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Bioactive potential of *Tridax procumbens* L. leaf extract against skin infection causing bacteria

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Abstract

Anti-bacterial activity of methanolic extract of *Tridax procumbens* L. leaf (MTP) was evaluated against the common skin infection causing bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) using various methods such as agar well method, modified agar well, disc diffusion method etc. and also the phytochemicals present in MTP were investigated. *P. aeruginosa* used in our study was a multidrug resistant strain. Phytochemical analysis of MTP revealed the presence of tannins, flavonoids and phenols. In agar well diffusion method at 25 mg/well concentration, *P. aeruginosa* showed a zone of inhibition (ZOI) of 3.3 cm, whereas *S. aureus* exhibited 1.7 cm of ZOI at same concentration of MTP. In modified agar well method, ZOI of *P. aeruginosa* and *S. aureus* were found to be 3.4 cm and 2 cm, respectively. In disc diffusion method *S. aureus* showed 0.8 cm of ZOI at 4 mg/disc concentration. *P. aeruginosa* had maximum ZOI i.e., 2.4 cm at 4 mg/disc and 2.1 cm at 3.2 mg/disc concentration. The colony forming units per ml (cfu/ml) were found to be reduced in a dose dependent manner in the MTP treated bacteria in comparison to control/wild bacteria, as evident from the results of both spread plate and absorbance method. MTP had more bactericidal activity against *P. aeruginosa*, in comparison to *S. aureus* in agar well, modified agar well and disc diffusion method.

Keywords: Skin infections, *S. aureus*, *P. aeruginosa*, *T. procumbens* L, MTP

Introduction

Tridax procumbens L. (Asteraceae) commonly called as coatbuttons or tridax daisy is an annual weed. It is a semi-prostrate, creeper herb found worldwide. Stem is ascending, branched and hairy. Leaves are simple, opposite, petiolate and hairy. The plant bears daisy like yellow-centered white or yellow flowers with 3-toothed ray florets. It is well known for its various therapeutic properties and frequently used for typhoid, diarrhoea, cough, asthma, epilepsy etc ^[1]. The whole plant was reported to treat various ailments, such as bronchitis, cataract, dysentery, diarrhoea, preventing hair loss and to check hemorrhage from cuts ^[2, 3]. The fresh leaves are very effective against bleeding from cutting wounds. It immediately stops bleeding and helps in healing. The young leaves are squeezed and rubbed on the affected parts 2 or 3 times per day. It is used as an antidote to arrow poison by applying the powdered leaves on the wound ^[4, 5]. Different parts of the plant and whole plant of *T. procumbens* L. has been reported for its antimicrobial activity against various species of bacteria. Antimicrobial activity is mostly investigated by disc diffusion method and agar well diffusion method ^[6].

Pharmacological studies have been shown that *T. procumbens* L. possess properties like anti-inflammatory, hepatoprotective, wound healing, immunomodulatory, antimicrobial, antiseptic, hypotensive, and cardioprotective effects ^[7, 8, 9]. The leaf extract has been extensively used in Indian traditional medicine as anticoagulant, anticancer, antifungal, and insect repellent ^[10]. Flavones, sterols, tannins, glycosides, luteolin, glucoluteolin, quercetin, polysaccharide and monosaccharide, saturated and unsaturated fatty acids, campesterol, stigmaterol, amyirin, sitosterol have been isolated from the plant. These extracts are found to have antibacterial properties ^[11]. The most important bioactive constituents of *T. procumbens* are alkaloids, tannins, flavonoids and phenolic compounds ^[12]. Dexamethasone luteolin, glucoluteolin, β -sitosterol quercetin, β -sitosterol-3- α - β -D-xylopyranoside and flavonoid procumbenetin have been isolated from leaves and flowers of *T. procumbens* ^[13]. Water soluble novel polysaccharides were reported ^[14]. Luteolin and glucoluteolin were isolated from the flowers of *T. procumbens* ^[15]. The sterols, hydrocarbons, saturated and unsaturated fatty acids were reported in *T. procumbens* ^[16].

