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SCREENING FOR ANTIMICROBIAL ACTIVITY OF NINE ETHANOLIC EXTRACTS OBTAINED FROM LEAVES OF *BEGONIA* PLANT: A POSSIBLE ALTERNATIVE IN THE TREATMENT OF INFECTIONS CAUSED BY *CITROBACTER FREUNDII*

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Many plants of the family *Begoniac*eae are used in the treatment of different diseases. Traditionally leaves of *Begonia* L. are used to decrease of fleeting pain in the limbs and joints, for blood purification, to reduce the body temperature, for treating anemia, for the treatment of respiratory infections, diarrhea, blood cancer, and skin diseases, peptic ulcer, conjunctivitis, colic and dyspepsia, dysentery and mouth ulcer. Moreover, the leaves of *Begonia* species are used for the treatment of cancer; besides, they possess anti-HIV activity. Some of the plants of the genus *Begonia* were previously reported for their antimicrobial activities. The antimicrobial activity of ethanolic extracts obtained from the leaves of *Begonia solimutata* L.B. Sm. & Wassh., *Begonia goegoensis* N.E.Br., *Begonia foliosa* Kunth, *Begonia* × *erythrophylla* Hérincq, *Begonia thiemei* C.DC., *Begonia peltata* Otto & Dietr., *Begonia heracleifolia* Cham. & Schltdl., *Begonia dregei* Otto & Dietr., and *Begonia mexicana* G. Karst. ex Fotsch was evaluated against the clinical strain of *Citrobacter freundii* strain. The testing of the antibacterial activity of the plant extracts was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. All ethanolic extracts obtained from leaves of *Begonia* species exhibited high activity against *C. freundii*. The most effective plants among species screened against *Citrobacter freundii* locally isolated were *B. thiemei*, *B. foliosa*, and *Begonia* × *erythrophylla* being highly active with the ethanolic extract (diameters of inhibition zone were ranged from 16.5 to 26 mm). The highly active antimicrobial effects of extracts obtained from *B. thiemei*, *B. foliosa* noted against *Citrobacter freundii* are worthy of highlighting. The identification of active compounds and their mode of action requires further investigation for antibacterial drug development.

Keywords: *Begonia*, leaf ethanolic extracts, antibacterial activity, inhibition zones, disc diffusion technique

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Introduction

Begonia L. is one of the most species-rich angiosperm genera with approximately 1,500 species currently recognized (Frodin, 2004). Representatives of *Begonia* genus are widely known as popular ornamental plants but the medicinal importance of its members is sparsely known. A few papers have been published, providing information regarding the medical properties of various *Begonia* species (Indrakumar et al., 2014; Amutha and Sreedevikumari, 2016; Shrestha et al., 2016). For instance, its leaves, flowers, and roots are used in diverse ailments in traditional and folklore remedies. Moreover, the leaves of *Begonia* species are used for the treatment of cancer and possess anti-HIV activity (Wu et al., 2004).

Plants belonging to the *Begonia* genus can be good candidates for as an alternative therapy in restricting the resistant infectious organisms. Some of the plants of the genus *Begonia* were previously reported for their antimicrobial activities (Holetz et al., 2002; Indrakumar et al., 2014; Amutha and Sreedevikumari, 2016; Shrestha et al., 2016). The antimicrobial activities of volatile compounds of intact plants of 24 *Begonia* species have been assessed against several pathogenic microorganisms (i.e. *Staphylococcus epidermidis*, *Escherichia coli*, and *Candida albicans*). As a result, 14 *Begonia* species, possessing well expressed phytoncide activity have been recommended to use as indoor plants, based on their ability to reduce microbial air pollution indoor by a factor of 1,5–3,0 in particular, by decreasing the *Staphylococcus aureus* load (Karpova et al., 2009, Tsybulia et al., 2011).

