AcademicPres

Available online: www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2014, 6(2):155-157

Notulae Scientia Biologicae

In vitro Antibacterial Activity Analysis of Leaves of *Limonia acidissima*

G. Kishor NAIDU*, B. SUJATHA, K. Chandra Sekhar NAIDU

*Department of Botany, Andhra University, Visakhapatnam (A.P.) 530 003, India; gandi.gkn@gmail.com (*corresponding author)*

Abstract

The present study was carried out the antibacterial activity and phytochemical screening of the hexane, chloroform and methanol extracts of leaves of *Limonia acidissima*. The antibacterial activity was evaluated against four Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) and five Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus, Staphylococcus aureus*, *Streptococcus pneumoniae*) by agar well diffusion method. Methanol extract showed good antibacterial activity with the high inhibition zones while chloroform extract exhibited mild to moderate activity and hexane extract was found to be less active. Phytochemical screening revealed the presence of various secondary metabolites like steroids, alkaloids, phenols, flavonoids, coumarins, saponins, tannins and triterpenoids. The results of the present study suggest that leaves of *Limonia acidissima* can be used to treating infectious diseases caused by *E. coli*, *P. vulgaris* and *S. pneumoniae*.

Keywords: Antibacterial activity, *Limonia acidissima*, minimum inhibition concentration, methanol extract

Introduction

Limonia acidissima Linn is an important medicinal plant and is a moderate-sized deciduous tree grown throughout tropical and temperate regions of the world. Tree with rough, spiny bark. Leaves are pinnate, with 5-7 leaflets, with a citrus scent when crushed. Leaves of the plant are used for vitiated conditions of vata and pita (Kirtikar and Basu, 2005). The decoction of the leaves used in the treatment of constipation, vomiting, cardiotonic, diuretic (Tarakeswara Naidu *et al.,* 2012). It has been reported that plant contains flavanoids, glycosides, saponins, tannins (Saima *et al.,* 2000), coumarins (Ghosh *et al.,* 1982) and tyramine derivatives (Parthasarathi *et al.,* 1991). Also the presence of alkaloids, steroids, glycosides, phenols, gum and mucilage, fixed oils and fats, resins and tannins(Thomas and Ponnammal, 2005). The leaves have hepatoprotective activity (Kamat *et al.,* 2003), antimicrobial (Bandara *et al.,* 1990) antifungal (Khewkhom *et al.,* 2008; Adikaram *et al.,* 2007) astringent, anti-inflammatory (Kim *et al.,* 2009) and insulin secretogouge activities (Gupta *et al.,* 2009). As the plant is reported to have various medicinal uses, authors have attempted to study the antimicrobial activity of the leaves.

Materials and methods

The present study was done in the Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India. Leaves of *Limonia acidissima* were collected from University premises.

Chemicals, Media and Antibiotic

Hexane, chloroform, methanol and Dimethyl sulphoxide (DMSO), were obtained from Rankem company, India. Nutrient broth and Nutrient agar were obtained from Hi-media, Mumbai, India. The antibacterial agent Ciprofloxacin was obtained from Axiom Laboratories Ltd., India.

Test organisms

Bacteria were selected for the antibacterial activity that include Gram-negative (*Escherichia coli* MTCC B1560, *Klebsiella pneumoniae* MTCC B4030, *Pseudomonas aeruginosa* MTCC B2297, *Proteus vulgaris* MTCC B7299) and Gram-positive (*Bacillus subtilis* MTCC B2274, *Enterococcus faecalis* MTCC B3159, *Micrococcus luteus* MTCC B1538, *Staphylococcus aureus* MTCC B3160, *Streptococcus pneumoniae* MTCC B2672) bacteria. All strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India and were maintained in nutrient agar.

Extraction procedure

Limonia acidissima leaves washed thoroughly under running tap water, dried on paper towel, then shade dried and finally crushed to fine powder in mixture grinder. The dried powder of the leaves was allowed to Soxhlet for sequential extraction with hexane, chloroform and methanol. The liquid extract was collected, filtered and evaporated to dryness using Rotary evaporator (Heidolph, Heizbad, Laborota 4001, Germany 2002). A semisolid or dried crude extracts of leaves so obtained was re-suspended in an inert solvent, DMSO. The extracts were dissolved in DMSO to reach final concentrations (50 mg/ml, 75 mg/ml and 100mg/ml), and were kept in refrigerator till used.

Antimicrobial activity

The antimicrobial activity of extracts of *Limonia acidissima* leaves was determined by using agar well diffusion technique

(Aniel Kumar *et al.,* 2010a). Nutrient agar plates were each seeded with 0.1 ml of an overnight culture of each bacterial (equivalent to 10^7-10^8 CFU/mL) strain. The 24 hrs broth culture of each bacterium used to seed sterile molten nutrient agar at 45 ° C, allowed to solidify at room temperature and well made by sterile standard cork borer and 50 µl solution of extract added to each well. Then bacterial plates incubated at 37 ° C for 24 hrs after which diameter of zones of incubation were measured (mm) by using HiAntibiotic ZoneScale-C (Himedia). Each assay was performed in at least triplicate and mean values (± standard error) are reported. Standard antibiotic strip of Ciprofloxacin (5 µg/disc) for each bacteria along with DMSO were used as positive and negative controls, respectively.