S. aureus is an important pyogenic organisms and lesion are localized in nature in contrast to streptococcal lesions, which are spreading in nature. Coagulase enhances virulence of *S. aureus* by inhibiting phagocytosis. It forms a wall of fibrin clot around the lesion. Thick creamy pus is formed in staphylococcal infections. Staphylococcal diseases may be classified as cutaneous and deep infections; food poisoning, nosocomical infections, skin exfoliative diseases and toxic shock syndromes ^[17].

P. aeruginosa is a ubiquitous aerobe that is present in water, soil and on plants. It is one of the most important secondary hospital acquired infection in burnt patients [18]. Infection caused by *P. aeruginosa* is particularly problematic because the organism is inherently resistant to many drug classes and is able to acquire resistance to all effective anti microbial drugs [19, 20]. Most people infected with these bacteria have cystic fibrosis [21]. *P. aeruginosa* could be isolated from injuries, sputum, stool and ulcerative keratitis [22, 23]. This microorganism produces a number of exoproducts, which have been implicated in the pathogenesis of *P. aeruginosa* infections. Among them, the activity of elastase, proteinase, alginate, exotoxin A, hemolysin and other virulence factors, increase their degree of pathogenicity [24, 25].

S. aureus (Gram positive) and *P. aeruginosa* (Gram negative), used in the present study were isolated from various patients suffering from skin infections. Bacteria are becoming multi drug resistant and causing dangerous infections due to their horizontal gene transfer capacity and rapid multiplying activity. So, it becomes highly necessary to find out an alternative way to overcome the infectious activities of microorganisms. This study aims to investigate the antibacterial activity of *T. procumbens* against *S. aureus* and *P. aeruginosa* and it is also necessary to find out the secondary metabolites present in the methanolic extract of *T. procumbens*, which are responsible for the antibacterial activity.

Materials and methods

Plant part collection and extract preparation

Leafy aerial parts of *Tridax procumbens* L. were collected from the campus area of Berhampur University, Bhanjabihar, Odisha during the month of June-July 2016. The plant parts were shade dried for 15 days and then ground. The powdered plant parts (57g) were subjected to soxhlet extraction in methanol for 72 hours at 50 °C. The filtered extract was concentrated and kept in incubator at 37 °C for complete solvent evaporation, stored at 4 °C for future use. Necessary amount was measured and dissolved in distilled water to prepare the desired concentration of fresh extract solution and used in our different experiments.

Phytochemical analysis

Qualitative test

A. Steroid test

1ml of extract was dissolved in 10ml of chloroform and equal volume of conc. H₂SO₄ acid was added from the side of the test tube.

B. Tannin test

4ml of extract was treated with 4ml of FeCl₃.

C. Saponin test

5ml of extract was mixed with 20ml of distilled water then agitated in graduated cylinder for 15min.

D. Anthocyanin test

2ml of aqueous extract was added to 2ml of 2N HCl and NH₃.

E. Coumarine test

3ml of 10% NaOH was added to 2ml of aqueous extract.

F. Glycosides test

Keller Killani test: Plant extract was treated with 2ml glacial acetic acid containing a drop of FeCl₃.

G. Flavonoid test

Alkaline reagent test: Extract was treated with 10% NaOH solution.

Quantitative test

Total phenolic compound (TPC)

The total phenolic content of the extract was determined by the folin- ciocalteu method. 200µl of crude extract (1mg/ml), were added to 3.16ml of distilled water, mixed thoroughly with 0.2ml of FC-reagent for 8min, followed by the addition of 0.6ml of 10% Na₂CO₃. The mixture was allowed to stand for 60min in the dark and absorbance was measured at 765nm. The TPC was calculated from the calibration curve, and the results were expressed as mg of galic acid equivalent for g dry weight. Statistical analysis was carried out with MS excel 2010 software and results are expressed as mean ± standard deviation.

Bacterial strains

Clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were procured from MKCG Medical College, Berhampur, Odisha. For routine use the cultures were maintained on Mueller hinton agar (MHA) plates. For long term storage, glycerol stocks were prepared by inoculating a single colony into Nutrient broth (NB) and incubated at 37 °C for 16 hr. To 0.8ml of culture, 0.2ml of 50% sterile glycerol was added, mixed thoroughly and stored at 4 °C for 1 hr and then stored at -20 °C.