The genus *Citrobacter* belongs to the family of Enterobacteriaceae and comprises 11 different species of facultatively anaerobic, motile, Gram-negative bacilli, which are oxidase-negative and typically utilize citrate as the sole carbon source (Hodges et al., 1978; Janda et al., 1994; Samonis et al., 2009). Among *Citrobacter* species, the most commonly isolated from human clinical specimens are *C. koseri* (formerly named *C. diversus*), *C. freundii*, *C. youngae*, *C. braakii*, and *C. amalonaticus*, while the majority of cases of infection are associated with *C. koseri* and *C. freundii* (Janda et al., 1994; Samonis et al., 2009). *Citrobacter freundii* is recognized as an emerging opportunistic pathogen and is known to cause a variety of ailments (e.g., urinary tract infections, wound infections, gastrointestinal infections, septicemia, meningitis), especially in immunocompromised patients and in-hospital settings (Brenner et al., 1993; Gupta et al., 2003; Samonis et al., 2009; Ranjan and Ranjan, 2013; Leski et al., 2016). This emergence has coincided with the finding that *C. freundii* is often resistant to multiple antibiotics, suggesting that both clinical and environmental strains may be important reservoirs of antimicrobial resistance determinants (ARDs) (Pepperell et al., 2002; Leski et al., 2016). *Citrobacter* infections were found to represent 0.8% of Gram-negative infections in a large surveillance study (Jones et al., 2000; Samonis et al., 2009). Moreover, in hospital settings, *Citrobacter* spp. have accounted for 3–6% of all isolates of Enterobacteriaceae (Lipsky et al., 1980; Samonis et al., 2009; Lavigne et al., 20117). The mortality rate of hospitalized patients with *Citrobacter* infections has been observed to be 6.8% (Mohanty et al., 2007).

Considering the points highlighted above and based on previous results obtained in our laboratory, the aim of the present study was to find out *in vitro* possible antimicrobial action of the ethanolic extracts from leaves of nine *Begonia* species against *Citrobacter freundii* strain locally isolated.

Materials and methodology

Collection of Plant Materials. Collection of Plant Material

The leaves of *Begonia* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. The leaves of *Begonia solimutata* L.B. Sm. & Wassh., *Begonia goegoensis* N.E.Br., *Begonia foliosa* Kunth, *Begonia* × *erythrophylla* Hérincq, *Begonia thiemei* C.DC. (syn. *Begonia macdougallii* Ziesenh.), *Begonia peltata* Otto & Dietr. (syn. *Begonia kellermanii* C.DC.), *Begonia heracleifolia* Cham. & Schltdl., *Begonia dregei* Otto & Dietr., *Begonia mexicana* G. Karst. ex Fotsch was sampled for our study. The antimicrobial screening of *Begonia* leaf extracts has been carried out.

Preparation of Plant Extracts

The leaves were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial test strain and growth conditions

The non-repetitive clinical strain of *Citrobacter freundii* isolated from patients with uretic infection was collected from Koszalin Hospital during March-April, 2019. The purity, as well as the identity of isolate, was confirmed in the laboratory conditions by standard microbiological methods and were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (2014).

Susceptibility testing of the isolate was performed by disk diffusion according to the Guidelines of Clinical and Laboratory Standard Institute (CLSI, 2014). The antibiotics tested were piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, cefuroxime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamicin, trimethoprim-sulphamethoxazole, ciprofloxacin, levofloxacin, tetracycline, tigecycline, and polymyxin B. Results were interpreted according to CLSI criteria. MIC was determined by *E*-test strips (according to manufacturer's instruction) and agar dilution method (according to the Guidelines of Clinical and Laboratory Standard Institute). The resistance breakpoints were the same as the ones defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2014).

The cultivation medium was trypticase soy agar (Oxoid, UK), supplemented with 10% defibrinated sheep blood. Cultures were grown aerobically for 24 h at 37 °C. The cultures were later diluted with a sterile solution of 0.9% normal saline to approximate the density of 0.5 McFarland standard. The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the specified concentration. *Citrobacter freundii* strain studied was susceptible to all antibiotics used.

