OPEN ACCESS

African Journal of Biotechnology

21 February 2018
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

AcademicJournals

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

Editorial Board

African Journal of Biotechnology

Table of Content: Volume 17 Number 8 21 February, 2018

ARTICLES

academicJournals

Vol. 17(8), pp. 232-238, 21 February, 2018
DOI: 10.5897/AJB2017.16355
Article Number: 72A7C2D56045
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antioxidant and antibacterial activities of essential oil of *Lippia sidoides* against drug-resistant *Staphylococcus aureus* from food

Renata Albuquerque Costa[1], Theodora Thays Arruda Cavalcante[1], Carla Thiciane Vasconcelos de Melo[1,2], Domingos Lucas Araújo Barroso[1], Hider Machado Melo[1], Mário Geraldo de Carvalho[3] and Francisco Eduardo Aragão Catunda Júnior[1,4*]

[1]Department of Biotechnology, INTA College, Sobral, Brazil.
[2]Medical School, Federal University of Ceará, Sobral, Brazil.
[3]Federal Rural University of Rio de Janeiro, Seropédica, Brazil.
[4]Center for Exact, Natural and Technological Sciences, State University of the Tocantina Region of Maranhão, Brazil.

Received 6 December, 2017; Accepted 2 February, 2018

Chemical composition, *in vitro* antioxidant and antibacterial activities of essential oil of *Lippia sidoides* (EOLS) and its major constituent was investigated. Chemical composition was analyzed in a gas chromatograph coupled to a mass spectrometer (GC/MS). Antioxidant activity (AA) was obtained using 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay. Antimicrobial activity was tested using disk-diffusion test against *Staphylococcus aureus* ATCC 25923 and nine *S. aureus* strains resistant to beta-lactams, tetracycline and quinolone. EOLS chemical composition revealed the presence of 15 components identified as monoterpenes hydrocarbon (1.56%), oxygenated monoterpenes (94.31%) and sesquiterpenes hydrocarbon (4.11%), and the major components were thymol (79.70%). All OELS concentrations analyzed showed AA varying from 19.09 ± 2.088% (100 µg/mL) to 74.32 ± 1.61% (3.200 µg/mL). For thymol, the most efficient AA (70.11 ± 3.43%) was found with higher concentration (6.400 µg/mL). All *S. aureus* strains (n = 10) were sensitive to OELS and thymol. When OELS was used, the halo size of the ATCC strain was 74.4 mm and for the resistant-strains, it ranged from 29 to 60.4 mm. Thymol bioactive was lower when compared with OELS: the ATCC strain showed a halo of 24.5 mm and a variation of 17.5 to 45 mm was observed between the halo sizes in resistant strains. The results suggest that OELS as compared to thymol has better potential for use as an antimicrobial and antioxidant agent.

Key words: Antimicrobials, Lippia, thymol, essential oil, 2,2-diphenylpicrylhydrazyl (DPPH), *Staphylococcus aureus*, oxygenated monoterpenes, antioxidant.

INTRODUCTION

The *Lippia sidoides*, a northeastern Brazil native plant, is utilized in popular medicine as a local antiseptic on the skin and mucosal tissues (Veras et al., 2012). Furthermore, other activities have been described: antifungal (Morais et al., 2016), amoebic (Santos et al., 2016), antimalarial (Mota et al., 2012), leishmanicidal (Farias-Junior et al., 2012), larvicidal (Silva et al., 2014) and acaricidal (Gomes et al., 2014), characterizing this

phanerogam as a source of bioactive compounds.

The search for new phytochemicals in plants including *L. sidoides* has also been directed to the worldwide problem related to emergency drug-resistant bacteria. Reports on antibiotic resistant bacteria point to the *Staphylococcus aureus* species recognized as foodborne pathogen (Rasooly et al., 2017) and one of the most frequently isolated (Li et al., 2016; Ge et al., 2017). In this context, the search for new active substances to combat the drug-resistant *S. aureus* is a current need.

This study aimed to evaluate the chemical composition, *in vitro* antioxidant and antibacterial activities of essential oil of *L. sidoides* and its major constituent, against *S. aureus* ATCC 25923 and nine drug-resistant *S. aureus* strains isolated from food.

MATERIALS AND METHODS

The leaves of *L. sidoides* Cham. (Verbanaceae) were collected in May 2014, from the Garden of Medicinal Plants, Farm of Faculdades INTA, City of Cariré, Ceará, Brazil (3°49'51 82''S and 40°24' 37 85''W). The plant was classified and a voucher specimen was deposited in the Francisco José de Abreu Matos Herbarium of the State University of Acaraú Valley under the number: HUVA 17480.

EOLS extraction

The leaves (1 kg) were placed in a 5 L round bottom beaker with 2 L of distilled water and subjected to hydrodistillation in a Clevenger apparatus for 2 h. The oil obtained was dried with anhydrous sodium sulphate, filtered, and then maintained in glass bottles under refrigeration until analysis.

EOLS analysis (gas chromatography/mass spectrometry)

The chemical composition of the essential oil was analyzed in a gas chromatograph coupled to a mass spectrometer (GC/MS-Shimadzu QP-2010 Plus) equipped with a Factor Four/VF 5 ms fused-silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness), using helium as carrier gas at 1 mL/min. The initial oven temperature was 35°C, which after being held constant for 2 min, was increased at a rate of 4°C/min to 180°C, followed by 10°C/min to 250°C, with a final isotherm (250°C) for 20 min. The sample injection volume was 1 µL (1:50 split mode). The injector and detector temperatures were both 250°C. The mass spectra were obtained in a range of 10 to 300 m/z by the electron impact technique at 70 eV.

Gas chromatography/flame ionization detector

Quantitative analysis of the essential oil and chemical composition was carried out in a gas chromatograph coupled to an HP 5890 Series II flame ionization detector (FID), using the same type of column as in the GC/MS analysis. The injector and detector temperatures were 240 and 300°C, respectively. The percentage of each constituent was calculated by the integral area under its respective peak in relation to the total area of all the sample constituents.

Identification of EOLS constituents

The various chemical constituents of the essential oil were identified by visual comparisons of their mass spectra with those in the literature and spectra supplied by the equipment database (NIST08), as well as by comparison of the retention indices with those in the literature (Adams, 2007). A standard solution of n-alkanes (C8-C20) was injected under the same chromatographic conditions as the sample and used to obtain the retention indices as described by Van Den Dool and Kratz (1963).

2,2-Diphenylpicrylhydrazyl (DPPH) radical scavenging assay

The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams et al. (1995) with some modifications. DPPH is a commercially available stable free radical, which is purple in colour. The antioxidant molecules derived from medicinal plants, when incubated, react with DPPH and convert it to di-phenyl hydrazine which is yellow in colour. The discoloration degree of purple to yellow was measured at 517 nm using *Spectramax paradigm*© (Molecular Devices). Sample stock solutions of essential oil of *L. sidoides* (8.0 mg/mL) and thymol (8.0 mg/mL) were diluted to final concentrations of 6.400, 3.200, 1.600, 800, 400, 200 and 100 µg/mL. Eugenol at the same final concentrations was used as a positive control, distilled water in 3% Tween 80 was used as a negative control and an ethanol and methanol solution in a ratio of 1:1 was prepared and used as a blank. For experimental procedure, 150 µL of a 0.27 mM DPPH ethanol : methanol (1:1) solution was added to 50 µL of sample solutions of different concentrations in 96-well plates and were allowed to react for 30 min at room temperature. After 30 min, the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity (AA) using the following formula: AA (%) = [(AB – AS)/ AB] x 100, where AB means absorption of DPPH sample and AS means absorption of sample solution.

Origin of strains

S. aureus ATCC 25923 and nine drug-resistant *S. aureus* strains isolated from shrimp *Litopenaeus vannamei* were used: S14 (ciprofloxacin-resistant), S46 (penicillin-resistant), S82 (oxacilin-resistant), S86 (oxacilin-resistant), S16 (oxacilin and cefepime-resistant), S27 (oxacilin and cefepime-resistant), S83 (oxacilin and cefepime-resistant), S102 (oxacilin, tetracycline and ampicillin-resistant), S26 (oxacilin, penicillin, and ampicillin-resistant) and S81 (cefepime, ceftriaxone, oxacilin and ampicillin-resistant).

Agar disc-diffusion assay

The antimicrobial susceptibility test by disk diffusion was done according to standard of Clinical and Laboratory Standards

*Corresponding author. E-mail: cearajr@gmail.com.

Table 1. Percentage concentrations of the constituients of essential oil of *Lippia sidoides* (EOLS).

Component	CRR	LRR	$RT_{CG/FID}$	%
p-Cymene	1029	1024	11.745	0.36
Limonene	1033	1029	11.978	0.73
1,8-Cineole	1037	1031	12.165	0.83
γ-Terpinene	1062	1059	13.582	0.47
E-4-Thujanol	1075	1070	14.320	0.45
6,7-Epoxymyrcene	1093	1092	15.528	0.42
Linalool	1102	1096	16.075	0.96
Ipsdienol	1148	1145	18.788	1.30
Citronellal	1157	1153	19.403	0.36
3-Thujen-2-one	1177	1171	20.747	0.11
Terpinen-4-ol	1184	1177	21.269	0.98
Methyletherthymol	1234	1235	24.512	0.43
Carvone	1253	1243	25.780	8.77
Thymol	1302	1290	29.196	79.70
E-Caryophyllene	1422	1419	35.827	4.11
Total				99.98

CRR= Calculated retention rate; LRR = literature retention rate; RT = retention time in the GC-FID.

Institute (CLSI 2012), with modifications. These strains were activated in two bacterial samplings in BHI broth (Difco©) grown for 24 and 18 h, respectively, in an oven at 37°C and adjusted to a 10^6 to 10^8 CFU/mL concentration in 0.85% saline solution equivalent to 0.5 McFarland and then inoculated on Mueller-Hinton agar (Difco©). Sterile 6 mm diameter white discs (Laborclin) were soaked with 20 µL of the tested substances and, after drying, were placed on plates containing Mueller-Hinton agar (Difco©) where bacteria were inoculated. Meropenen (30 µg) was used as positive control and sterile distilled water was used as negative control. After 24 to 48 h of plate's incubation at 35 ± 2°C under aerobic conditions, the inhibition halos were measured.

Statistical analysis

All analyses were performed in triplicates and the results were presented as mean (%) ± standard error mean (S.E.M). Data were analyzed by the software, GraphPad Prism 5.0, ANOVA followed by Student-Newman Keuls as *post hoc* test. The values of $p < 0.05$ were considered statistically significant.

RESULTS

Table 1 shows EOLS chemical composition. Fifteen components representing 99.98% of the essential oil were determined. Three monoterpenes hydrocarbon (1.56%), 11 oxygenated monoterpenes (94.31%) and one sesquiterpene hydrocarbon (4.11%) were identified. The major components were thymol (79.70%), carvone (8.77%) and E-caryophyllene (4.11%), totalizing 92.58%.