Biochemical characterization and antibiotic sensitivity test

Biochemical characterization and antibiotic sensitivity test was carried out according to standard laboratory procedure followed by the Department of Microbiology, MKCG medical college, Berhampur. Briefly, one isolated colony was inoculated into sterile saline or peptone water. A sterile cotton swab was dipped into the dilute culture medium. Excess saline was squeezed and swabbed completely on agar plate. Different antibiotic discs (Himedia, Mumbai) were aseptically taken and placed properly on agar plate leaving appreciable gap between two discs. Plates were incubated at 37 °C for 18hr. The clear zone formed around the disc was a measure of the susceptibility of the organism to the antibiotic at a specific concentration.

Antibacterial test

The effect of MTP against the clinically isolated skin infection causing bacteria (*S. aureus*, *P. aeruginosa*) was determined by disc diffusion, agar well diffusion, modified agar well diffusion method. The cfu/ml was determined in wild and drug treated bacteria by two methods.

- Bacterial colony counting by spread plate method
- Bacteria counting by absorbance method (OD_{600nm})

Disc diffusion method

Discs (4mm diameter) were prepared using whatman filter paper no-1. Different doses of MTP (4, 3.75, 3.5, 3.25 mg/disc) were added on it and left in incubator for some time for complete drying. MHA plates were prepared and swabbed with sterilized cotton bud containing bacterial culture. The MTP treated discs were aseptically placed on them and the plates were incubated over night. The clear zone of inhibition was measured in cm.

Agar well diffusion method

A single colony was suspended in 1ml of sterilized saline or peptone water, the colony was mixed properly by vortex and incubated at 45 °C for 15min. MHA plates were prepared. 0.1ml of the bacterial broth was poured onto the plate and swabbed with sterilized cotton bud. The excess broth from the plate was discarded. Then the plate was kept in the incubator for 15min. Then wells were dug into it and different doses of the MTP (25, 22.5, 20, 17.5 mg/well) were loaded into the well. The plates were left at room temperature for 1hr for drug diffusion into media and then incubated overnight at 37°C. The zone of inhibition was measured in cm.

Modified agar well diffusion method

In order to find sensitivity of different bacteria (*S. aureus*, *P. aeruginosa*), on a single plate, this method was used. A MHA plate was prepared. After solidification, a well was dug in the center. Different organisms were streaked on the plate from periphery towards center. Then a particular dose of the MTP (25 mg/well) was loaded into the well. Then the plate was left at room temperature for 1hr for drug diffusion and then incubated overnight at 37 °C. The inhibition zone or distance between the periphery of the well and the bacteria growth start point was measured in cm.

Cfu/ml determination in control and drug treated bacteria

Bacterial colony count by spread plate method

The stock solution of the MTP (500 mg/ml) was prepared. 3ml of NB was taken in different test tubes and different doses of extract were added to each test tube and then 100µl of fresh bacterial culture was added to each test tube. A control tube was also prepared by inoculating only bacteria without extract and incubated for 4hr. Aliquots of the culture were taken in eppendorf tube, contents of the eppendorf were diluted by adding distilled water (10µl of culture+990µl of distilled water).

MHA plates were prepared and 100µl of the diluted content of the eppendorf tubes was added to the plate, evenly spread on the plate using L rod. The plates were incubated overnight at 37 °C and colonies were counted.

Bacterial counting by absorbance method (OD_{600nm})

The stock solution of MTP was prepared. 3ml of NB was taken in different test tube and different doses of extract were added to each test tube and to each test tube 100µl of fresh bacterial culture was added. A control tube was also prepared by inoculating only bacteria without extract. Then the tubes were incubated for 18hr and OD value was determined at 600nm. Blank tubes were prepared similarly without bacterial culture and with only MTP to serve as blank while taking OD in order to measure the turbidity resulted by bacterial growth but not due to the extract.

Salt agglutination test

Different concentration of (NH₄)₂SO₄ solution were prepared (1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M). On a clean slide one drop of control culture or extract treated culture was taken and added with different concentration of (NH₄)₂SO₄ and observed for agglutination (if any).

Result

Plant extract

When 57g of powdered shade dried plant parts were subjected to exhaustive soxhlet extraction followed by extract concentration, a dark green colour powder was obtained with a mean yield of 13.05% (7.439g).