Determination of the antibacterial activity of plant extracts by the disk diffusion method

The testing of the antibacterial activity of the plant extracts was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). The strain was inoculated onto Mueller-Hinton (MH) agar plates. Sterile filter paper discs impregnated with extracts were

applied over each of the culture plates. Isolates of bacteria were then incubated at 37 °C for 24 h. The plates were then observed for the zone of inhibition produced by the antibacterial activity of ethanolic extract obtained from the leaves of various extracts of plants belonging to the *Begonia* genus. A negative control disc impregnated with sterile ethanol was used in each experiment. At the end of the period, the inhibition zones formed were measured in millimeters using the vernier. For each extract, eight replicates were assayed. The plates were observed and photographs were taken. The susceptibility of the test organisms to the plant extracts was indicated by a clear zone of inhibition around the holes containing the plant extracts and the diameter of the clear zone was taken as an indicator of susceptibility. Zone diameters were determined and averaged.

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of extracts tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 8.0 software (StatSoft, Poland) (Zar, 1999). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 10–15 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Antimicrobial activities of various ethanolic extracts obtained from leaves of various plants belonged to the *Begonia* genus against *C. freundii* measured as an inhibition zone diameter are presented in Figure 1 and 2. The present study has shown that all ethanolic extracts obtained from leaves of *Begonia* species exhibited high activity against *C. freundii*. The diameter of the inhibition zone for *B. solimutata* was (15.5 ±1.1) mm, for *B. goegoensis* – (18.1 ±1.2) mm, for *B. foliosa* – (21.5 ±1.5) mm, for *Begonia* × *erythrophylla* – (18.8 ±1.2) mm, for *B. thiemei* – (22.5 ±1.5) mm, for *B. peltata* – (16.8 ±1.3) mm, for *B. heracleifolia* – (13.2 ±1.1) mm, for *B. dregei* – (11.8 ±0.8) mm, and for *B. mexicana* – (12.5 ±0.6) mm (Figure 1 and 2).

Detailed data regarding the zones of inhibition by the various plant extracts were recorded and presented in Figure 2.

It should be noted that the most effective plants among species screened against *Citrobacter freundii* locally isolated were *B. thiemei*, *B. foliosa*, and *Begonia* × *erythrophylla*, being highly active with the ethanolic extract (diameters of inhibition zone were ranged from 16.5 to 26 mm). The highly active antimicrobial effects of extracts obtained from *B. thiemei*, *B. foliosa* noted against *Citrobacter freundii* are worthy of highlighting.

In our previous study (Tkachenko et al., 2016), we have also demonstrated that the ethanolic extracts obtained from leaves of *Begonia* species had moderate activity against *Escherichia coli*. The diameters of inhibition zone for *B. solimutata* were 14 mm, 11.5 mm for *B. goegoensis*, 13 mm for *B. foliosa*, 13.5 mm for *Begonia* × *bunchii*, 15 mm for *B. thiemei*,

19 mm for *B. peltata*, 12 mm for *B. heracleifolia*, 11.5 mm for *B. dregei*, and 16 mm for *B. mexicana*. The highest antimicrobial effect was recorded for *B. peltata*, *B. mexicana*, and *B. thiemei*. The most antimicrobial effective plant against *E. coli* was *B. peltata*, being highly active with the ethanolic extract (diameter of inhibition zone was 19 mm). The highly active antimicrobial effects noted against *E. coli* are worthy of highlighting (Tkachenko et al., 2016). Moreover, the ethanolic extract from the leaves of *B. goegoensis* exhibited strong activity against *Pseudomonas aeruginosa* (inhibition zone diameter ranged from 12 mm to 13 mm), while methanolic leaf extract screened revealed less profound activity (within 11–12.5 mm) in our previous study (Tkachenko et al., 2017). Moreover, it has been observed that ethyl acetate, hexane and dichloromethane extracts obtained from leaves of *B. goegoensis* revealed no antibacterial activity against *P. aeruginosa* and β-lactamases producing *P. aeruginosa* (MBL-positive *P. aeruginosa*) strains. MBL-positive *P. aeruginosa* was also susceptible to ethanolic and methanolic extracts (inhibition zone diameter ranged from 12.5 mm to 15.5 mm) (Tkachenko et al., 2017).