All EOLS concentrations analyzed showed antioxidant activity (AA) varying from 19.09 ± 2.088 (100 µg/mL) to 74.32 ± 1.61% (3.200 µg/mL) as compared to the negative control (0%), as shown in Figure 1A. No statistical difference was observed between concentrations of 3.200 (74.32 ± 1.61%) and 6.400 µg/mL (68.88 ± 6.27%) meaning that the best antioxidant activity was visualized at the concentration of 3.200 µg/mL. Antioxidant activity decreased with decrease in concentrations to 200 µg/mL (26.84 ± 1.028%), and there was no difference in the low concentration of 100 µg/mL (19.09 ± 2.088%).

Figure 1B shows antioxidant activity of different concentrations of thymol. The best activity (70.11 ± 3.43%) was found with higher concentration (6.400 µg/mL) as compared to the negative control (0%). Despite the fact that other concentrations exhibit antioxidant activity, their action decreased considerably from 3.200 (29.65 ± 1.53%) to 400 µg/mL (15.65 ± 3.88%). Concentrations of 200 and 100 µg/mL did not present antioxidant effect as compared to the negative control.

Antioxidant activity for eugenol, as a positive control is shown in Figure 1C. As expected, eugenol showed antioxidant activity in all concentrations analyzed and there was no statistical difference between them, with maximal value of 100 ± 0.46% at concentration of 6.400 µg/mL.

All *S. aureus* strains (n = 10) were sensitive to EOLS and thymol. Figure 2A shows that there was a significant difference ($p < 0.05$) between the halo size of the ATCC strain (74.4 mm) and the halo size of the resistant strains (29 to 60.4 mm).

Thymol bioactive was lower when compared with EOLS (Figure 2B). The ATCC standard strain showed a halo of

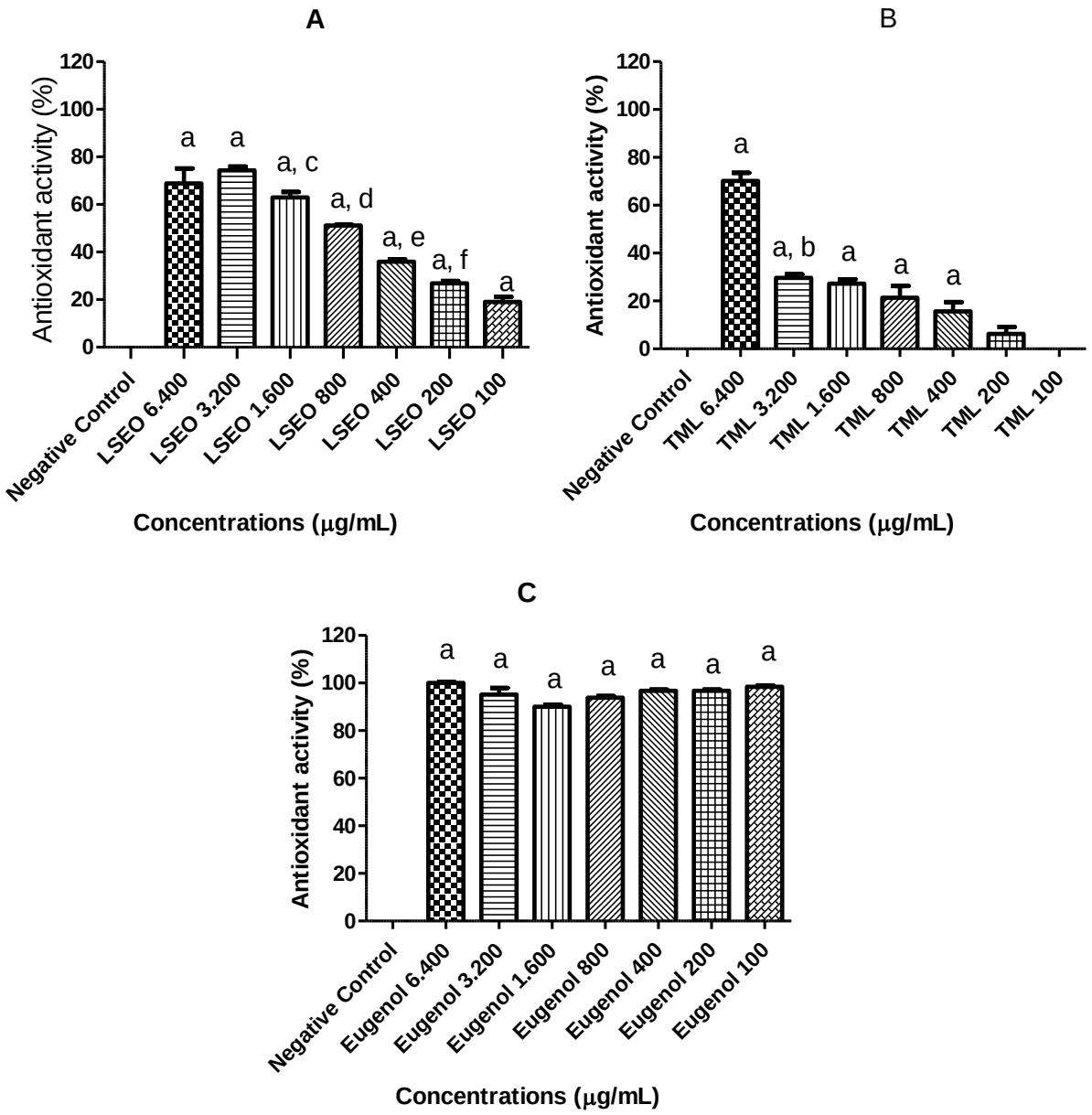


Figure 1. Percentage of antioxidant activity of essential oil of *Lippia sidoides* (EOLS), Thymol (TML) and Eugenol using DPPH radical scavenging assay. (a) Percentage of antioxidant activity of different concentrations of EOLS (10, 100, 200, 400, 800, 1.600, 3.200 and 6.400 µg/mL); (b) Percentage of antioxidant activity of different concentrations of TML (10, 100, 200, 400, 800, 1.600, 3.200 and 6.400 µg/mL); (c) Percentage of antioxidant activity of different concentrations of Eugenol (10, 100, 200, 400, 800, 1.600, 3.200 and 6.400 µg g/mL). ANOVA followed by Student-Newman-Keuls as the *post hoc* test. [a]p<0.05 vs. negative control; [b]p<0.05 vs. 6.400; [c]p<0.05 vs 3.200; [d]p<0.05 vs. 1.600 and [e]p<0.05 vs. 800

24.5 mm and a variation of 17.5 (S16) to 45 mm (S102) was observed between the halo sizes in resistant strains. Significant variation ($p < 0.05$) was observed between the halo size of the standard strain and the halos of eight of the resistant strains.

Antibacterial activity of meropenem, as a positive control, is shown in Figure 2C and all strains were sensitive to this carbepenem. 39.3 mm of halo was observed when ATCC strain was used and halos ranging from 24.9 (S82) to 48.3 (S102) when tested resistant strains were used. Significant variation ($p < 0.05$) was observed between the halo size of the standard strain and the halos of all strains.

DISCUSSION

The occurrence of thymol (79.7%) as the major

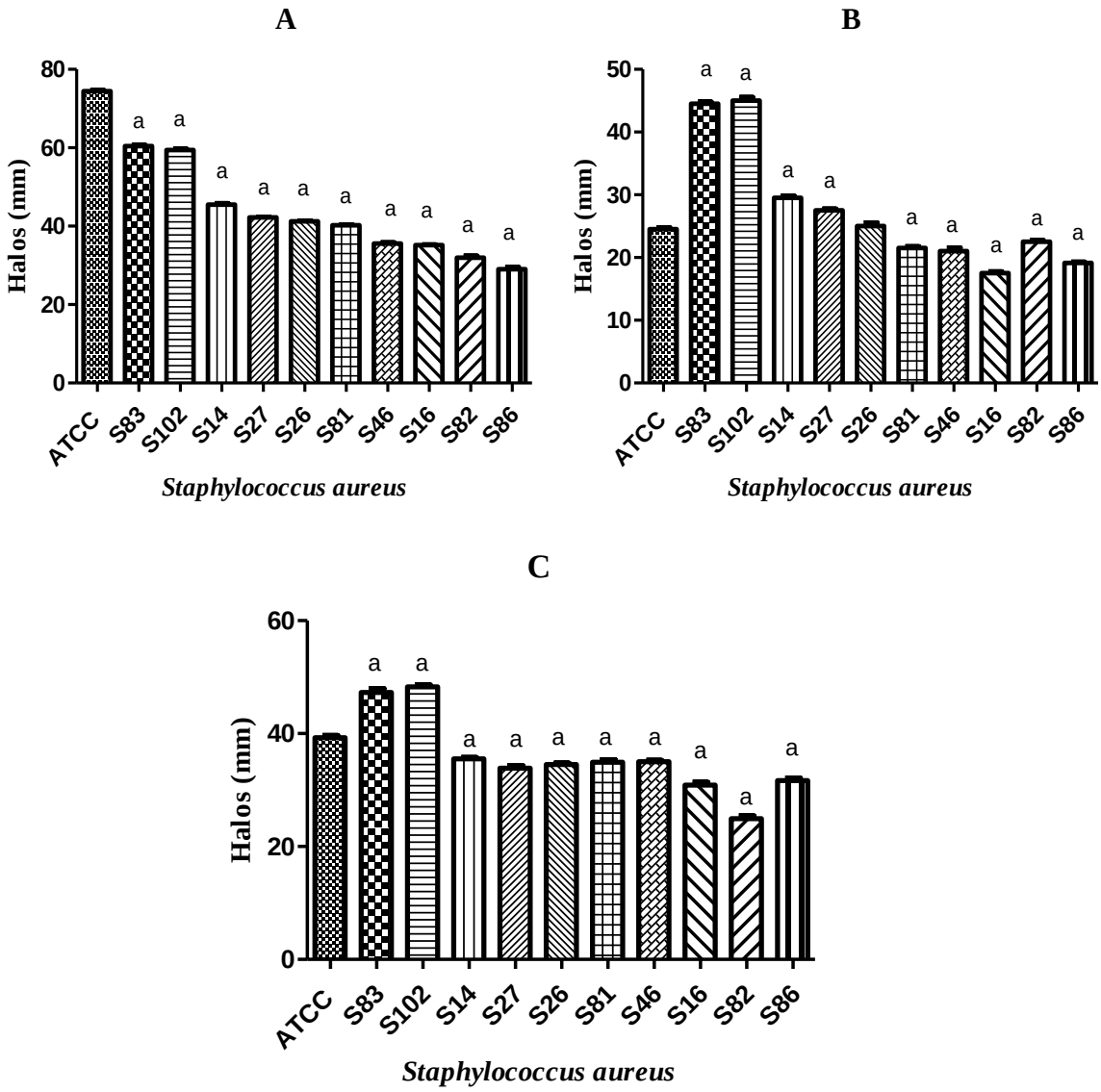


Figure 2. Antibacterial activity of essential oil *Lippia sidoides* (EOLS), Thymol (TML) and Meropenem using agar disc-diffusion assay. (a) Antibacterial activity of essential oil *L. sidoides* (EOLS) against *S. aureus* ATCC 25923 and *S. aureus* drug-resistant strains; (b) Antibacterial activity of Thymol against *S. aureus* ATCC 25923 and *S. aureus* drug-resistant strains; (c) Antibacterial activity of Meropenem against *S. aureus* ATCC 25923 and *S. aureus* drug-resistant strains. ANOVA followed by Student-Newman-Keuls as the *post hoc* test. [a]$p<0.05$ vs. *S. aureus* ATCC 25923.

constituent of EOLS observed in the present study (Table 1) is in accordance with literature reports (Guimarães et al., 2015). In this context, thymol (2-isopropyl-5-methylphenol) is considered the main monoterpene phenol in essential oils isolated from plants belonging to the Verbenaceae family, including *L. sidoides* (Marchese et al., 2016).

