Appraisal of Cymbopogon citratus (Lemon grass) for Antibacterial Activity Against Uropathogens

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ABSTRACT

Urinary tract infections (UTI) are one of the major public health concerns in both genders, but variations in the anatomy, physiology and behaviour of urogenital and reproduction tract make women more susceptible. UTI is more prevalent and severe in women of all ages and in older men because of multi-drug resistant strains and high recurrence, it has become an important socioeconomic burden. Due to the microbial resistance, several life-threatening side effects, repeated high doses, high cost and low effectiveness of these antibiotics motivated the researchers to explore natural remedies for UTI therapy. The purpose of the research was to evaluate the antibacterial effect of Cymbopogon citratus (C. citratus), against uropathogens isolated from UTI patients, mainly include Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa) Klebsiella pneumoniae (K. pneumoniae) and Escherichia coli (E. coli). Isolates were confirmed through conventional biochemicals techniques. Ethanolic extract of C. citratus was evaluated against isolates through disc diffusion method and minimum inhibitory concentration was also determined. Ethanolic extract of C. citratus was phytochemically characterized through high profile liquid chromatography (HPLC). Antibacterial susceptibility was determined by measuring zone of inhibition (ZOI) and E. coli, S. aureus, P. aeruginosa and K. pneumoniae showed average 14.0, 13.0, 13.0 and 8mm ZOI against ethanolic extract, respectively. HPLC showed flavonoids and phenolics components present in ethanolic extract of C. citratus. In mouse model C. citratus also decreased the significant number of uropathogens. This study reports the role of lemon grass for treating UTI and provides new remedy for the treatment of UTI.

INTRODUCTION

Microbial infections, including Escherichia coli (E. coli), Enterococcus faecalis and Klebsiella species have been found to be the main causes of urinary tract infection (UTI). Various signs including painful urination or dysuria, haematuria, urinary urgency, burning, frequent urination, nausea and vomiting observed in UTI (Anderson et al., 2004). UTIs are among the most common conditions requiring medical treatment with 6-10% of all young women with bacteriuria. The incidence of UTIs increases (25-50%) with age in females aged 80 years and older have bacteriuria (Hung et al., 2009). UTIs occur as a result of interactions between the uropathogens and host. The uropathogens initially bind to the epithelial surface, colonize and spread throughout the mucosa causing tissue damage. Pathogens can ascend into the urinary bladder after the initial colonization period, leading to symptomatic or asymptomatic bacteriuria. Further progression may lead to renal impairment and
pyelonephritis. Specific virulence factors residing on the uropathogen’s membrane are responsible for bacterial resistance to the normally effective defense mechanisms of the host. Bacterial adhesions and their associated epithelial binding sites have recently been identified and natural mechanisms of anti-adherence are currently being investigated (Jafari et al., 2012). According to statistical calculations, the association between UTIs caused by E. coli in the female (P<0.05) was significant (Jahandeh et al., 2015). Reported research showed 80% of UTIs are caused by E. coli and 10-15% caused by S. saprophyticus. In United States and other region of the world Enterococci, Klebsiella, Enterobacter and Proteus mirabilis rarely cause uncomplicated cystitis and pyelonephritis (Ronald, 2002).

 Eternal bacterial resistance in bacteria leads to the alterations in antibiotics for the control of bacterial infections (Younas et al., 2019). Moreover, antibiotics impose many side-effects on the host gut flora, hypersensitivity and immunosuppression (Patel, 2007). The pharmaceutical industries developed latest generations of antibiotics for the treatment of resistant bacterial strains (Barrata et al., 1998). Antibiotics resistant strains were reported first time in Brazil from 1980s and now it persists worldwide. Until now various strategies has been developed including modified drugs but natural bioactive antimicrobial agents proved to be a good alternate for the treatment of bacterial infections with very few limitations (Buckova et al., 2018).