Minimum inhibitory concentration (MIC) was determined by the broth dilution method (Aniel Kumar *et al.,* 2010b). A quantity of 0.6g of each extract was dissolved in 300 ml sterile nutrient broth, which yields an initial concentration of 2000 µg/ml. subsequently, two-fold serial dilution was made from the stock to obtain following concentrations 1000, 500, 250, 125, 62.5 µg/ml. Different concentrations of leaf extract in hexane, chloroform and methanol was tested separately for each bacterium and inhibition zone of microbial growth in the plates containing test solutions was judged by comparison with blank control plates. MIC is defined as the lowest concentration of test samples that result in a complete inhibition of visible growth. Experiments were carried out in triplicate.

Results and Discussion

The inhibition zones and MIC values of leaves of *Limonia acidissima* are presented in Tab. 1, Tab. 2, respectively. Methanol extract showed the high antibacterial activity while chloroform extract showed mild to moderate activity and hexane extract found to less effective. Methanol extract showed the highest zone of inhibition against *Proteus vulgaris* followed by *E. coli* and *S. pneumoniae*, while chloroform and hexane extracts against had the high zone of inhibition *Proteus vulgaris* followed by *S. pneumoniae*. Hexane extract did not shown inhibition values against *M. luteus*, *Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* whereas chloroform extract against *Bacillus subtilis*. On comparison standard antibiotic ciprofloxacin, methanol extract exhibited higher inhibition zones against *E. coli*, *P. vulgaris*, *S. pneumoniae* and similar value against *E. faecalis* while chloroform extract showed higher activity against *Proteus vulgaris*. DMSO, a negative control did not show any inhibition zones and indicated that it is not interfering zone formation.

The low MIC values of methanol extract were <62.5 µg/ml against *Proteus vulgaris* and *S. pneumoniae* while 62.5 µg/ml against *E. coli*; 125 µg/ml against *M. luteus*, *S. aureus*. Chloroform extract showed low MIC against *Proteus vulgaris, S. pneumoniae*, it was 62.5 µg/ml whereas hexane extract found to be low MIC against *E. coli, S. pneumoniae* and it was 250 µg/ml.

Plants are important sources of potentially useful substances for the development of new therapeutic agents. Various phytochemical compounds which are naturally present in plants as secondary metabolites have been implicated in plants as the conferment of antibacterial activities (Kishor Naidu *et al.,* 2013). The inhibition of bacterial growth *in vitro* by the extracts of *Limonia acidissima* plant parts such as bark, leaf, rind, pulp and seed showed the presence of alkaloids, flavonoids, steroids, saponins, glycosides, phenols, gum and mucilage, fixed oils and fats, resins and tannins(Thomas and Ponnammal, 2005).

Methanol proved as the most effective solvent for extracting broad spectrum of antimicrobial compounds from plants (Vlachos *et al.,* 1996). It is worth mentioning to note that a correlation was observed between the extract solubility and antibacterial activity of different fractions. This suggests that in sequential extraction, maximum antibacterial compounds were soluble in polar solvent as methanol

Tab. 1. Antimicrobial activity of extracts of *L. acidissima* leaves

Bacteria	Hexane extract	Chloroform extract	Methanol extract	Ciprofloxacin	DMSO
E. coli	12±0.21	18±0.82	24±0.13	20±0.04	-
K. pneumoniae	10±0.04	12±0.23	17±0.45	20±0.04	-
P. aerginosa	-	10±0.45	12±0.06	20±0.03	-
P. vulgaris	16±0.45	23±0.54	26±0.32	18±0.12	-
B. subtilis	-	-	18±0.24	22±0.02	-
E. faecalis	12±0.30	16±0.90	22±0.56	22±0.13	-
M. luteus	-	10±0.34	20±0.40	19±0.08	-
S. aureus	-	14±0.07	19±0.03	23±0.32	-
S. pneumoniae	14±0.08	20±0.41	24±0.36	23±0.02	-

Note: the values are expressed as mean ±SE
-: negative

extract displayed the highest antibacterial activity followed by chloroform and hexane extracts. These results further confirm that significant antibacterial compounds are polar in nature as evidenced by the higher degree of antibacterial activity of methanol extracts of *Limonia acidissima* leaves. These active compounds may act alone or in combination to inhibit bacterial growth and conferred the strong antibacterial activity.