Veras et al. (2012) evaluated the chemical composition of EOLS by the GC-MS analysis and revealed the following main constituents: thymol (84.9%) and p-cymene (5.33%). In addition, 12 compounds were characterized by Botelho et al. (2007) in EOLS, having the major constituents: thymol (56.7%) and carvacrol (16.7%). Monoterpenes and sesquiterpenes were well represented in the chemical composition of EOLS characterized by Mota et al. (2012).

On the other hand, isoborneol, bornyl acetate, *α*-humulene, *α*-fenchene and 1.8-cineole have already been reported as the major components of the EOLS. This fact suggests the existence of two chemotypes of this species (Morais et al., 2012, 2016).

Difference in the chemical composition of EOLS (Table 1) with the essential oils described above may be related to the culture conditions of *L. sidoides* specimens.

For Castro et al. (2011), the chemical composition of essential oils may be influenced by climate, season, geographical conditions, harvest time and distillation technique. The antioxidant activity of species of the genus *Lippia* has been reported (Fabri et al., 2011). These authors reported that the AA of methanolic extracts of the *L. sidoides* leaves is related to the presence of flavonoids and coumarins.

In the present study, AA EOLS is not related only to the presence of thymol, since AA above 70% at concentrations of 6.400 and 3.200 of LSOE was verified (Figure 1A), and there was a drastic reduction of thymol AA in the concentration of 3.200 (29.65%) (Figure 1B). Thus, EOLS AA can be attributed to the presence of thymol and other polar phenolic compounds (Damasceno et al., 2011). Due to the antioxidant activity of naturally occurring substances in plants, attention on the protective activity of these natural antioxidants against chronic disorders caused by oxidative process has increased (Garcia et al., 2012).

The results of the present study also indicated bacterial activity (Figure 2). It is important to note that inhibition halos for EOLS were higher than those formed by thymol for all tested strains (Figure 2A and B), showing that the EOLS (mixture of 15 substances) may present different mechanisms of action with a substance potentiating the action of another. The consequence of this possible event is a greater activity of the essential oil as compared to the activity of its component individually tested. In accordance with the current data, the microbicidal action of essential oil of *L. sidoides* against *S. aureus* isolated from food (homemade cheese) was reported (Castro et al., 2011). This fact highlights the potential of this essential oil against foodborne pathogens.

In the present study, EOLS was able to inhibit the growth of all drug-resistant strains (Figure 1A). Oliveira et al. (2006) observed the action of EOLS against *S. aureus* from clinical material resistant to beta-lactams, aminoglycosides, macrolides, licosamides and quinolones, with inhibition halos varying from 15 to 21 mm. EOLS was more efficient than meropenem (positive control) when the size of the inhibition halos was observed (Figure 2A and C). This data is important and reveals the potential of EOLS, since meropenem is considered as a carbepenem, which is a drug commonly used and relatively resistant to hydrolysis by most β-lactamases, target penicillin-binding proteins, and generally have broad-spectrum antibacterial effect (El-Gamal et al., 2017).

The major constituent of EOLS, thymol, also had an effect on the ATCC and drug-resistant strains (Figure 2B). The effect of safe plant metabolites, thymol, on regulation of human pathogenic growth has been reported (Gutierrez et al., 2017). As in the current study, thymol was bioactive against *S. aureus* isolated from dairy and meat products, which would be of interest for use in the food industry (Rúa et al., 2011).

Thymol is a natural ingredient used as flavor or preservative agent in food products. This monoterpene, in higher concentrations, disrupted *S. aureus* cell membrane integrity and reduced cell viability. Furthermore, it induced a mild destabilization in the DNA secondary structure. Thus, antibacterial effect of thymol is related to destruction of bacterial cell membrane and binding directly to genomic DNA (Wang et al., 2017).

The data show a promising effect of thymol on drug-resistant strains (Figure 2B). Miladi et al. (2016) reported that thymol may serve as potential source of efflux pump inhibitor in food-borne pathogens (Miladi et al., 2016). Plant-derived antibacterial compounds may be of value as a novel means for controlling antibiotic-resistant bacteria in food animals and their products. It has been reported that thymol is effective against *S. aureus* blaZ and is able to reduce the minimum inhibitory concentration of ampicillin, penicillin and bacitracin (Palaniappan and Holley, 2010).

Conclusion

The results of this study suggest that EOLS, as compared to thymol, presents better potential for use as an antimicrobial and antioxidant agent. In addition, this essential oil has a promising antimicrobial effect against beta-lactam, tetracycline and ciprofloxacin-resistant *S. aureus*, making it of interest for application as a preservative in the food industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Adams RP (2007) Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. 4th edn. Allured Publ. Corp., Carol Stream, IL.

Botelho MA, Nogueira NA, Bastos GM, Fonseca SG, Lemos TL, Matos FJ, Montenegro D, Heukelbach J, Rao VS, Brito GA (2007). Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. Braz. J. Med. Biol. Res. 40:349-356.

Brand-Williams W, Cuvelier ME, Berset C (1995). Use of a free radical method to evaluate antioxidant activity. LWT Food Science and Technology. 28:25-30.

Castro CE, Ribeiro JM, Diniz TT, Almeida AC, Ferreira LC, Martins ER, Duarte ER (2011). Antimicrobial activity of *Lippia sidoides* Cham. (Verbenaceae) essential oil against *Staphylococcus aureus* and *Escherichia coli*. Rev. Bras. Plantas Med. 13:293-297.

Clinical and Laboratory Standards Institute (CLSI) (2012). Performance standards for antimicrobial disk susceptibility tests; approved standard – eleventh edition. CLSI document M02-A10. Wayne, PA.

Damasceno EIT, Silva JKR, Andrade EHA, Sousa PJC, Maia JGS (2011). Antioxidant capacity and larvicidal activity of essential oil and extracts from *Lippia grandis*. Rev. Bras. Farmacogn. 21:78-85.

El-Gamal MI, Brahim I, Hisham N, Aladdin R, Mohammed H, Bahaaeldin A (2017). Recent updates of carbapenem antibiotics. Eur. J. Med. Chem. 131:185-195.

Fabri RL, Nogueira MS, Moreira J dos R, Bouzada ML, Scio E (2011). Identification of antioxidant and antimicrobial compounds of *Lippia* species by bioautography. J. Med. Food. 14:840-846.

Farias-Junior PA, Rios MC, Moura TA, Almeida RP, Alves PB, Blank AF, Fernandes RP, Scher R (2012). *Leishmanicidal activity of carvacrol-rich essential oil from* Lippia sidoides *Cham*. Biol. Res. 45:399-402.

Garcia EJ, Oldoni TLC, Alencar SM de, Reis A, Loguercio AD, Grande RHM (2012). Antioxidant activity by DPPH assay of potential solutions to be applied on bleached teeth. Braz. Dent. J. 23:22-27.

Ge B, Mukherjee S, Hsu CH, Davis JA, Tran TT, Yang Q, Abbott JW, Ayers SL, Young SR, Crarey ET, Womack NA, Zhao S, McDermott PF (2017). MRSA and multidrug-resistant *Staphylococcus aureus* in U.S. retail meats, 2010-2011. Food Microbiol. 62:289-297.

Gomes GA, Monteiro CM, Julião L de S, Maturano R, Senra TO, Zeringóta V, Calmon F, Matos R da S, Daemon E, Carvalho MG (2014). Acaricidal activity of essential oil from *Lippia sidoides* on unengorged larvae and nymphs of *Rhipicephalus sanguineus* (Acari: Ixodidae) and *Amblyomma cajennense* (Acari: Ixodidae). Exp. Parasitol. 137:41-45.

Guimarães LG de L, Silva ML da, Reis PC, Costa MT, Alves LL (2015). General characteristics, phytochemistry and pharmacognosy of *Lippia sidoides*. Nat. Prod. Commun. 10:1861-1867.

Gutiérrez S, Morán A, Martínez-Blanco H, Ferrero MA, Rodríguez-Aparicio LB (2017). The usefulness of non-toxic plant metabolites in the control of bacterial proliferation. Probiotics Antimicrob. Proteins. doi: 10.1007/s12602-017-9259-9.

Li L, Ye L, Yu L, Zhou C, Meng H (2016). Characterization of extended spectrum b-lactamase producing Enterobacteria and Methicillin-Resistant *Staphylococcus aureus* isolated from raw pork and cooked pork products in South China. J. Food. Sci. 81:M1773-7.

Marchese A, Orhan IE, Daglia M, Barbieri R, Di Lorenzo A, Nabavi SF, Gortzi O, Izadi M, Nabavi SM (2016). Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chem. 210:402-414.

Miladi H, Zmantar T, Chaabouni Y, Fedhila K, Bakhrouf A, Mahdouani K, Chaieb K (2016). Antibacterial and efflux pump inhibitors of thymol and carvacrol against food-borne pathogens. Microb. Pathog. 99:95-100.

Morais SR de, Oliveira TL, Bara MT, Conceição EC da, Rezende MH, Ferri PH, Paula JR de (2012). Chemical constituents of essential oil from *Lippia sidoides* Cham. (Verbenaceae) leaves cultivated in Hidrolândia, Goiás, Brazil. Int. J. Anal. Chem. 2012:363919.

Morais SR de, Oliveira TL, Oliveira LP de, Tresvenzol LM, Conceição EC da, Rezende MH, Fiuza TS, Costa EA, Ferri PH, Paula JR de (2016). Essential oil composition, antimicrobial and pharmacological activities of *Lippia sidoides* Cham. (Verbenaceae) from São Gonçalo do Abaeté, Minas Gerais, Brazil. Pharmacogn. Mag. 12: 262-270.

Mota KL, Lobo LT, Costa JM, Costa LS, Rocha HA, Rocha e Silva LF, Pohlit AM, Neto VF (2012). *In vitro* and *in vivo* antimalarial activity of essential oils and chemical components from three medicinal plants found in northeastern Brazil. Planta Med. 78:658-664.

Oliveira FP de, Lima E de O, Siqueira Júnior JP de, Souza EL de, Santos BHC, Barreto HM (2006). Effectiveness of *Lippia sidoides* Cham. (Verbenaceae) essential oil in inhibiting the growth of *Staphylococcus aureus* strains isolated from clinical material. Rev. Bras. Farmacogn. 16:510-514.

Palaniappan K, Holley RA (2010). Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. Int. J. Food Microb. 140:164-168.

Rasooly R, Do PM, Hernlem BJ (2017). Rapid Cell-Based assay for detection and quantification of active staphylococcal enterotoxin type D. J. Food. Sci. 82:718-723.