The herbal medicines have greater antimicrobial activity due to presence of different bioactive chemicals like Allicin, flavonoids, terpenoids, tannins, alkaloids etc. (Fayyaz et al., 2019; Mahmood et al., 2019; Abbas et al., 2019). Different plant materials have been reported to possess immunostimulant (Mahmood et al., 2014), antiviral (Aslam et al., 2014), antibacterial (Arshad et al., 2017; Yasmin et al., 2020) and antiprotozoan activity (Abbas et al., 2017; Zhang et al., 2020), while some plant material extracts have been reported to consist up of biofuel (Fatima et al., 2016). C. citratus plant extract also reported as an excellent source of various bioactive compounds which can be used to treat UTIs. It can be utilized as a remedy for ophthalmia, intestinal sickness, elephantiasis pneumonia and vascular scatters. Analysts found that C. citratus has characteristics of nervous disease preventing agent, bactericidal, antiseptic, astringent, antioxidant, fungicidal and sedative characteristics (Ronald, 2002).

 The use of medicinal plants as key drugs to sustain human health is emphasized by the World Health Organization (WHO). Brazil, Latin America and Argentina have gradually increased the use of medicinal plants. About 80% of people in developing countries use medicinal plants as conventional remedies. Therefore, these plants should be studied in order to better understand their properties, safety and efficiency (Cimanga et al., 2002). Many medicinal plants has been investigated phychemically for the bioactive compounds and their therapeutic use bioactive compounds including tannins and phenols are growth inhibitors for pathogenic bacteria (Cui et al., 2016). Identifying new and effective strategies as an alternative treatment will be the foremost priority. Medicinal plants and essential oil of plants showed significant bactericidal and antioxidant activity (Naik et al., 2018). Multi drug resistant bacteria cause long term acute infections which fails antibiotic therapy for the control of pathogens (Patel, 2007).

 Recent studies on medicinal plants and their therapeutic use is quite helpful to solve the antibiotic resistance issue of bacteria. The infectious diseases are leading cause of diseases and one third of the total deaths are attributed to infectious diseases. The multi-drug resistant bacteria are reported to be associated mainly with human diseases epidemics. The emerging antibiotic resistance against antibiotic trigger the use of herbal medicine as an alternative.

 This present study was conducted to evaluate the bioactive compounds of C. citratus and their antimicrobial activity against UTIs causing bacteria. C. citratus readily grow in Asia and no such previous work related to antimicrobial activity against UTIs causing bacteria is available in literature. This study helped to evaluate the role of C. citratus for treating UTIs and provide new dimension in medical field.

MATERIALS AND METHODS

Plant extract and Phytochemical Analysis: Cymbopogon citratus plants were collected from local nurseries and confirmed from a Botanist. Leaves were air dried and crushed to powdered form. Ethanolic extract (80%) of C. citratus leaves was made through conventional method by using soxhlet apparatus (Redfern et al., 2014). High performance liquid chromatography (HPLC) was performed for the phytochemical analysis of ethanolic extract of C. citratus. By adding the plant extract sample in HPLC grade at 0.1mg/µl concentration and strained through 0.2 millipore membrane filter. It was then subjected on RP-18 column. The fractions correlating to maximum peaks with fixed retention time were collected by using a fraction collector. It was performed by using two LC-10AT pumps (Shimadzu).

Collection of Bacterial culture and In-vitro antibacterial analysis: UTIs causing bacteria including gram positive (Staph. aureus, K. pneumoniae) and gram-negative bacteria (E. coli and P. aeruginosa) cultures and confirmation was done through biochemical testing. Antibacterial activity of ethanolic extract of C. citratus was performed through disc diffusion method. Bacterial culture compared with 0.5% MacFarland standard having 1.5 × 10⁸ CFU/ml grown as a lawn culture on Mueller-Hinton agar plates. Filter paper discs dipped in 50ul extract for overnight and used to evaluate their antibacterial activity against selected uropathogens. Discs placed on Mueller-Hinton agar plates with bacterial lawn and incubated at 37°C for 24 hrs. Zones of inhibition (ZOI) were measured for results. Recommended antibiotics discs used as positive control and PBS dipped filter paper disc used as negative control for each bacterium (Cui et al., 2016). ZOI were compared with negative and positive control.

Minimum Inhibitory Concentration (MIC): MIC of C. citratus ethanolic leaves extract was performed in micro dilution plate. 50ul of extract with two- fold serial dilution
and 50 µl of nutrient broth were added from well no 3 to 12 in micro dilution plate. Then 20 µl of bacterial inoculums was added in each well. Well no 1 and 2 were maintained as control negative and positive containing nutrient broth (50µl) + bacterial inoculum (20µl) and antibiotic + nutrient broth (50µl) + inoculum (20µl) respectively. After incubation of 24 hours at 37°C, turbidity change was observed.