Phytochemical analysis

The presence of tannins, flavonoids and phenolic compounds were confirmed. The total phenolic content of the MTP, calculated from the calibration curve (R²=0.9842) was 31.5±0.032 galic acid equivalents/g (data not shown).

Antibiotic sensitivity test

S. aureus was found to be sensitive to amikacin (Ak), ampicillin (A/S), linezolid (LZ), cefpodoxime (CPD), vancomycin (VA), cefepime (CPM), amoxycylac (AMC), levofloxacin (LE) and ofloxacin (OF) and it was resistant to only one drug – erythromycin (E) (Plate 1a).

P. aeruginosa strain used in the study were found to be drug resistant strain showing resistance to ceftazidime (CAZ), cephaloxime (CTX), levofloxacin (LE), tobramycin (TOB), carbenicillin (CB), moxifloxacin (MO) and piperacillin+tazobactam (PIT). It was sensitive to imipenen+cilastin (IC), amikacin (Ak), and ceftriaxone (CTR) (Plate 1b).

Antibacterial activity of MTP

Disc diffusion method

In disc diffusion method, the MTP was found to be more effective against clinically isolated *P. aeruginosa* in a dose dependent manner (Plate 2b) and *S. aureus* was found to be resistant to MTP (Plate 2a, table 1).

Table 1: Effect of *Tridax procumbens* against *S. aureus* and *P. aeruginosa*

Conc. in mg/disc	ZOI of <i>S. aureus</i> in cm	ZOI of <i>P. aeruginosa</i> in cm
4	0.8	2.4
3.75	0.7	2.3
3.5	0.6	2.2
3.25	R	2.1

*results are expressed as the mean value of triplicate set of experiments

Agar well diffusion method

Different doses of MTP (25, 22.5, 20, 17.5 mg/well) were used to determine the antibacterial activity against *S. aureus* (Plate 3a) and *P. aeruginosa* (Plate 3b). The extract was found to be effective against both the strains. *P. aeruginosa* was found to be comparatively sensitive than *S. aureus*. The extract produced growth inhibition zone around the wells in a dose dependent manner (Table 2 and Plate 3).

Table 2: The growth inhibitory effect of MTP against *S. aureus* and *P. aeruginosa*

Conc. in mg/well	ZOI of <i>S. aureus</i> in cm	ZOI of <i>P. aeruginosa</i> in cm
25	1.7	3.3
22.5	1.6	3.3
20	1.6	3.2
17.5	1.5	3.0

*results are expressed as the mean value of triplicate set of experiments

Modified agar well diffusion method:-

MTP (25 mg/well) was used to determine its antibacterial activity against *S. aureus* and *P. aeruginosa* on a single plate for better comparison and avoid plate to plate variation. *P. aeruginosa* was found to be comparatively sensitive than *S. aureus* (table 3 and plate 4).

Table 3: Bioactivity of MTP against different bacterial strain

Type of organism	ZOI (25mg/well)
<i>P. aeruginosa</i>	3.4
<i>S. aureus</i>	2.0

*results are expressed as the mean value of triplicate set of experiments

Cfu/ml determination in control and MTP treated bacteria

Spread plate method

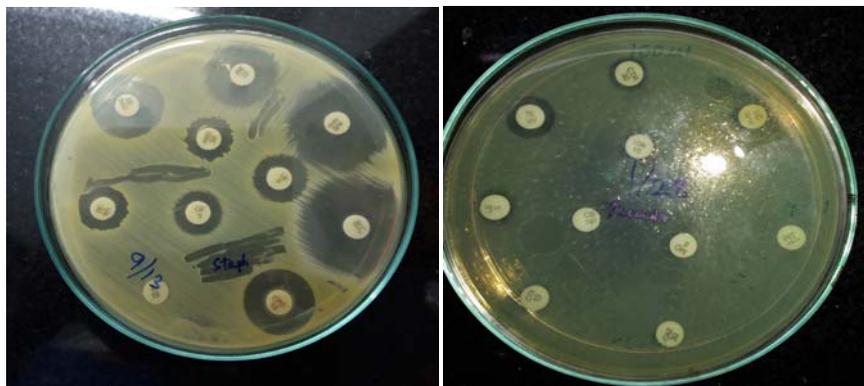
The cfu/ml in control was determined to be 1.2×10^7 but cfu/ml in drug treated bacteria was found to decrease in a dose dependent manner as shown in figure 1.

