Streptomycin	Gentamycin	Chloramphenicol	Oxacillin	Vancomycin	Ciprofloxacin	Methicillin
<i>Escherichia coli</i>	—	—	—	—	—	—	—
<i>Enterobacter aerogenes</i>	—	—	—	—	—	—	—
<i>Pseudomonas aeruginosa</i>	—	—	—	—	—	—	—
<i>Citrobacter freundii</i>	—	—	—	—	+	—	—
<i>Proteus vulgaris</i>	—	—	—	—	—	—	—
<i>Klebsiella pneumoniae</i>	—	—	+	—	—	—	+

— above 8.5 mm resistant, ++ below 2.5 mm sensitive, + below 1.5 mm intermediate

Table 4 Antibacterial activity of medicinal plants against diabetic foot ulcer pathogens

Plants	Organisms				
	<i>Enterobacter aerogenes</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
<i>Ocimum Sanctum</i>	—	—	+	—	+
<i>Azadirachta indica</i>	+	—	++	—	—
<i>Acalypha indica</i>	—	++	—	+++	—
<i>Cyanodon dactylon</i>	+	—	—	+++	—
<i>Calotropis gigantea</i>	—	+	—	—	+
<i>Leucas aspera</i>	+	—	++	+++	—
<i>Tragia involucrata</i>	—	—	—	—	—
<i>Psidium guajava</i>	—	—	+	—	++
<i>Thymus vulgaris</i>	++	+	—	—	—
<i>Tridax procumbens</i>	—	+	++	—	+++

— below 13 mm resistant, + below 3.5 mm intermediate, ++ below 2 mm sensitive, +++ below 0.5 mm highly sensitive

subjected to column chromatography, TLC and NMR studies.

Antibacterial activity of spices against urinary tract pathogens

Table 4 showed that methanolic extract of *A. sativum* was found to be effective against the organism isolated from urinary tract infections such as *E. aerogenes*, *P.*

aeruginosa, *E. coli*, *C. freundii* and *K. pneumoniae*. The extract of *Z. officinale* inhibited *E. coli*, *C. freundii*, *S. aureus*, *E. aerogenes*, *P. nigrium*, and *A. cepa* extract did not show any inhibitory activity Table 5.

Column chromatography

In this column chromatography, the methanolic extract of *T. involucrata* showed three fractions. All the three

Table 5 Antibacterial activity of selected spices against urinary tract microflora pathogens

Plants	Organisms						
	<i>Enterobacter aerogenes</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Proteus vulgaris</i>	<i>Staphylococcus aureus</i>	<i>Citrobacter freundii</i>	<i>Klebsiella pneumoniae</i>
<i>Allium sativum</i>	—	—	—	+	+	—	—
<i>Myristica fragrans</i>	++	+	++	—	—	+	+++
<i>Zingiber officinale</i>	—	+	—	+	—	—	++
<i>Piper nigrum</i>	+	+	+	—	—	+	+
<i>Allium cepa</i>	—	+	++	—	—	+	—

— below 13 mm resistant, + below 3.5 mm intermediate, ++ below 2 mm sensitive, +++ below 0.5 highly sensitive

Table 6 Antibacterial activity of column fractions from methanolic extract of *T. involucrata* against foot ulcer pathogens

S. no.	Bacteria	Fractions	Zone of inhibition (mm)
1	<i>Staphylococcus aureus</i>	1	3.4
		2	2.5
		3	0.5
2	<i>Enterobacter aerogenes</i>	1	0.08
		2	1.6
		3	0.9
3	<i>Proteus vulgaris</i>	1	1.2
		2	1.8
		3	0.5
4	<i>Pseudomonas aeruginosa</i>	1	3.1
		2	+
		3	—
5	<i>Escherichia coli</i>	1	2.1
		2	2.5
		3	0.7

fractions were checked for antimicrobial activity of diabetic foot ulcer micro flora pathogens. The fraction 1 was found to be highly effective (Tables 6, 7).