Earlier, it was reported that methanolic extracts of *L. acidissima* plant parts were tested against *Escherichia coli* and *Staphylococcus aureus* showed varying degrees of antimicrobial activity (Thomas and Ponnammal, 2005). Stem bark had antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterobacter eloacae, Klebsiella erogenes, Aspergillus niger* and *Candida albicans* (Rahman

Tab. 2. MIC values of the *L. acidissima* leaves

Bacteria	Hexane extract	Chloroform extract	Methanol extract
E. coli	250	250	62.5
K. pneumoniae	>1000	500	500
P. aerginosa	>1000	>1000	>1000
P. vulgaris	500	125	<62.5
B. subtilis	>1000	>1000	250
E. faecalis	1000	500	500
M. luteus	>1000	>1000	125
S. aureus	>1000	1000	125
S. pneumoniae	250	125	<62.5

and Gray, 2002) whereas Chloroform extract of the leaves exhibited antifungal activity (Khewkhom *et al.,* 2008) and in the present study stated that *Proteus vulgaris* was the most sensitive to the hexane, chloroform and methanol extracts of *L. acidissima* leaves and suggest that extracts of *L. acidissima* may be used to treat human diseases caused by microorganisms. Therefore, *L. acidissima* may be a future drug candidate to prove its efficacy as a preventive and therapeutic agent against *E. coli*, *P. vulgaris* and *S. pneumoniae*. Therefore, further studies needed to isolation and identification of compounds responsibility for antibacterial activity.

Conclusion

In conclusion, the results provide a scientific base for the traditional use of *L. acidissima* as an antimicrobial agent. It is suggested that, *L. acidissima* may possess promising therapeutic action in the treatment of infectious diseases caused by the species like *E. coli*, *P. vulgaris* and *S. pneumoniae*. Further work will emphasize the isolation and characterization of active compounds responsible for antibacterial activity.

References

Adikaram NKB, Abhayawardhane Y, Gunatilaka AAL, Bandara BMR, Ijeratne EMK (2007). Antifungal activity, acid and sugar content in the wood apple (*Limonia acidissima*) and their relation to fungal development. Plant Pathol (38):258-265.

Aniel Kumar O, Mutyala Naidu L, Raja Rao KG (2010a). Antibacterial evaluation of snake weed (*Euphorbia hirta* L.). J Phytol 2(3):08-12.

Aniel Kumar O, Mutyala Naidu L, Raja Rao KG (2010b). *In vitro* antibacterial activity in the extracts of *Andrographis paniculata* Burm. F Intern J PharmTech Res 2(2):1383-1385.

Bandara BMR, Hewage CM, Jayamanne DHLW, Karunaratne V. Asikaram NKB, Bandara KANP, Wijesundara DSA (1990). Biological activity of some steam distillates from leaves of ten species of rutaceous plants. J Natn Sci Coun Sri Lanka 18:71-77.

Ghosh P, Sil P, Majumdar SG, Thakur S (1982). A Coumarin from *Limonia acidissima*. Phytochem 21:240-241.

Gupta R, Johri S, Saxena AM (2009). Effect of ethanolic extract of *Feronia elephantum* Correa fruits on blood glucose level in normal and streptozotocin-induced diabetic rats. Nat Prod Rad 8:32-36.

Kamat CD, Khandelwal KR, Bodhankar SL, Ambawade SD, Mhetre NA (2003). Hepatoprotective activity of leaves of *Feronia elephantum* Correa (Rutaceae) against carbon tetrachloride-induced liver damage in rats, J Natural Remed 3:148-154\

Khewkhom N, Greger H, Shangchote S (2008). Antimicrobial activity of 16 plant extracts of the Rutaceae family against phytopathogenic fungi. Agr Sci J 39(3):119-123.

Kim KH, Ha SK, Kim SY, Kim SH, Lee KR (2009). Limodissimin A: A New dimeric coumarin from *Limonia acidissima.* Bull Korean Chem Soc 30:2135-2137.

Kirtikar KR, Basu BD (2005). Indian Medicinal Plants. Dehradun, India, International Book Distributors 1:478-79.

Kishor Naidu G, Naidu KCS, Sujatha B (2013). *In vitro* Antibacterial activity and phytochemical analysis of leaves of *Gymnema sylvestre*. Intern J Pharm Tech Res 5(3):1315-1320.

Parthasarathi G, Prabal S, Srabani D, Swapnadip T, Kokke WCMC, Akihisa T, Shimizu N, Tamura T, Matsumoto (1991). T. Tyramine Derivatives from the Fruit of *Limonia acidissima.* J Nat Prod 54:1389-1393.

Rahman MM, Gray AI (2002). Antimicrobial constituents from the stem bark of *Feronia limonia*. Phytochemistry 59:73-77.

Saima Y, Das AK, Sarkar KK, Sen AK, Sur P. (2000). An antitumor pectic polysaccharide from *Feronia limonia*. Int J Biol Macromol 27:333-335.

Tarakeswara Naidu M, Chandra Babu N, Aniel Kumar O, Venkaiah M (2012). Herbal remedies for rheumatoid arthritis used by the tribes of Vizianagaram district, Andhra Pradesh. J Non-Timb Fores Prod 19(4):303-308.

Thomas A, Ponnammal NR (2005). Preliminary studies on phytochemical and antibacterial activity of *Limonia acidissima* L. plant parts. Anci Sci Life 25:1-4.

Vlachos V, Critchely AT, Von Holy (1996). Establishment of protocol for testing antimicrobial activity in South African Macroalgae. Microbios 88:115-123.