Rúa J, Fernández-Álvarez L, Castro C de, Del Valle P, Arriaga D de, García-Armesto MR (2011). Antibacterial activity against foodborne Staphylococcus aureus and antioxidant capacity of various pure phenolic compounds. Foodborne Pathog. Dis. 8:149-157.

Santos IG, Scher R, Rott MB, Menezes LR, Costa EV, Cavalcanti SC, Blank AF, Aguiar J dos S, Silva TG da, Dolabella SS (2016). Amebicidal activity of the essential oils of *Lippia* spp. (Verbenaceae) against *Acanthamoeba polyphaga* trophozoites. Parasitol. Res. 115:535-540.

Silva DT, Silva LL, Amaral LP, Pinheiro CG, Pires MM, Schindler B, Garlet QI, Benovit SC, Baldisserotto B, Longhi SJ, Kotzian CB, Heinzmann BM (2014). Larvicidal activity of Brazilian plant essential oils against Coenagrionidae larvae. J. Econ. Entomol. 107:1713-1720.

Van Den Dool H, Kratz PD (1963). A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. J. Chromatogr. 11:463-471.

Veras HN, Rodrigues FF, Colares AV, Menezes IR, Coutinho HD, Botelho MA, Costa JG (2012). Synergistic antibiotic activity of volatile compounds from the essential oil of *Lippia sidoides* and thymol. Fitoterapia. 83:508-512.

Wang LH, Zhang ZH, Zeng XA, Gong DM, Wang MS (2017). Combination of microbiological, spectroscopic and molecular docking techniques to study the antibacterial mechanism of thymol against *Staphylococcus aureus*: membrane damage and genomic DNA binding. Anal. Bioanal. Chem. 409:1615-1625.

academicJournals

Vol. 17(8), pp. 239-248, 21 February, 2018
DOI: 10.5897/AJB2017.16359
Article Number: 68CE78656049
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Chlorella vulgaris DPSF 01: A unique tool for removal of toxic chemicals from tannery wastewater

P. S. Subashini and P. Rajiv*

Department of Biotechnology, Karpagam Academy of Higher Education, Coimbatore, India.

Received 7 December, 2017; Accepted 25 January, 2018

The present study investigated the removal of toxic pollutants and reduction of heavy metals from tannery wastewater using *Chlorella vulgaris*. The physiochemical parameters like pH, electrical conductivity (EC), biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), total dissolved solids (TDS), chloride, total hardness (TH), bicarbonate, magnesium, ammoniacal nitrogen, phosphate and heavy metals (Cu, Cr, Zn, Ni and Fe) were analysed using standard methods. Functional group of toxic chemicals in tannery wastewater and *C. vulgaris* treated wastewater were analysed by Fourier transforms infrared spectroscopy (FT-IR) and gas chromatography – mass spectrometry (GC-MS). 20 to 60% of chemicals (bicarbonate, chloride, nitrogen, phosphate and magnesium) were reduced by the treatment using *C. vulgaris* within 28 days. FT-IR and GC-MS analysis reveals that the functional group of azo compounds was not in *C. vulgaris* treated wastewater. Thus, the results obtained conclude that *C. vulgaris* can be used as a suitable tool for the removal of toxic chemicals of tannery wastewater.

Key words: *Chlorella vulgaris*, toxic chemicals, tannery wastewater, physio-chemicals, Fourier Transforms Infrared Spectroscopy (FT-IR), Gas Chromatography – Mass Spectrometry (GC-MS).

INTRODUCTION

Nowadays, industries are releasing huge amount of wastewater without treatment and causing major water pollution and diseases. There are so many conventional methods such as chemical (chlorination) and physical (sedimentation process) available for wastewater treatments but having drawbacks (Suresh et al., 2015). India is ranked third in leather production in the world and 88% of tannery industries are in Tamilnadu, Uttar Pradesh and West Bengal. The maximum tannery industries are located near river basins in Tamilnadu. During leather production, there are various toxic chemicals that are used and wastewater is directly discharged into rivers without treatment. The largest organic and inorganic pollutants present in the urban and rural wastewater is due to industrial and anthropological activities (Bernhardt et al., 2008).

The biotechnological based treatments are useful for overcoming these problems. Bioremediation is a

*Corresponding author. E-mail: rajivsmart15@gmail.com. Tel/Fax: +914222611146.

worldwide acceptable technology in wastewater treatment. In bioremediation, bacteria, fungi and algae have been used. Algae are the best because it is less expensive and potential source of wastewater treatment compared to bacteria and fungi (Sheehan et al., 1998).

Microalgae are universally acknowledged in the purification of wastewater (Ayodhaya, 2013). Removal of organic and inorganic pollutants from wastewater by algae is known as phycoremediation. Phycoremediation is safe, efficient and eco-friendly for the removal of toxic materials including heavy metal from wastewater (Ding et al., 2014). The biomass of algae can be used for bio-fuel production after the wastewater treatment (Yadavalli et al., 2014). Various microalgae are used in the treatment of wastewater including *Scenedesmus* spp. (Ajayan and Selvaraju, 2012), *Chlorella marina* (Chellam and Sampathkumar, 2012), *Chlorella vulgaris* (Chu et al., 2008), *Chlamydomonas* and *Digdigma proteus* (Rehman et al., 2007), *Oscillatoria*, *Ulotrix* and *Phormodium* (Rai et al., 2005; Balaji et al., 2015).

This work aimed at evaluating the growth of *C. vulgaris* in tannery wastewater and its efficiency in reducing the pollution load of wastewater by examining the pH, EC, BOD, COD, TS, TDS, Chloride, TH, bicarbonate, magnesium, ammoniacal nitrogen, phosphate and heavy metals (Cr, Cu, Fe, Zn and Ni) of tannery wastewater before and after treatment.

MATERIALS AND METHODS

Collection of tannery wastewater and analysis of physio-chemical parameters

Tannery wastewater was collected from the wastewater outlet of tannery industries located in the Erode (Latitude - 11.3410° N, Longitude - 77.7172° E) District, Tamilnadu, India. The collected samples were stored in sterile polythene bottles at 4°C. The physio-chemical parameters of tannery wastewater such as pH, electrical conductivity, biological oxygen demand, chemical oxygen demand, total solids, total dissolved solids, chloride, total hardness, bicarbonate, magnesium, ammoniacal nitrogen and phosphate and heavy metals (Cr, Cu, Fe, Zn and Ni) were assessed using standard methods (Clesceri et al., 1989).

Collection of microalgae and chemicals

C. vulgaris DPSF01 was collected from the Department of Marine Science, Bharathidasan University, Tiruchirappalli, Tamil nadu, India. It was grown in the Bold's Basal Medium (BBM) at 20 to 23°C under fluorescent lights (with 12 h light: 12 h dark photoperiods) (Nichols, 1973). The chemicals used for the preparation of media were purchased from MERCK, Mumbai, India.

Phycoremediation

The experimental designs were T_1 (100% Raw tannery wastewater), T_2 (75% of tannery wastewater diluted with tap water), T_3 (60% of tannery wastewater diluted with tap water), T_4 (45% of tannery wastewater diluted with tap water), T_5 (30% of tannery wastewater diluted with tap water) and T_6 (15% of tannery wastewater diluted with tap water) (Cindrella et al., 2016). The culture of *C. vulgaris* was centrifuged at 10,000 rpm for 10 min and the supernatant was removed. The pellet of the algal cells were washed with sterile water and resuspended to inoculate into respective dilution. The density of *C. vulgaris* was about 30×10^3 cells/mL. The culture was grown for 28 days at a constant temperature of 15 to 20°C with the photoperiod of 12 h light and 12 h dark. At different time intervals (7^{th} day, 14^{th} day, 21^{st} day and 28^{th} day) the samples were collected and stored for further analysis (Ajayan and Selvaraju, 2011).

Analysis of algal growth

The algal growth was indirectly analysed by algal cell count method and estimated using hemocytometer during the treatment of tannery wastewater at different intervals (7^{th} day, 14^{th} day, 21^{st} day and 28^{th} day) according to Lenore (1998).

Estimation of chlorophyll

Chlorophyll (*a* and *b*) was estimated according to Arnon (1949). 20 mL of the culture was centrifuged at 10,000 rpm for 10 min. The collected pellet was mixed with 90% acetone. The mixture was centrifuged at 5000 rpm for 10 min; the absorbance value of supernatant was measured using UV-spectrometer (UV–2450, Shimadzu) at 663 nm (Chlorophyll *a*) and 645 nm (Chlorophyll *b*).

Analysis of physio-chemical properties

20 mL of sample was taken and centrifuged at 10,000 rpm for 20 min; the pellet was discarded and the pH of supernatant analysed by pH meter (ELICO Model-107). The electrical conductivity of supernatant was assessed by digital EC meter (ELICO Model-180) (Lauber et al., 2009).

The assessment of pH, EC, BOD, COD, TS, TDS, Chloride, Total Hardness, Bicarbonate, Magnesium, Ammoniacal nitrogen and Phosphate were followed by APHA (1989) method. The assessments were determined on seven days interval from 1^{st} day to 28^{th} day. Heavy metals (Zn, Cu, Fe, Ni and Cr) were assessed at different time intervals using Atomic Absorption Spectrophotometer (1983-400 HGA 900/AS 800 Perkin Elmer) and multi-Element Standard (MERCK-112837) (Fraile et al., 2005).

Statistical analysis

Experiments were carried out with three replications. Results are represented with means ± standard errors for three independent experiments.

FTIR and GC-MS analysis

The functional groups of toxic chemicals from tannery wastewater before and after treatment were analysed by Fourier Transforms Infrared Spectroscopy (Perkin-Elmer 1725_X). The treated and untreated wastewater were dissolved in methanol-water (9:1) (v/v) and kept in a shaker overnight at room temperature. After the incubation period, the sample was filtered by using filter paper (Whatman No. 42, Maidstone, England). The filtrate was dried in

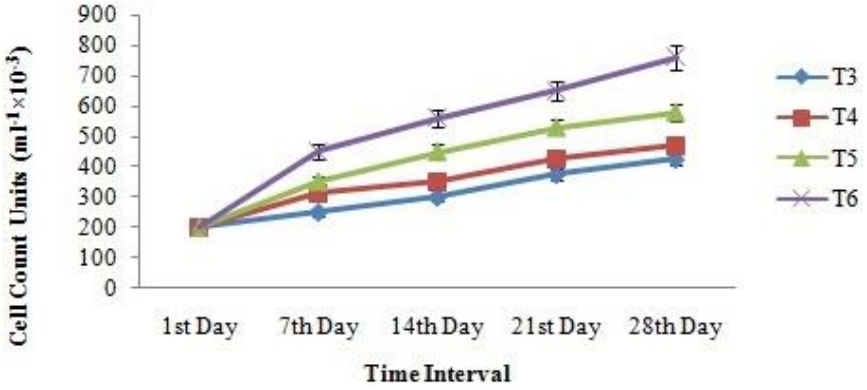


Figure 1. Cell count of *Chlorella vulgaris* on different concentration of tannery wastewater.