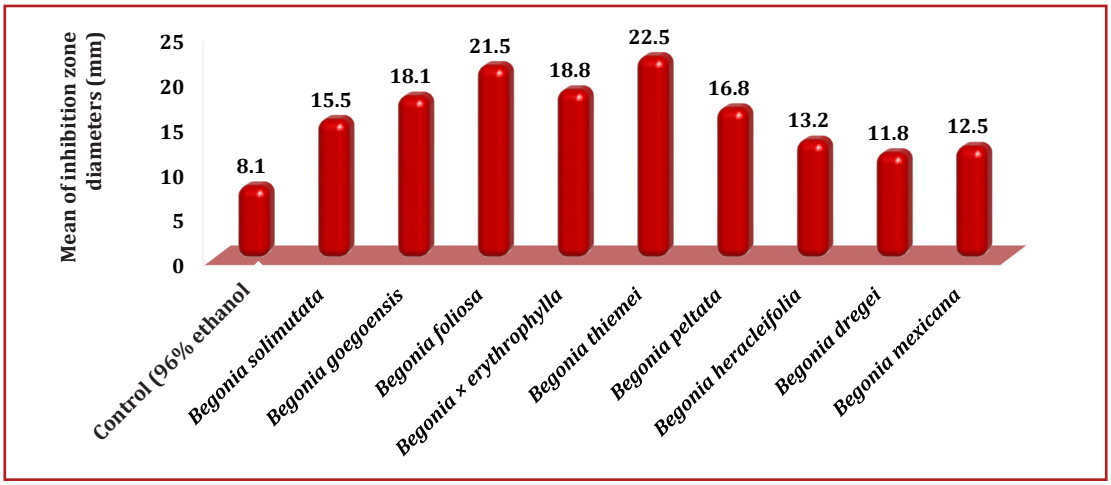

Figure 1 Antimicrobial activity of various extracts obtained from leaves of various plants belonged to the *Begonia* genus against *Citrobacter freundii* measured as inhibition zone diameter

Similar antimicrobial effect of various plants belonged to *Begonia* genus was also demonstrated by other researchers. For example, Siregar et al. (2018) have demonstrated the antibacterial potency of simple fractions of ethyl acetate extract of *Begonia baliensis* Girm. from Bukit Sangyang, Penebel, Tabanan-Bali. The chemical compounds of ethyl acetate extracts were isolated and separated by column chromatography. The obtained fractions were analyzed for antibacterial activity by disc diffusion assay against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus epidermidis* and *Staphylococcus aureus*. The chromatographic column yielded 14 simple fractions, whereas antibacterial test results showed 5 active fractions. Fraction 3 was active against *S. epidermidis*, fraction 5 against *E. coli* and *S. epidermidis*, while fractions 10, 11 and 12 were active only against *Bacillus subtilis* (Siregar et al., 2018).