Absorbance method

$OD_{600} = 1 \times 10^9$ of bacteria. In control the no. of bacteria was found to be 1.05×10^9 and in drug treated $d_1(4\text{mg/ml})$ the no.of bacteria was found to be 5.9×10^8 and in $d_2(16\text{mg/ml})$ it was found to be 2.4×10^7 for *P. aeruginosa* and 0.98×10^9 for wild and 6.2×10^8 and 3.2×10^7 respectively for MTP treated *S. aureus* (figure 2).

Salt agglutination test

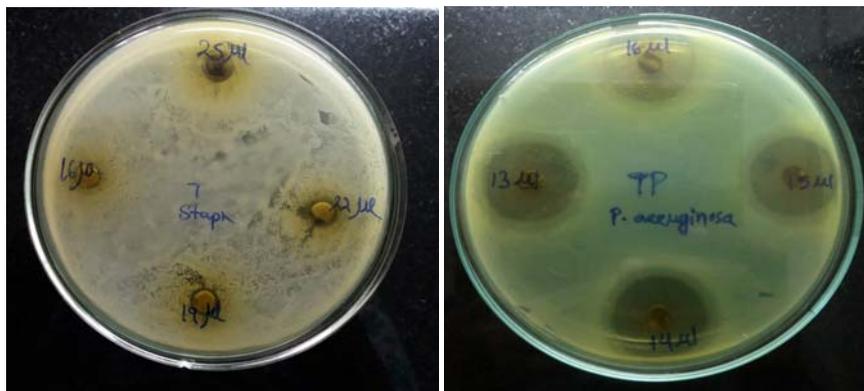
When $(\text{NH}_4)_2\text{SO}_4$ was treated with control culture, salt agglutination occurred at third step [2M of $(\text{NH}_4)_2\text{SO}_4$] onwards, whereas in extract treated bacteria salt agglutination occurred at fourth step [2.5M of $(\text{NH}_4)_2\text{SO}_4$] onwards.



A

B

Plate 1: Antibiotic sensitivity test (a) *S. aureus* (b) *P. aeruginosa*



A

B

Plate 2: Antibacterial activity of MTP by disc diffusion method (a) *S. aureus* (b) *P. aeruginosa*



A

B

Plate 3: Antibacterial activity of MTP by agar well diffusion method (a) *S. aureus* (a) *P. aeruginosa*.

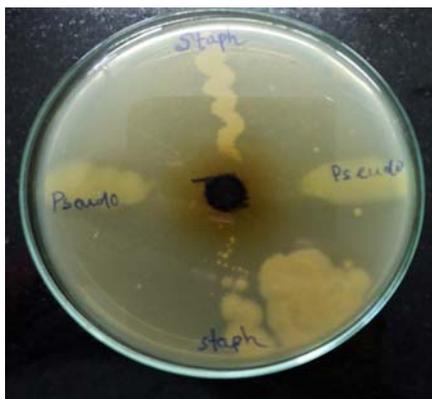


Plate 4: Antibacterial activity of MTP by modified agar well diffusion method *S. aureus* and *P. aeruginosa*.