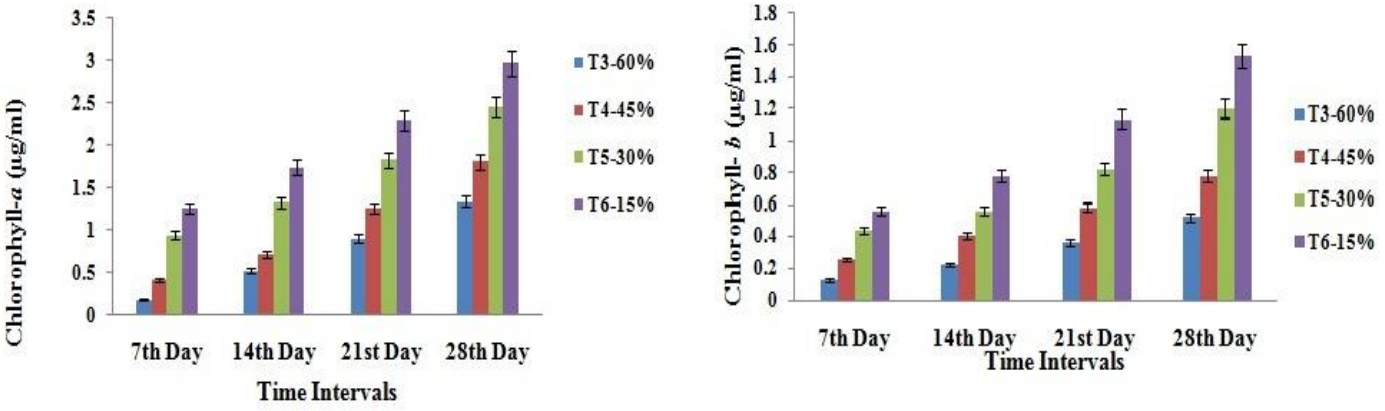


Figure 2. Analysis of chlorophyll-*a* and *b* of *Chlorella vulgaris* on different concentration of tannery wastewater.

hot air oven and the pellet was collected. The samples were analysed under the wavelength ranging between 400–4000 cm^{-1} (Kishore et al., 2015). Degradation of azo compounds was determined by GC-MS Thermo MS DSQ II, gas carrier helium (1.0 mL/min), capillary column (Ajay Kumar Pandey and Vinay Dubey, 2012).

RESULTS AND DISCUSSION

Measuring the algal growth

The maximum cell growth was found to be 760 × 10^3 cells mL^{-1} in T_6 on the 28th day whereas the lower growth was observed in T_3 and T_4 (Figure 1). The maximum growth of micro-algae in T_5 and T_6 treatment was due to heavy metal resistant mechanism and highest dilution of tannery wastewater (Rehman, 2011). The micro-algae were unable to grow in T_1 and T_2 treatments due to high amount of heavy metals and lower dilution of tannery wastewater (Ajayan et al., 2015).

Estimation of chlorophyll

The yield of chlorophyll '*a'* and '*b'* were high in T_5 and T_6 treatments on 28th day (Figure 2a and b). The Chlorophyll '*a'* reached a maximum level of 2.97 µg/mL in T_6 whereas T_5 had 2.46 µg/mL on 28th day. Chlorophyll *b* was 1.54 and 1.20 µg/mL in T_6 and T_5, respectively. The present study proves that *C. vulgaris* was able to decolourise the tannery wastewater by dominant production of chlorophyll *a* and *b* (Hanumantha et al., 2011).

Physio-chemical analysis of treated and untreated tannery wastewater

Tables 1 and 2 present the physio-chemical parameters

Table 1. Physio-chemical properties of tannery wastewater before and after treatment.

S/N	Parameters	Raw Effluent	Treated Effluent (15% dilution)
1	pH	5.5±0.3	7.78±0.20
2	EC (dsm^{-1})	13.01±0.4	2.19±0.16
3	Biological Oxygen Demand (mg L^{-1})	1560±2.64	333±4.58
4	Chemical Oxygen Demand (mg L^{-1})	2920±3.60	1314±10.39
5	Total Solids (mg L^{-1})	7152±5.03	2578±14.15
6	Total Dissolved Solids (mg L^{-1})	6370±2.88	2348±7.83
7	Chloride (mg L^{-1})	590±5.23	180±6.69
8	Total hardness (mg L^{-1})	1288±1.52	428±8.25
9	Bicarbonate (mg L^{-1})	750±5.29	177±7.26
10	Magnesium (mg L^{-1})	54±2.96	26±4.35
11	Ammoniacal Nitrogen (mg L^{-1})	17±4.48	8.12±0.60
12	Phosphate (mg L^{-1})	18±2.34	10.68±1.63

of raw tannery wastewater and algal treated tannery wastewater respectively. The pH of the tannery wastewater increased from 5.5 to 7.78 in all the treatments (T_3, T_4, T_5 and T_6). Due to the mechanism of photosynthesis, the microalgae reduced the concentration of dissolved CO_2 hence the pH of tannery wastewater rose from acidic to alkaline on treatment (Borowitzka, 1998). The electrical conductivity of tannery wastewater reduced (from 11.51 to 2.19 dSm^{-1}) after the treatment. The reduction of BOD (1560 to 333 mg L^{-1}) and COD (2920 to 1314 mg L^{-1}) in *C. vulgaris* treated wastewater confirms the carbon dioxide sequestration. Balakumar et al. (2014) reported the carbon dioxide sequestration and reduction of green house gases from tannery wastewater using algal biomass. High amounts of xenobiotics compounds contribute in increasing the COD, which was reduced to about 90% by *Chlorella* (Sharma and Khan, 2013). The reduction of total solids (7152 to 2578 mg L^{-1}) and total dissolved solids (6370 to 2348 mg L^{-1}) during the treatment increases the cell count of *C. vulgaris*.

Algae are able to reduce TDS to base level by the mechanism of biosorption and adsorption (Nandha et al., 2010). The lowest total hardness was found in T_6 treatment (428 mg L^{-1}) on the 28th day while the 10 to 50% of total hardness were reduced by the treatment of *C. vulgaris* in tannery wastewater. Similar results were observed in lake and pond water treatment using *Chlorococcum humicola* (Sivasubramanian et al., 2012). The other chemical constituents (bicarbonate, chloride and magnesium) of tannery wastewater decreased from the 7th to the 28th day using the cultivation of *C. vulgaris*. The bicarbonate (177 mg L^{-1}), chloride (180 mg L^{-1}) and magnesium (26 mg L^{-1}) were very low on 28th day, because of the utilization of nutrients by *C. vulgaris* for their growth.

50% of ammoniacal nitrogen was removed by the *C. vulgaris* which determines the denitrification and nitrification process by micro-algae (Durai and Rajasimman, 2011). Phosphate has been used for the production of ATP, phospholipids and nucleic acid hence phosphate was reduced (18 to 10.68 mg L^{-1}) by the treatment of *C. vulgaris* in tannery wastewater (Becker, 1994).

Efficiency of *C. vulgaris* on heavy metals removal

Table 3 shows the reduction of heavy metals from tannery wastewater by *C. vulgaris* during the treatment process. Heavy metals (71% of Copper, 50% of Zinc, 45% of Iron, 40% of Chromium and 20% of Nickel) were reduced by *C. vulgaris*. It shows that *C. vulgaris* is resistant to the toxicity of heavy metals in tannery wastewater. After 28 days, the reduction of heavy metals were in the following order; Copper > Zinc > Iron > Chromium > Nickel. Mehta and Gaur (2005) indicated the removal of heavy metals from wastewater by pre-treatment of algae using $CaCl_2$. The uptake of nickel by algae was stimulated by copper ions due to similar ionic properties and increased permeability of plasma membrane (Mehta et al., 2000). 50% of zinc metal was removed by *C. vulgaris* in tannery wastewater. Similar results were reported by Dinesh Kumar et al. (2015). Chromium is predominantly present in tannery wastewater and 40% of chromium was removed by *C. vulgaris.* Hammouda et al. (2015) showed that 56.3% of chromium was removed by *Chlorella* when tannery wastewater was mixed with domestic wastewater.

FTIR analysis of treated and untreated tannery wastewater

FTIR spectrum of raw and treated tannery wastewater

Table 2. Evaluation of physio-chemical parameters during phycoremediation of tannery wastewater.

Treatments	1st day	7th day	14th day	21st day	28th day
Physical parameter					
pH					
Control	5.5±0.3	5.5±0.1	5.5±0.12	5.5±0.8	5.5±0.7
T_3 (60%)	6±0.05	6.15±0.08	6.22±0.10	6.28±0.12	6.36±0.15
T_4 (45%)	6.32±0.02	6.40±0.08	6.47±0.10	6.55±0.12	6.64±0.18
T_5 (30%)	7±0.03	7.17±0.05	7.25±0.08	7.27±0.10	7.30±0.15
T_6 (15%)	7.43±0.05	7.48±0.10	7.58±0.12	7.65±0.15	7.78±0.20
Electrical conductivity (dSm^{-1})					
Control	13.01±0.4	13.01±0.2	13.01±0.4	13.01±0.3	13.01±0.6
T_3 (60%)	11.51±0.10	10.91±0.15	10.11±0.12	9.89±0.08	9.55±0.15
T_4 (45%)	9.49±0.09	8.78±0.13	8.22±0.09	7.47±0.10	6.71±0.11
T_5 (30%)	7.46±0.07	6.84±0.14	6.27±0.08	5.45±0.14	4.57±0.16
T_6 (15%)	5.33±0.15	4.65±0.13	3.92±0.15	3.05±0.14	2.19±0.16
Chemical parameter					
Biological oxygen demand (mg L^{-1})					
Control	1560±2.64	1560±2.88	1560±1.15	1560±1.73	1560±2.64
T_3 (60%)	1535±2.88	1473±2.51	1405±6.65	1336±7.93	1266±7.57
T_4 (45%)	1467±7.23	1335±6.35	1210±11.26	1080±10	954±11.37
T_5 (30%)	1379±8.14	1194±12.49	1014±3.21	836±5.29	647±8.14
T_6 (15%)	1251±11.15	1015±8.73	787±11.93	562±15.39	333±4.58
Chemical oxygen demand (mg L^{-1})					
Control	2920±3.60	2920±4.04	2920±4.72	2920±2.00	2920±6.88
T_3 (60%)	2887±8.11	2815±10.11	2744±6.08	2671±10.14	2602±13.89
T_4 (45%)	2822±8.08	2685±13.69	2543±8.14	2407±5.81	2265±6.24
T_5 (30%)	2705±8.73	2507±10.58	2301±10.97	2075±13.22	1839±9.13
T_6 (15%)	2527±9.52	2235±7.37	1922±8.62	1617±10.92	1314±10.39
Total solids (mg L^{-1})					
Control	7152±5.03	7152±5.50	7152±3	7152±3.84	7152±5.78
T_3 (60%)	6814±8.83	6356±11.05	5846±7.05	5328±6.38	4746±4.33
T_4 (45%)	6410±8.95	5858±4.09	5290±6.42	4737±9.24	4152±7.02
T_5 (30%)	5937±5.85	5304±11.56	4662±6.69	3995±4.91	3345±12.53
T_6 (15%)	5399±5.20	4673±11.93	3967±11.66	3266±7.93	2578±14.15
Total dissolved solids (mg L^{-1})					
Control	6370±2.88	6370±3.38	6370±4.37	6370±3.51	6370±5.29
T_3 (60%)	6058±3.71	5530±2.51	5047±3.75	4527±2.40	4014±7.12
T_4 (45%)	5636±5.85	5145±7.68	4647±5.60	4141±6.65	3605±5.45
T_5 (30%)	5068±4.97	4553±5.60	4067±6.00	3591±4.93	3104±7.21
T_6 (15%)	4399±7.05	3893±10.36	3411±6.65	2887±7.81	2348±7.83
Total hardness (mg L^{-1})					
Control	1288±1.52	1288±5.36	1288±3.60	1288±4.91	1288±5.81
T_3 (60%)	1265±4.84	1224±4.16	1186±4.33	1146±7.05	1106±3.92
T_4 (45%)	1179±4.58	1106±6.43	1037±6.17	969±5.81	900±4.61
T_5 (30%)	1050±5.77	958±7.05	866±8.08	774±4.35	675±6.11
T_6 (15%)	887±6.33	772±6.35	662±4.16	550±7.50	428±8.25

Table 2. Contd.