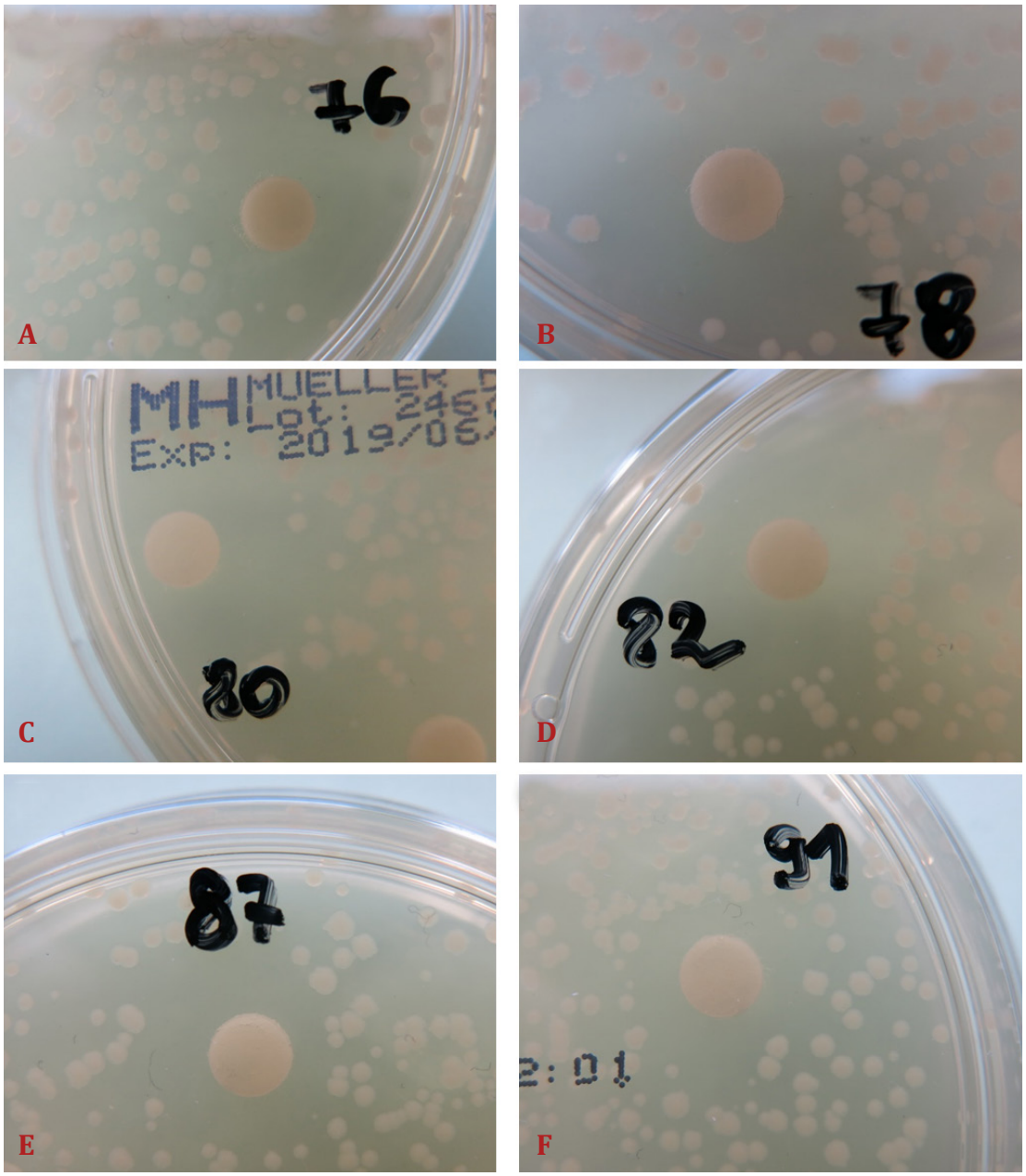

Figure 2 Inhibition zones induced by various ethanolic extracts obtained from leaves of *Begonia goegoensis* (A), *B. foliosa* (B), *Begonia* × *erythrophylla* (C), *B. thiemei* (D), *B. peltata* (E), and *B. heracleifolia* (F) against *Citrobacter freundii* growth

Antimicrobial properties of *Begonia fischeri* var. *palustris* plantlets were assessed by Karpova et al. (2019). Flavonoid composition of the leaves of *in vitro* plantlets and greenhouse stock plants had no substantial differences. Significant differences between flavonoid contents of the leaves [13.6 and 15.5 mg/g of dry weight (DW), respectively], were not found.