Thin layer chromatography

Fraction 1 of column chromatography was separated in thin layer chromatography. It showed single layer of yellow pigmented region. This pigmented portion was taken for further analysis.

Nuclear magnetic resonance (NMR)

¹H NMR analysis of the same methanol extract of leaf of *T. involucrata* showed a peak at 0.86–1.68 may be aliphatic alicyclic compounds 2.17 and 2.5 showed the hydroxyl protons and methyl protons respectively. At 3.45 the presence of methoxy protons between 4.19 and 4.22 reveals the presence of CH protons and 7.07–7.7 shows the

Table 7 Antibacterial activity of column fractions from methanolic extract of *T. involucrata* against Urinary tract pathogens

S. no.	Bacteria	Fractions	Zone (mm)
1	<i>Enterobacter aerogenes</i>	1	4.2
		2	2.1
		3	0.7
2	<i>Pseudomonas aeruginosa</i>	1	3.1
		2	3.4
		3	0.5
3	<i>Escherichia coli</i>	1	2.4
		2	2.7
		3	1.1
4	<i>Proteus vulgaris</i>	1	4.4
		2	2.3
		3	0.4
5	<i>Staphylococcus aureus</i>	1	1.1
		2	0.8
		3	0.5
6	<i>Citrobacter freundii</i>	1	1.3
		2	0.7
		3	0.5
7	<i>Klebsiella pneumonia</i>	1	1.2
		2	0.4
		3	0.3

presence of aromatic cyclic compounds as well as some phenolic groups (Table 8).

Discussion

The result of the present study clearly showed that, extract from selected medicinal plants and spices are caused antibacterial activity against tested pathogens. The effectiveness of the active compounds present in the plant extracts showed growth inhibition which appears in clear areas surrounding the disc. This antibacterial activity might be due to the active components which are present in the

Table 8 Possible functional groups and their structures present in the methanol extract of *T. involucrata* analyzed by ^1H NMR

Peak in PPM	Possible type of group of compound	Possible structure
0.86–1.68	Aliphatic alicyclic compounds	$\text{H}_3\text{C}-\overset{\text{H}_2}{\underset{ }{\text{C}}}-\overset{\text{H}_2}{\underset{ }{\text{C}}}-\text{R}$
2.17	Hydroxyl protons	$\text{H}_3\text{C}-\overset{\text{H}_2}{\underset{ }{\text{C}}}-\overset{\text{H}_2}{\underset{ }{\text{C}}}-\text{OH}$
2.5	Methyl protons
3.45	Methoxy protons	$\text{H}_3\text{CO}-\overset{\text{H}_2}{\underset{ }{\text{C}}}-\overset{\text{H}_2}{\underset{ }{\text{C}}}-\text{OCH}_3$
4.19–4.22	CH protons	$\begin{array}{c} \text{R} \\ \\ \text{R}_1-\text{CH} \\ \\ \text{R}_2 \end{array}$
7.07–7.70	Aromatic cyclic compounds

plant extracts (Anita et al. 2012). Plants produce a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceuticals industry and it is expected that extracts showing target sites other than those used by antibiotics will be active against uropathogens. Similarly the present study on *A. indica* inhibited *E. aerogenes*, *E. coli* and *O. sanctum* was found to inhibit the growth of *E. aerogenes*, *P. aeruginosa* and *E. coli*. Similarly the water extract of *O. sanctum* showed related antimicrobial activity against

some clinical bacterial isolates like *E. coli*, *S. aureus* and *S. typhi* (Alamgir et al. 2003). Out of five spices tested, three were found to inhibit the organisms isolated from urinary tract. *T. involucrata* has antibacterial properties of nine different compounds and studied against *Escherichia coli*, *Proteus vulgaris* and *Staphylococcus aureus* using the disc-diffusion method at 50 $\mu\text{g/ml}$ concentrations (Samy et al. 2006). The results of the present study, nutmeg was found to inhibit the organisms such as *E. aerogenes* and *E. coli* only. Similarly, the antibacterial effect of nutmeg extract