Chloride (mg L^{-1})					
Control	590±5.23	590±4.93	590±5.68	590±6.42	590±4.72
T_3 (60%)	578±8.71	566±8.71	551±5.56	538±2.51	524±7.37
T_4 (45%)	560±5.13	527±5.48	492±7.21	461±6.08	425±5.29
T_5 (30%)	535±4.58	476±4.91	426±7.75	368±5.77	311±8.38
T_6 (15%)	477±7.68	401±5.20	330±6.08	252±5.68	180±6.69
		Bicarbonate (mg L^{-1})			
Control	750±5.29	750±3.05	750±4.50	750±5.56	750±2.64
T_3 (60%)	735±5.50	715±7.68	687±7.57	661±6.65	634±4.40
T_4 (45%)	708±4.16	657±5.92	602±5.48	549±6.35	497±5.54
T_5 (30%)	643±6.35	568±5.36	495±4.09	423±5.68	348±8.08
T_6 (15%)	571±6.35	473±4.63	373±3.92	272±5.89	177±7.26
		Magnesium (mg L^{-1})			
Control	54±2.96	54±4.40	54±3.78	54±4.66	54±2.64
T_3 (60%)	53±4.93	51±4.35	48±2.60	44±4.05	41±3.78
T_4 (45%)	51±4.35	47±2.64	42±4.05	39±5.23	33±4.16
T_5 (30%)	49±2.60	43±3.48	36±3.60	31±4.04	24±4.16
T_6 (15%)	45±4.72	41±3.78	37±3.78	32±3.48	26±4.35
		Ammoniacal Nitrogen (mg L^{-1})			
Control	17±4.48	17±4.72	17±3.21	17±2.64	17±4.72
T_3 (60%)	16.5±3.06	15.62±2.98	14.39±2.34	12.5±2.01	10.48±0.36
T_4 (45%)	16.15±3.09	15.22±2.72	14.1±2.57	12.51±2.95	10.29±1.66
T_5 (30%)	16.02±2.59	14.33±2.04	12.78±1.95	11.03±1.31	9.37±1.76
T_6 (15%)	15.97±1.76	13.99±1.82	11.97±1.14	10.08±1.51	8.12±0.60
		Phosphate (mg L^{-1})			
Control	18±2.34	18±2.15	18±1.62	18±2.05	18±1.66
T_3 (60%)	17.80±1.56	17.55±0.86	17.02±1.92	16.92±1.17	16.51±3.28
T_4 (45%)	17±4.72	16.56±3.30	15.96±3.04	15.12±2.67	14.62±2.53
T_5 (30%)	16.01±3.11	15.18±2.71	14.26±2.61	13.29±1.70	12.67±3.11
T_6 (15%)	14.98±2.56	13.91±1.72	12.86±3.19	11.98±2.15	10.68±1.63

are shown in Figure 3a and b. A peak at 3510 cm^{-1} represents NH_2 group of aromatic amines. The region between 3420–3250 cm^{-1} indicates the presence of OH group of alcohols and phenols. A broad peak at 2250 cm^{-1} indicates C≡C of alkynes. The FTIR data of raw tannery wastewater (Figure 3a) shows the presence of azo group from the region between 1539 to 1580 cm^{-1}. The wave number 1315 cm^{-1} shows the presence of SO_2 in sulfones (Figure 3a).

The peak value between 3200 to 3600 cm^{-1} represents the stretching vibration of O-H and N-H group; the peak value 1258 cm^{-1} indicates the stretching of phosphodiester (>P=O) in nucleic acid of microalgae (Dilek et al., 2012); whereas the peak value of 2756 cm^{-1} indicates the stretching of OH group of carboxylic acid on treated water. A net negative charge formed by the carboxyl, hydroxyl, amino and sulphydryl groups on the cell surface confirms the high affinity for the binding of heavy metals (Deng et al., 2007; Gupta and Rastogi, 2008). The region between 1080 to 1040 cm^{-1} denotes the presence of SO_3H in sulfonic acid. Gardea-Torresdey et al. (1990) reported that the carboxyl group has a higher metal binding capacity followed by –OH, $–SO_3H$ and $–P_2O_3$.

In Figure 3b (treated tannery wastewater), there was no peak value between 1539– 1580 cm^{-1} indicating the absence of azo group. The comparative FTIR spectra analysis of treated and untreated tannery wastewater reveals that the absorption peak of azo compound groups was not present in the treated wastewater.

Table 3. Heavy metals analysis of raw and treated tannery wastewater.

Treatments	7th Day	14th Day	21st Day	28th Day
		Chromium (ppb)		
Control	98.69±4.85	98.69±4.20	98.69±3.30	98.69±4.96
T_3 (60%)	95.36±2.63	90.96±2.51	86.38±3.58	82.16±4.65
T_4 (45%)	92.56±2.77	86.93±1.65	81.16±1.23	75.66±3.64
T_5 (30%)	89.67±2.36	80.57±1.16	71.55±2.16	62.44±2.84
T_6 (15%)	85.35±3.19	74.23±2.20	66.02±3.65	51.86 ±1.35
		Copper (ppb)		
Control	70.61±2.69	70.61±2.21	70.61±2.50	70.61±1.06
T_3 (60%)	67.66±3.97	60.75±2.28	51.54±3.64	45.62±2.78
T_4 (45%)	64.70±2.43	52.23±2.05	44.36±2.38	38.27±3.86
T_5 (30%)	61.71±3.03	45.16±2.35	37.12±3.08	30.85±2.17
T_6 (15%)	58.79±2.97	35.18±1.35	30.83±2.15	20.68±0.99
		Iron (ppb)		
Control	66.32±1.76	66.32±2.43	66.32±2.49	66.32±2.53
T_3 (60%)	64.06±2.21	60.13±3.22	55.53±2.79	50.59±1.44
T_4 (45%)	61.96±2.36	57.03±3.05	51.33±3.11	43.67±1.47
T_5 (30%)	59.71±3.54	55.58±3.09	50.38±1.44	41.56±1.52
T_6 (15%)	57.25±3.10	50.51±2.50	44.27±2.31	35.52±2.24
		Zinc (ppb)		
Control	36.34±2.60	36.34±2.56	36.34±3.03	36.34±2.85
T_3 (60%)	35.22±3.10	34.10±2.28	31.26±1.42	28.66±1.77
T_4 (45%)	33.10±2.07	32.06±1.73	27.35±2.04	25.38±2.12
T_5 (30%)	34.32±2.27	33.32±1.29	25.32±2.13	21.26±1.40
T_6 (15%)	32.78±2.13	29.12±1.56	23.79±2.45	18.48±1.68
		Nickel (ppb)		
Control	12.86±1.38	12.86±1.10	12.86±0.62	12.86±0.52
T_3 (60%)	12.69±1.55	12.54±0.95	12.37±1.49	12.18±1.04
T_4 (45%)	12.50±1.08	12.16±1.46	11.80±1.30	11.46±1.35
T_5 (30%)	12.32±1.38	11.51±1.34	10.91±0.87	10.76±1.12
T_6 (15%)	12.17±1.04	11.52±1.34	10.82±1.13	10.12±1.44

Degradation of azo compounds by *C. vulgaris*

GCMS analysis confirmed the degradation of azo compounds by *C. vulgaris*. Figure 4a represents the GCMS analysis of raw tannery wastewater. Azo compounds such as 1H-1,2,4-triazole-3-yl-N-[2-(3-methylphenoxy)ethyl] carboxamide (Mol-wt–246; RT-30.99), 1,6-dihydroimidazo[4,5-d]imidazole (Mol-wt–108; RT-5.58) and 4-{4-(3,5-dimethylphenyl)-2-[4-(methylsulfonyl)phenyl]-1,3-thiazol-5-yl}-2,6-dimethylpyridine (Mol-wt–416; RT-31.45) were present in tannery wastewater. All the above compounds were absent in *C. vulgaris* treated wastewater (Figure 4b). The degradation of azo compounds by azo reductase in *C. vulgaris* was due to the breakage of N=N bond (Lin and Liu, 1992).

Conclusion

Results of this investigation concluded that in tannery wastewater, *C. vulgaris* has a remarkable potential to survive as well as uptakes nutrients and heavy metals from tannery wastewater. Appreciable reduction of BOD and COD in tannery wastewater provides a space for the survival of other aquatic organisms. The mechanism of adsorption of heavy metals and uptake of nutrients from