Aqueous ethanolic extracts of plants showed antimicrobial effects against reference strains of *Bacillus subtilis*, *Streptococcus pyogenes* and *Staphylococcus aureus*. The concentration of flavonoids in acetone and ethanol extracts of exudative compounds of the leaves of *in vitro* plantlets was 0.02 and 2.0 mg/g DW, respectively (Karpova et al., 2019).

Jeeva and Antonisamy (2012) have investigated the antibacterial activity and phytochemical properties of *Begonia floccifera* Bedd. methanolic flower extracts against the selected pathogens (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, *Serratia marcescens*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*) by the disc diffusion method. The results of the phytochemical screening revealed that phenol, tannins, xanthoproteins, steroids, phytosterols, triterpenoids, sapogenins, coumarins, and carbohydrates are comprised in the methanolic extracts of *B. floccifera*. The antibacterial activity has been observed in the methanolic extracts of *B. floccifera* against the tested bacteria with varied activity. The maximum diameter of inhibition zone was 28 mm for *B. cereus*, 25 mm for *S. aureus*, 15 mm for *E. coli*, 13 mm for *P. mirabilis*, 7 mm for *K. pneumonia*. The other pathogens viz., *P. aeruginosa*, *S. typhi*, *S. marcescens*, *Enterobacter* spp., *E. faecalis*, and *S. pyogenes* showed the minimal susceptibility to inhibition by extracts tested. Thus, methanolic flower extracts of *B. floccifera* can be used to treat nausea, vomiting, diarrhea, urinary tract infections, nosocomial infections, pneumonia, septicemias, etc. (Jeeva and Antonisamy, 2012).

Phytochemical investigation of the various extracts of the leaves of *Begonia malabarica* Lam. resulted in the isolation and identification of six known compounds, viz. friedelin, epi-friedelinol, β-sitosterol, luteolin, quercetin, and β-sitosterol-3-β-d-glucopyranoside in the study of Ramesh et al. (2002). The aqueous and organic solvent extracts were also tested against ten human pathogenic bacteria and four fungal strains by the agar well diffusion method. All the extracts were devoid of antifungal activity against the tested fungi. The hexane extract did not show any activity. The aqueous extracts showed activity against the Gram-negative bacteria except for *Vibrio parahaemolyticus*. The chloroform and methanol extracts showed activity against all the tested bacteria (Ramesh et al., 2002). Shobi et al. (2018) have studied the antibacterial activity of di-butyl phthalate isolated from *B. malabarica*. It was extracted with various solvents and bioactive compounds were isolated using chromatographic techniques. One of the bioactive compounds isolated from it was a colorless or pale yellow oily compound that is soluble in chloroform. The structure of the compound was elucidated as di-butyl phthalate with the help of spectral data. The compound is reported to have antibacterial and anticancer properties. Dibutyl phthalate showed a 9 mm zone of inhibition against *Staphylococcus epidermidis*, *Strephtococcus pneumoniae*, *Escherichia coli*, *Micrococcus luteus*, *Klebsiella pneumonia*e, *Shigella flexneri*, *Vibrio cholerae* and *P. aeruginosa* at the concentration of 100 mg/ml. Similar zone of inhibition recorded at all the concentrations of *E. coli* and *Pseudomonas aeruginosa*. Eight mm zone of inhibition was recorded against *Strephtococcus pneumoniae* at 50, 25 and 12.5 mg/ml. *Staphylococcus epidermidis*, *Klebsiella pneumonia*e, and *Shigella flexneri* also showed an 8 mm zone of inhibition at 25, 12.5 and 6.25 mg/ml concentrations (Shobi et al., 2018).