against *E. coli* that most of them were sensitive to the extract at 100 and 75 % concentrations. The methanolic extract of *T. involucrata* was checked by column and TLC. Then purified compounds were characterized using proton NMR spectroscopy. According to proton NMR spectroscopy, studies of methanolic extract reveals that phenolic compounds can be present in plant species. Similarly, the present study ¹H NMR analysis of the methanol extract of *T. involucrata* showed presence of aliphatic group of compounds which might be responsible for the bioactive properties of the plant extract. Indeed, NMR spectroscopy is by far the most powerful spectroscopic technique for obtaining detailed structural information about organic compounds in solution (Saraf 2010). These compounds have the ability to reduce the growth of resistant foot ulcer microflora and further work of this compound to exploit for pharmaceutical purposes.

Conclusion

The conclusion of the present work the methanol extract of plants have great potential as antimicrobial compounds against microorganisms when compared to spices extract and so they can be used in the treatment of foot ulcer and urinary tract infection caused by resistant microorganisms.

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Conflict of interest All contributing authors declare no conflicts of interest.

References

Alamgir M, Khan MTH, Jabbar S, Shabriar M, Choudhuri MSK (2003) Bioactivity studies of the aqueous extract of *Ocimum sanctum* Lin. Hamalay Med 46:34–39

- Anita P, Anthoni Samy A, Raj JS (2012) In vitro antibacterial activity of *Aegiceras corniculatum* and *Bruguiera cylindrical* against isolated bacterial urinary tract infections. Int J Pharm Res Dev 3:1120–1125
- Balandrin MFJ, Kjöcke A, Wurtele E (1985) Natural plant chemicals: sources of industrial and medicinal materials. Science 228:1154–1160
- Beg ZA, Ahmad I (2004) Effect of *Plumbago zeylanica* extract and certain curing agents on multidrug resistant bacteria of clinical origin. World J Microbiol Biotechnol 16:841–844
- Bowler PG, Davies BJ (2001) The microbiology of infected and non-infected leg ulcers. Int J Dermatol 38:573–578
- Dawson G, Gerstein BK (2002) Assessment and management of foot ulcers for people with diabetes. Can Diabetes Assoc 4:4–15
- Dubey RC (2007) The Practical book of microbiology, 2nd edn. Times Books, New York, pp 37–39
- Ellof JN (1998) Which extract should be used for the screening and isolation of antimicrobial components from plants. J Ethnopharmacol 60:1–6
- Kirbag S, Zengin F, Kursat M (2009) Antimicrobial activities of extracts of some plants. Pak J Bot 41(4):2067–2070
- Nascimento G, Locatelli J, Freitas C, Silva L (2000) Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Braz J Microbiol 31:247–256
- Pandey AK, Chowdhry PK (2006) Propagation techniques and harvesting time on productivity and root quality of *Withania somnifera*. J Trop Med Plant 7:79–81
- Porter IA, Bordie J (1969) Boric acid preservation of urine samples. Br Med J 2:353–355
- Samie A, Obi CL, Bessong PO, Namrita L (2005) Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. Afr J Biotechnol 4(12):1443–1451
- Samy RP, Gopalakrishnakone P, Houghton P, Ignacimuthu S (2006) Purification of antibacterial agents from *Tragia involucrata*: a popular tribal medicine for wound healing. J Ethnopharmacol 107(1):99–106
- Saraf A (2010) Phytochemical and antimicrobial studies of medicinal plant *Costus speciosus* (Koen.). Eur J Chem 7(1):405–413
- Talwalkar PG (2000) Diabetic wound practice. TSIM Special Issue Diabetic's Wound 13:1–11