Minimum Bactericidal Concentration (MBC): To check the MBC, wells showing no visible growth in MIC, 20µl of mixture were transferred onto fresh nutrient agar plate with micropipette by spread plate method and incubated at 37°C for 24 hours.

**In-vivo testing of extract in mouse model:**
Experimental mouse model as reported previously (Hung et al., 2009; Cui et al., 2016) was used to evaluate the in-vivo effect of 80% ethanolic leaves extract of *C. citratus*. Albino mice (7-9 weeks) of age were injected with 1× 10⁷ CFU in 50µl PBS bacterial culture and after three days treatment for 6 days with 50 µl *C. citratus* extract. *C. citratus* extract was injected intraperitoneally. Three days post treatment mice were sacrificed and bacterial titer were measured from homogenized tissues of bladders. 0.2 ml of homogenized tissues were poured on LB medium and bacterial titer after 24 hours was measured by using the formula:

\[ \text{CFU per ml} = \text{number of colonies} \times 10^{\text{dilution}} \times \text{total volume} \]

**RESULTS**

**Confirmation of bacteria:** The bacteria isolated from UTI patient were characterized through conventional biochemical methods presented in Table 1.

**Phytochemical Anaylysis of extract:** HPLC showed the phenolics and flavonoids compounds present in ethanolic leaves extract of *C. citratus*. The quantity and positive control are presented in Table 2. Fig. 1 presents the retention time of different flavonoids and phenolic compounds. Structures of compounds drawn through chembio draw 16 software are shown in Fig. 2.

### Table 1: Biochemical identification of bacterial strains

| Bacterial Strains | Selective media | Gram staining | Catalase test | Oxidase test | Triple sugar iron test | Citrate test | Methyl red |
|-------------------|-----------------|---------------|---------------|--------------|------------------------|-------------|------------|
| Staph. aureus     | Manitol salt agar | +ve.          | +ve.          | -ve          | A/A                   | +ve.        | +ve.       |
| E. coli           | MacConkey agar   | -ve.          | +ve.          | -ve          | A/A, gas+H₂S          | -ve.        | +ve.       |
| P. aeruginosa     | Cetrimide agar   | -ve.          | +ve.          | +ve          | K/K, gas-no H₂S       | +ve         | -ve.       |
| K. pneumoniae     | MacConkey agar   | -ve.          | +ve.          | -ve          | A/A, gas, No H₂S      | +ve         | -ve.       |

**Fig. 1:** Chromatogram of HPLC analysis of Lemon grass extract.

**Fig. 2:** Chemical structures of different alkaloids isolated from Lemon grass extract.
Table 2: Antibiotic susceptibility testing, zone of inhibition at 50ug of C. citratus extract in three replicates and their mean with comparison to positive control

| Bacterial spp. | Zone of inhibition (mm) | Mean | Antibiotic (positive control) |
|---------------|-------------------------|------|-------------------------------|
| E. coli       | 15                      | 13   | 15                            |
| K. pneumoniae | 11                      | 8    | 7                             |
| Staph. Aureus | 15                      | 11   | 14                            |
| P. aeruginosa | 11                      | 16   | 13                            |

In-vitro antibacterial activity of extract: Plant extract of C. citratus was evaluated through disc diffusion method. ZOI compared with positive controls (antibiotics), ampicillin is used for S. aureus, E. coli, K. pneumonia and gentamycin was used for P. aeruginosa. C. citratus has proved to be more effective against P. aeruginosa, Staph. aureus and E. coli and less effective against K. pneumoniae in disc diffusion method. The largest ZOI were observed against E. coli (14mm) and P. aeruginosa (13mm). Table 2 showed all the mean ZOI against uropathogens.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): MIC of plant extract performed in micro dilution plate. Minimum concentrations inhibiting the growths of E. coli, Staph. aureus, P. aeruginosa and K. pneumoniae were 6.25, 0.8, 3.125 and 6.25µl, respectively. All the details of MIC are given in Table 3. 20µl from each MIC determined well evaluated through spread plate method on agar plates and all showed same MBC values as MIC.