Other researchers also demonstrated the necessitates of using medicinal plants as an alternative therapy in restricting the resistant infectious organisms. In the study of Narayanan et al. (2011), the antibiotic resistances of organisms isolated from urinary tract infected patients were evaluated using the National Committee for Clinical Laboratory Standards (NCCLS) method and Multiple Antibiotic Resistance (MAR) index values, and the MAR values were also calculated for plant extracts. The 10 common medicinal plants collected from Kolli Hills, Namakkal, South India were extracted using the chloroform, methanol, acetone, ethanol and saponification procedure. The efficacy of the extracts on the uropathogens was tested by agar disc diffusion method in order to analyze the inhibitory activity of plant extract on the organisms. *Azadiracta indica* A. Juss., *Tinospora cordifolia* (Wild.) and *Euphorbia hirta* Linn. exhibited high inhibitory activity against most of the 11 tested organisms followed by *Cassia javanica* Linn. and *Phyllanthus niruri* Linn. The maximum zone size of 46.3 mm was exhibited by methanol extract of *P. niruri* Linn. against *Pseudomonas aeruginosa*. *Asparagus racemosus* Willd. and *Eupatorium triplinerve* Vahl had the least activity against resistant pathogens. Saponified lipids of most of the plants exhibited maximum antibacterial activity. Among the tested organisms, *P. aeruginosa* and *Staphylococcus epidermidis* were the most susceptible and *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter koseri*, and *Citrobacter freundii* were the least inhibited by most of the extracts of medicinal plants (Narayanan et al., 2011).

Variation in the chemical profile of extracts could influence their biological activities. Therefore, it was important to evaluate the chemical composition of extracts to correlate with their antimicrobial activities. A study conducted by Kalpanadevi and Mohan (2012) has shown that the extracts of *B. malabarica* and *B. floccifera* leaves contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. *In vitro* assay systems confirmed *B. malabarica* and *B. floccifera* whole plants as natural antioxidants. The phenolics and flavonoids could be the reason for its antioxidant activity. The preliminary phytochemical studies revealed the presence of flavone, sterol, triterpene in hexane, chloroform, and methanol extracts; phenol in chloroform and methanol extracts of *B. malabarica* and quinone, saponin, tannin, and starch in methanol extract. All the extracts did not answer for alkaloids (Ramesh et al. 2002). Preliminary phytochemical screening of *B. floccifera* and *B. malabarica* conducted by Ariharan et al. (2012) showed the presence of vitamin C in the leaves of both plant species assayed. Apparently, the antimicrobial activity of the leaf extracts of these *Begonia* species screened against pathogenic strains of Gram-positive (*Staphylococcus aureus*, *S. epidermidis*) and Gram-negative (*Pseudomonas aeurogenosa*, *Salmonella typhimurium*) bacteria could be due to the presence of this phytoconstituent. Additionally, the flavonoids content (including glycosides of quercetin and kaempferol), anthocyanins and ascorbic acid in overground part of plants of 7 species and cultivars of genus *Begonia* L. (*B. bahiensis*, *B. bowerae*, *B. carolineifolia*, *B. fischeri*, *B. heracleifolia*, *B. 'Erythrophylla'*, *B. 'Helen Teupel'*) were determined by Karpova et al. (2009). The flavonoids content was 24–650 mg% of dry weight, including glycosides of quercetin – 3–76 mg%. Kaempferol glycosides were detected only in species of section Gireoudia (1.2–5.7 mg%). The contents of anthocyanins were between 60 and 157 mg%, ascorbic acid – 5–43 mg% of fresh weight. These results suggest that studied plants of *Begonia*

species can be considered as the sources of biologically active compounds with antioxidant and antimicrobial activities (Karpova et al., 2009).

Conclusions

This *in vitro* study corroborated the antimicrobial activity of the selected plants belonged to *Begonia* genus. All these plants were effective against *Citrobacter freundii* strain locally isolated. This study also showed that *B. thiemei*, *B. foliosa*, and *Begonia* × *erythrophylla* could be potential sources of new antimicrobial agents. The identification of active compounds and their mode of action requires further investigation for antibacterial drug development.

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