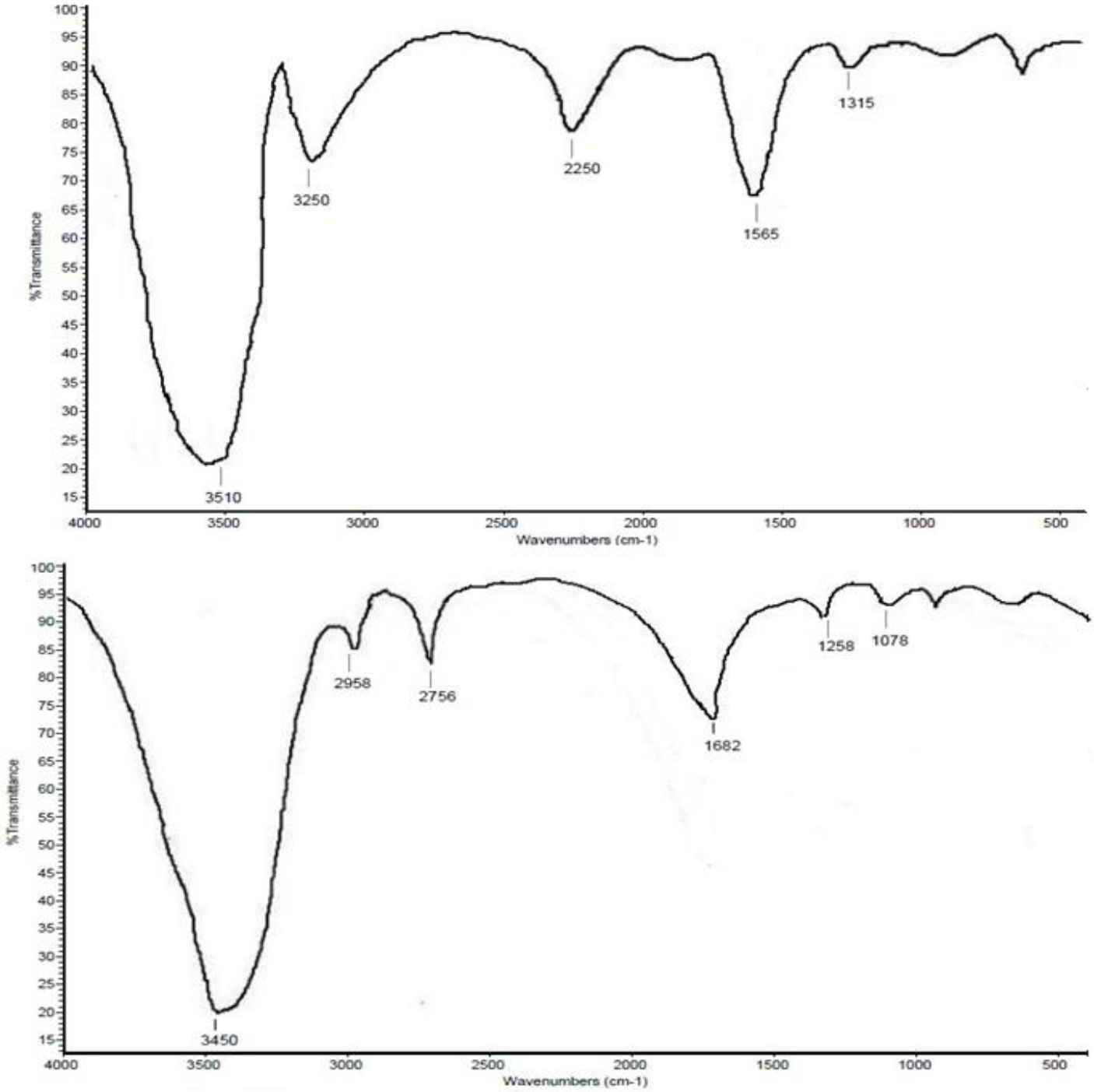


Figure 3. FT-IR spectra of raw and algal treated tannery wastewater.

wastewater will be studied in future.

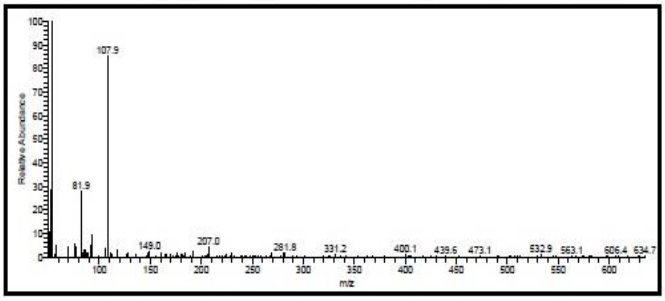
CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

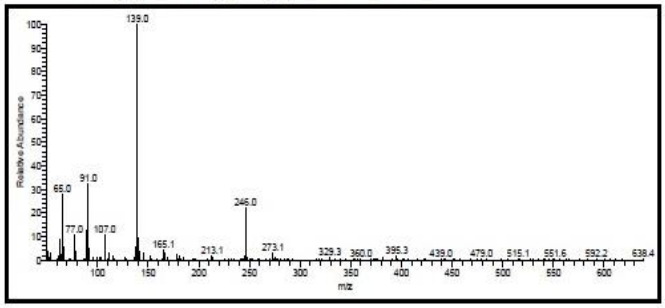
ACKNOWLEDGMENT

The research was supported by Bharathidasan University, Department of Environmental Science which provided the *C. vulgaris*.

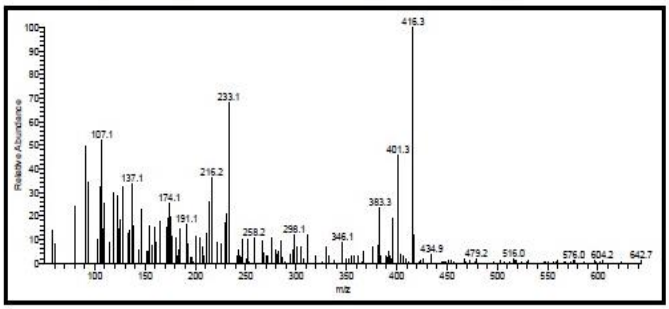
(a) Mass spectrum of 1,6-Dihydroimidazo[4,5-d] imidazole



(b) Mass spectrum of 1H-1,2,4-triazol-3-yl-N-[2-(3-methylphenoxy)ethyl]carboxamide

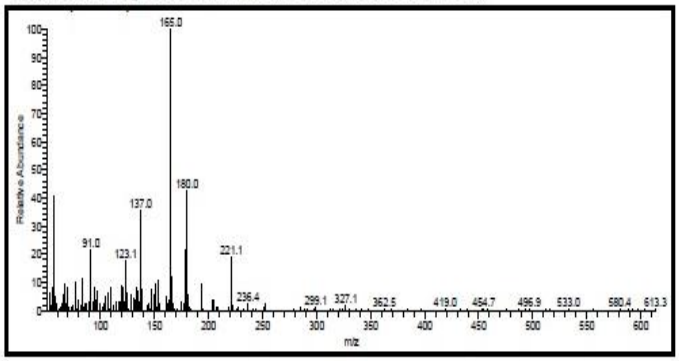


(C) Mass spectrum of 4-(4-(3,5-Dimethylphenyl)-2-[4-methyl sulfonyl)phenyl]-1,3-thiazol-5-yl}2,6-dimethyl pyridine

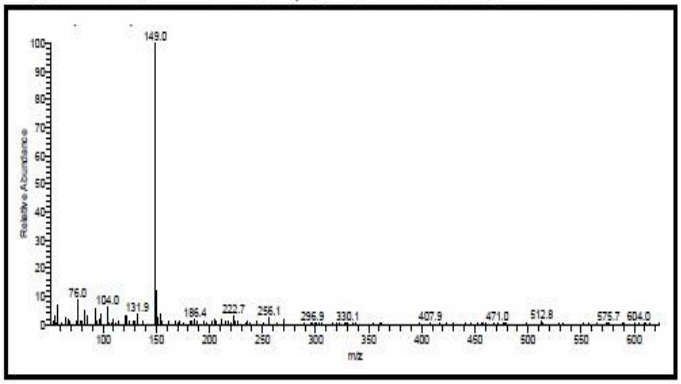


(I)

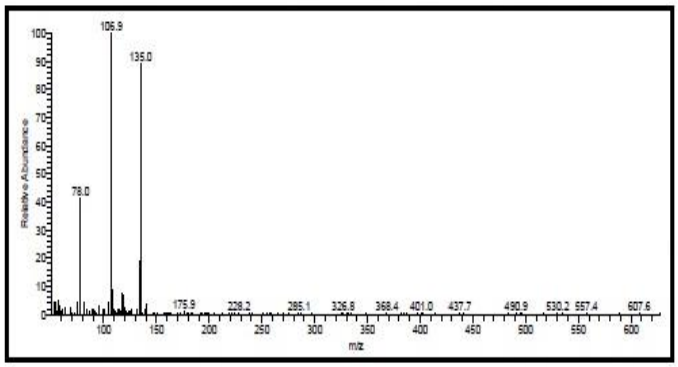
(a) Mass spectrum of 1,6-Methanofluorene



(b) Mass spectrum of,4-(2-propen-1-xyloxy) Benzeneamine



(c) Mass spectrum of 2,3,4,4a,5,6,- Hexahydroquinoline



(II)

Figure 4. GC-MS analysis of i) raw and ii) treated tannery wastewater.

REFERENCES

Ajayan KV, Selvaraju M (2011). Reflector based chlorophyll production by *spirulina platensis* through energy save mode. Bioresour. Technol. 102(16):7591-7594.

Ajayan KV, Selvaraju M (2012). Heavy metal induced antioxidant defense system of green microalgae and its effective role of phycoremediation of tannery effluent. Pak. J. Bio. Sci. 15:1056-1062.

Ajay KP, Vinay D (2012). Biodegradation of azo dye reactive red BL by *Alcaligenes Sp*. AA09. Int. J. Eng. Sci. 1(12):54-60.

Ajayan KV, Muthusamy S, Pachikaran U (2015). Phycoremediation of tannery wastewater using microalgae *Scenedesmus* species. Int. J. Phytorem. 17:907-916.

Arnon DI (1949). Copper enzymes in isolated chloroplasts, polyphenoxidase in beta vulgaris. Plant physiol. 24:1-15.

Ayodhaya DK (2013). Bioremediation of wastewater by using microalgae: an experimental study. Int. J. Life. Sci. Pharma. Rev. 2(3):340-346.

Balaji S, Kalaivani T, Sushma B (2016). Charaterization of sorption and differential stress response of microalgae isolates against tannery effluents from Ranipet industrial area – an application towards phycoremediation. Int. J. Phytorem. 18(8):747-753.

Balakumar K, Sharpudin J, Mubarak BH (2014). Comparative study of carbon sequestration by algae in domestic sewage and industrial effluent. Int. J. Emer. Technol. Adv. Eng. 4:807-809.

Becker EW (1994). Microalgae: Biotechnology and Microbiology. Cambridge Univesity Press, Cambridge, UK.

Bernhardt ES, Lawrence EB, Walsh CJ (2008). Understanding, managing and minimizing urban impacts on surface water nitrogen loading. Annals of the New York Academy Sciences, Year in Ecology and Conservation Biology, 61-96.

Borowitzka MA (1998). Limits to growth. In: Wong, Y.S., Tam, N.F.Y., (Eds.), Wastewater treatment with algae. Springer-Verlag, New York, pp. 203-226.

Chellam CT, Sampathkumar P (2012). Bio removal of nutrients in tannery effluent water using marine microalgae, *Chlorella marina*", *Proceedings of International Forestery and Environment Symposium*, Sri Lanka, Published by Department of Forestry and Environmental Science, University of Sri Jayewardenepura, P 17.

Chu WL, See YC, Phang SM (2009). Use of immobilised *Chlorella vulgaris* for the removal of colour from textile dyes. J. Appl. Phycol. 21:641-648.

Cindrella D, Naseera K, Anirudh R (2016). Bioremediation of tannery wastewater by a salt-tolerant strain of *Chlorella vulgaris*. J. Appl. Phycol.. 29(1):235-243.

Clesceri LS, Greenberg AE, Trussel RR (1989). Standard Methods for the Examination of Water and Wastewater (17th Edition), American Public Health Association, Washington, DC.

Deng L, Zhu X, Wang, X (2007). Biosorption of copper (II) from aqueous solutions by green alga *Cladophora fascicularis*. Biodegradation 18:393-402.

Dilek D, Abel UU, Tulay O (2012). Fourier transform infrared (FTIR) spectroscopy for identification of *Chlorella vulgaris* Beijerinck 1890 and *Scenedesmus obliqus* (Turpin) Kutzing 1833. Afr. J. Biotechnol. 11(16):3817-3824.

Ding J, Zhao F, Cao Y (2015). Cultivation of microalgae in diary farm wastewater without sterilization. Int.J. Phytorem. 17(3):222-227.