In-vivo testing of extract in mouse model: After 6 days of treatment, all the animals were sacrificed and bladders were homogenized in PBS. Bacterial titer was significantly decreased in both gram negative and positive uropathogens are presented in Table 5.

DISCUSSION

Antimicrobial effects of medicinal plants against UTIs causing bacteria and resistant strains proved to be an effective alternative to drugs. Increasing multidrug resistance in uropathogens demanded a safe substitute to effective alternative to drugs. In current study proved the antimicrobial resistance in uropathogens required a safe substitute to effective alternative to drugs. Increasing multidrug UTIs causing bacteria and resistant strains proved to be an significantly decreased in both gram negative and positive uropathogens were more resistant against C. citratus extract than gram negative bacteria. Staph aureus showed least MIC value of 0.8ul that inhibit the growth of bacteria in 96 well plate. As compared to other studies may the difference in techniques lead to the minor variability of the results. P. aeruginosa showed less resistant against 80% ethanolic leaves extract than ethanolic extract, which is in agreement of Khan et al. (2012).

In current study HPLC showed the flavonoids and phenolic compounds were present in ethanolic extract that were most effective against uropathogens. Active compounds that include flavonoids, geranyl acetate, phenolic compounds, steroids and saponin were already reported in C. citratus oil by Duraiapandian et al. (2006), Mothana et al. (2010), Hindumathy et al. (2011).

Various essential plant oils in synergism showed greater effect against uropathogens as compared to antibiotics as reported in previous studies (Sienkiewicz et al., 2012; Buckova et al., 2018). Ethanolic extract of C. citratus leaves can be recommended as alternative remedy for antibiotics to avoid the more damage from resistance strains. But further in vivo studies on animal models should be conducted for further improvement/refinement.

Table 3: Minimum inhibitory concentration (MIC) of different volumes of Lemon grass extracts against uropathogens

| Well number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-------------|---|---|---|---|---|---|---|---|---|----|----|----|
| Plant extract dilution | -ve control | +ve control | 50 µl | 25 µl | 12.5 | 6.25 | 3.125 | 0.8 | 0.4 | 0.2 | 0.1 | 0.05 |
| E. coli     | ✓ | x | x | x | x | x | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| S. aureus   | ✓ | x | x | x | x | x | x | x | x | ✓ | ✓ | ✓ |
| P. aeruginosa | ✓ | x | x | x | x | x | x | x | ✓ | ✓ | ✓ | ✓ |
| K. pneumonia | ✓ | x | x | x | x | x | ✓ | x | ✓ | ✓ | ✓ | ✓ |

Table 4: Quantitative phytochemical analysis of extract and concentrations of different compounds

| No. of series | Compound name | Retention time (mVs) | Area (mVs) | Area % | Amount (ppm) |
|---------------|---------------|----------------------|------------|--------|--------------|
| Standard      | T- butanol (standard) | 2.773 | 31.461 | 1.2 | |
| Flavonoids    | Quercetin     | 3.313 | 26.600 | 2.1 | 2.43 |
|               | Gallic acid   | 4.487 | 22.447 | 1.6 | 0.73 |
| Phenolic compounds | Syringic acid | 16.307 | 93.263 | 7.1 | 2.23 |
|               | Ferulic acid  | 21.720 | 99.395 | 7.6 | 1.14 |
|               | Sinapic Acid  | 26.613 | 156.00 | 11.9 | 2.212 |
|               | Gallic acid   | 4.487 | 22.447 | 1.6 | 0.73 |
Table 5: In-vivo testing of C. citratus 80% ethanolic leaves extract

| Animals* | Bacterial inoculum | Bacterial titer after treatment* | Mean |
|----------|-------------------|-------------------------------|------|
| 1 | E.coli | 1 × 10^5 CFU/ml | 1 × 10^5 |
| 2 | K. pneumoniae | 1 × 10^6 CFU/ml | 1 × 10^5 |
| 3 | E. coli | 1 × 10^7 CFU/ml | 1 × 10^5 |

*Three replicates of animal for each uropathogens were tested; **50ul of 80% ethanolic leaves extract of C. citratus for each animal.

Authors contribution: JL, XCH, DW, KMW and AR conceived and designed the study. TF executed the experiment. WB, FMZ and ML analyzed the data. All authors have interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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