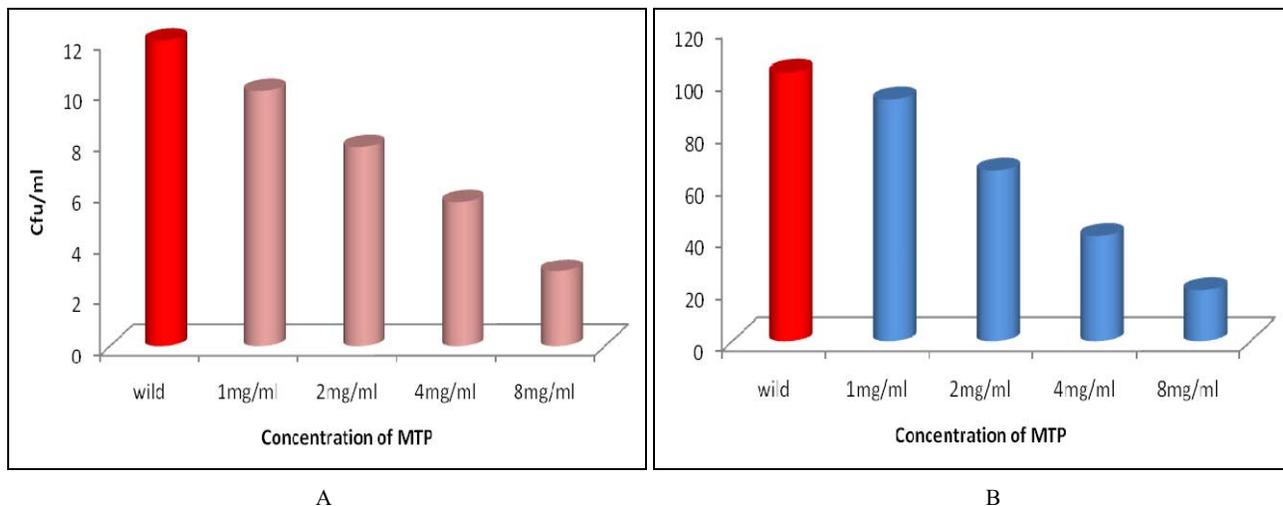


Fig 1: Antibacterial activity of MTP by spread plate method (A) *S. aureus* (B) *P. aeruginosa*. *results are expressed as the mean value of triplicate set of experiments

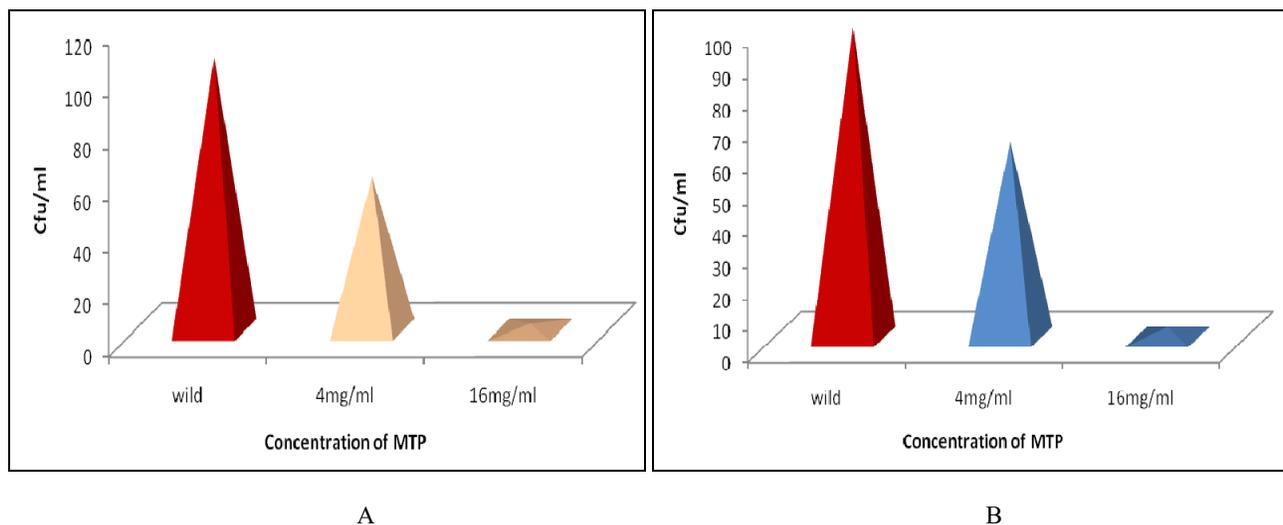


Fig 2: Inhibition study of MTP by absorbance method (A) *S. Aureus* (B) *P. Aeruginosa*. *results are expressed as the mean value of triplicate set of experiments

Discussion

Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of their chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-

emerging infectious diseases [26]. Many aromatic plants have been used traditionally in folk medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeast [27]. Therefore, researchers are increasingly turning their attention to folk medicine looking for new leads to develop better drugs against microbial infections [28]. Medicinal plants are very effective against several diseases

like diarrhoea, constipation, skin disease, cancer, jaundice etc. and they have potential to act against multi-drug resistant pathogenic bacteria and fungus.

The phytochemicals are divided into primary metabolites such as sugar and fat, which are found in all plants and secondary metabolites are compounds, which are found in smaller range of plants, serving a most specific function. For example some secondary metabolites are toxins used to deter predation and other are pheromones used to attract insect for pollination. It is this secondary metabolites and pigments that can have therapeutic action in human and which can be refined to produce drug.

Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent. Aniel and Naidu, 2011 have reported antimicrobial activity of ethanol and methanol extracts of root, stem, flower, leaf and whole plant of *T. procumbens* L. against *E. coli*, *K. pneumoniae*, *Proteus vulgaris*, *B. subtilis* and *S. aureus* [29]. The methanolic extract was found to be most effective against all tested microorganisms [30]. Therefore, we used this solvent system for extract preparation and methanol is also known to be an excellent medium for extraction of maximum phytoconstituents.

Agar well diffusion method had maximum ZOI in methanolic extract against *Klebsiella pneumonia* while minimum against *S. aureus* [31]. During the study, it was found that, methanolic extract showed more activity against *P. aeruginosa* (3.3cm ZOI) than *S. aureus* (1.7cm ZOI) which is shown in table 2 and plate 3.

Gram positive bacteria and Gram negative bacteria like *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Micrococcus sp.*, *Staphylococcus aureus*, *Citrobacter sp.* and *Serratia marcescens* showed a reduction in their growth on treatment with the different solvent extracts of TP. The degree of inhibition as measured by the disc diffusion method, reported that the Gram -ve bacteria (*P. aeruginosa*) were more inhibited than the Gram +ve bacteria (*S. aureus*). Various workers also reported similar findings from different plant sources [32-34]. In this present study it was also found that in the disc diffusion method the *S. aureus* was more resistant than *P. aeruginosa* (table 1, plate 2).

It may be possible to have result variation in different plates. So modified agar well method was done to find out the sensitivity of bacteria where we can inoculate multiple bacteria on a single plate. It was observed that *S. aureus* (2cm of ZOI at 25mg/well of MTP) is less sensitive than *P. aeruginosa* (3.4cm of ZOI at 25mg/well of MTP) (table 3, plate 4).

Antibacterial activity of MTP was also done in broth culture medium where bacteria can grow uniformly and get exposed to the extract evenly and the growth of bacteria was evaluated by two methods; spread plate and absorbance method (OD_{600nm}). In both spread plate and absorbance method numbers of bacteria were more in wild and gradually decreased in a dose dependent manner in MTP treated bacteria. It was also found that cfu/ml was more reduced in *P. aeruginosa* in comparison to *S. aureus* (figure 1 and 2).

Pathogenic bacteria agglutinate at a high salt concentration, which is a measure of bacterial surface hydrophobicity. MTP treated bacteria were shown to lose their surface hydrophobicity as the extract treated bacteria started to agglutinate with 2.5M or more of (NH₄)₂SO₄, whereas the control bacteria agglutinated at 2.0M or more of (NH₄)₂SO₄. From our results it was presumed that the extract treated

bacteria might not be able to attach to host cell surface as pathogenic bacteria attach to host cell by hydrophobic interactions. So, loss of surface hydrophobicity can reduce their infectious activity. However, further study is needed to confirm our results.

It is a well-established fact that *P. aeruginosa* is one of the leading Gram -ve organism associated with nosocomial infection [35]. The antibacterial activity of ethanolic extract of TP can be attributed to the presence of flavonoids and tannins, which are substances known to have several mechanism of actions such as inhibition of DNA gyrase, inhibition of cytoplasmic membrane function, inhibition of energy metabolism etc [36]. Plants rich in tannins have antibacterial potential as they react with proteins to form stable water soluble compounds by directly damaging its cell membranes and thus killing the bacteria [37].

The *S. aureus* strain used in the present study is comparatively more sensitive to the tested antibiotics in comparison to *P. aeruginosa*, which was comparatively resistant to most of the antibiotics. Our results showed that MTP can be used effectively in the treatment of multidrug resistant strains causing different types of skin infection. Future study is going on to isolate and characterize the active ingredients present in MTP and also to understand their mode of action against these pathogenic bacteria.

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