Dinesh KS, Santhanam P, Jayalakshmi T (2015). Excessive nutrients and heavy metal removal from diverse wastewaters using marine microalgae *Chlorella marina* (Butcher). Ind. J. Geo-Marine Sci. 44(1).

Durai G, Rajasimman M (2011). Biological treatment of tannery wastewater - A review. J. Environ. Sci. Technol. 4:1-17.

Fraile A, Penche S, González F (2005). Biosorption of copper, zinc, cadmium and nickel by *Chlorella vulgaris*. Chem. Ecol. 21(1):61-75.

Gardea-Torresdey JL, Becker-Hapak MK, Hosea JMD (1990). Effect of chemical modification of algal carboxyl groups on metal ion binding. Environmen. Sci. Technol. 24(9):1372-1378.

Gupta VK, Rastogi A (2008). Equilibrium and kinetic modelling of cadmium (II) biosorption by nonliving algal biomass *Oedogonium sp*. from aqueous phase. J. Hazar. Mater. 153(1-2):759-766.

Hammouda O, Abdel-Raouf N, Shaaban M (2015). Treatment of mixed domestic-industrial wastewater using microalgae *Chlorella* sp. J. Am. Sci. 11(12):303-315.

Hanumantha RP, Ranjith KR, Raghavan BG (2011). Application of phycoremediation technology in the treatment of wastewater from a leather-processing chemical manufacturing facility. Water Sol. 37(1):7-14.

Kishore J, Patil RT, Hitendra KL (2015). Bioprecipitation and biodegradation of fabric dyes by using *Chara Sp.* And *Scenedesmus obliquus*. J. Chem. Pharma. Res. 7(8):783-791.

Lauber CL, Hamady M, Knight R (2009). Pyrosequencing-based assessment of soil ph as a predictor of soil bacterial community composition at the continental scale. Appl. Environ. Microbiol. 75(15):5111-5120.

Lenore SC (1998). Standard Methods for the Examination of Water and Wastewater, (20th Edition), American Public Health Association, Washington, DC, USA.

Lin J, Liu H (1992). Degradation of azo dyes by algae. Environ. Pollu. 75(3):273-278.

Mehta SK, Tripathi BN, Gaur JP (2000). Influence of pH, temperature, culture age and cations on adsorption and uptake of Ni by *Chlorella vulgaris*. Euro. J. Protist. 36(4):443-450.

Mehta SK, Gaur JP (2005). Use of algae for removing heavy metal ions from wastewater: progress and prospects. Crit. Rev. Biotechnol. 25(3):113-152.

Nandha S, Sarangi PK, Abraham J (2010). Cyanobacterial remediation of industrial effluents II. Paper mill effluents. New York Sci. J. 3(12):37-41.

Nichols HE (1973). Growth media freshwater. In: Stein, J., (Ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University press, Cambridge, pp. 7-24.

Rai UN, Dwivedi S, Tripathi RD (2005). Algal biomass: an economical method for removal of chromium from tannery effluent. Bull. Environ. Contamin. Toxicol. 75(2):297-303.

Rehman A, Farah RS, Shakoori AR (2007). Heavy metal resistant Distigma proteus (Euglenophyta) isolated from industrial effluents and its possible role in bioremediation of contaminated wastewaters. Wor. J. Microbiol. Biotechnol. 23(6):753-758.

Rehman A (2011). Heavy metals uptake by Euglena proxima isolated from tannery effluents and its potential use in wastewater treatment. Russ. J. Ecol. 42(1):44-49.

Sharma GK, Khan SA (2013). Bioremediation of sewage wastewater using selective algae for manure production. Int. J. Environ. Eng. Manag. 4(6):573-580.

Sheehan J, Dunahay T, Benemann, J (1998). A look back at the US Department of energy's Aquatic sciences program-Biodiesel from algae. Golden, CO, National Renewable Energy institute, Report No. NREL/TP-580-24190.

Suresh KK. Hans UD, Eun JW (2015). Microalgae - A promising tool for heavy metal remediation. Ecotoxicol. Environ. Saf. 113:329-352.

Sivasubramanian V, Subramanian VV, Muthukumaran M (2012). Algal technology for effective reduction of total hardness in wastewater and industrial effluents. J. Phycol. Soc. (India). 42(1):51-58.

Yadavalli R, Rao CS, Rao RS (2014). Dairy effluent treatment and lipids production by *Chlorella pyrenoidosa* and *Euglena gracilis*: Study on open and closed systems. Asia-Pacific J. Chem. Eng. 9(3):368-373.

academicJournals

Vol. 17(8), pp. 249-254, 21 February, 2018
DOI: 10.5897/AJB2017.16160
Article Number: 1BFCD8956051
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Efficacy of lantana (*Lantana camara*) extract application against aphids (*Brevicoryne brassicae*) in rape (*Brassica napus*) over varied periods of time

Culver Mvumi* and Precious R. Maunga

Faculty of Agriculture, Zimbabwe Open University, Manicaland Region, Stand No. 992 C Avenue, Mutare, Zimbabwe.

Received 20 July, 2017; Accepted 25 January, 2018

Contact bioefficacy of lantana (*Lantana camara* L.) leaf extract (LLE) against rape aphids (*Brevicoryne brassicae* L.) in three rape (*Brassica napus* L.) seed brands at given time (hours) periods was explored. Samples of pounded young leaves mixed with distilled water (3 kg l^{-1}, 2 kg l^{-1} and 1 kg l^{-1}) were prepared. Dimethoate (7.5 ml 10 l^{-1}) and distilled water were the positive and negative controls, respectively. Treatments were arranged in a randomized complete block design (RCBD); 3 x 5 factorial arrangement, replicated 5 times. Adult aphids (10) were introduced to each plant. Aphid mortality at 6, 12, 18 and 24 h after application of treatments was evaluated. There was no significant interaction between seed brand and treatment at all times. Lantana treatments showed significant mortality effect ($p < 0.001$) on aphids at all times. The highest LLC (3 kg l^{-1}) showed the highest aphid mortality at each period of time after application, and of all the periods, the longest time after application (24 h), showed the highest mortality (9.67). It is recommended that smallholder farmers use 3 kg l^{-1} LLC to control aphids on rape, and allow 24 h after application to get the greatest kill.

Key words: Lantana leaf extract, rape, aphid mortality, hours after application.

INTRODUCTION

Rape (*Brassica napus* L.) is a major vegetable crop cultivated worldwide. It is known for its fast growth whereby prolific leaf production has been evidenced, and both fresh and dried leaves are utilized as relish (Oldham, 1999). In Southern Africa, and particularly in underdeveloped countries like Zimbabwe, the vegetable's nutritional value (Nyakudya et al., 2010) has considerable potentials for ameliorating some of the most widespread and debilitating nutritional disorders (Ijarotimi et al., 2003). The production of rape and other leaf vegetables for local and export markets is one of such profitable agricultural enterprise, and in Africa South of the Sahara, smallholder vegetable production is a fast expanding enterprise due to the increasing demand from the rapidly

*Corresponding author. E-mail: culvermvumi@gmail.com.

increasing populations (Kuntashula et al., 2004).

However, rape is attacked by a number of pests; amongst these, rape aphids (*Brevicoryne brassicae* L.), which also attack cabbage (*B. oleracea* var. *oleracea*), are the most serious (Khan et al., 2015). Three rape seed brands (Prime seeds, Avanos and Starke Aryes) are mainly grown in Southern Africa but how they comparably respond to lantana sprays when infested by aphids is unknown. Aphids have high reproductive potential. Each adult produces up to 60 nymphs which develop and reproduce in about one week (Ulusoy and Olmez, 2006). Attack by aphid colonies and the aphid damage due to sap sucking, can cause yield losses from 80% to complete crop failure, if the attack and damage start at seedling stage (Singh and Bakhetia, 1987).

Synthetic chemical insecticides have been used for many years in the control of aphids and are currently available (Shiberu and Negeri, 2016). Nevertheless, use of synthetic chemicals promotes development of pesticide resistance in the target pests (Belmain et al., 2013), pest resurgence and emergence of secondary pests. Synthetic pesticides affect non-target insect species (Shiberu and Negeri, 2016), cause food and groundwater contaminations, pollution and pesticide residue, which accumulate in beneficial organisms and human beings (Georghiou, 2000). They have high costs and associated toxicity risks which discourage rape growers from integrating them in their pest management system (Canhilal et al., 2006). Farmers always complain of local unavailability of synthetic products, poorly labeled or packaged products, and also frequently adulterated and sometimes, some products being sold after expiry date (Stevenson et al., 2012). Dayan et al. (2009) reported that there is a need to develop cheaper and safer alternatives for insect pest control, including plant-based products. Biopesticides are very important to the rural poor who are vulnerable and marginalized. Many smallholder rape growers are, herewith, shifting focus to more reliable, sustainable and environmentally friendly agents of pest control, the biopesticides. Indeed, because rape is a fast-growing vegetable, and yet highly susceptible to aphids, which are highly reproductive, it is necessary to determine a biopesticide which, at a highly efficacious concentration, can potentially control the pest in a short time so as to cherish the maximum leaf production of the crop.

Rajashekar et al. (2014) reported lantana to be efficacious against storage pests, while Muzemu et al. (2011) reported that different plant extracts are biopesticidal against rape aphids. Lantana has wide variety of chemical substances, including triterpenes, mono and sesquiterpenes, iridoid and phenyl ethanoid glycosides, nafthoquinones and flavonoids, among other compounds (Ghisalberti, 2000). The lantadene content of lantana leaves is variable, and potentially toxic plants contain at least 80 and 200 mg kg^{-1} of lantadenes A and B, respectively (Ghisalberti, 2000). In practice, this is equivalent to a dosage of 40 g of fresh material per kilogram of weight.

Lantana grows widely as wild species throughout the tropical, sub tropical and temperate parts of world and is locally and cheaply available, but there is little literature on the control of aphids using lantana. The current research sought to determine the efficacy of lantana leaf extract (LLE) in controlling aphids in rape by assessing adult aphid mortality over varied periods of time (hours) when the aphids are exposed to the treatment.

MATERIALS AND METHODS

Study site

The knockdown and toxic effects of lantana leaf extract as a biopesticide control of aphids was investigated in different periods of time (hours) in Watsomba, Mutasa District, Manicaland, Zimbabwe. The climate is tropical; the weather is always cool in early summer (August to September), while October is the hottest month with minimum temperatures averaging 16°C and maximum often reaching 32°C. The location in terms of Global Positioning System (GPS) is: latitude 18°40´50.99"S 32° 37´59.99"E.

Fertilizers

Basal fertilizer rates were used at a rate of 70 kg N ha^{-1}, 160 kg P_2O_5 ha^{-1} and 100 kg K_2O ha^{-1}. Top-dressing fertilizer was ammonium nitrate (AN), applied to rape at a rate of 34.5 kg N ha^{-1}, 2 times at 3-week interval from 2 weeks after transplanting.

Nursery establishment

Certified seed was obtained from Farm Supplies Company in Mutare city (location: 18°58´ 14.5200" S 32°40´ 15.0960" E) and sown on nursery bed of 6 m long × 1 m wide where the seedlings were raised. Sowing was done on 1 August 2016. Planting depth was 12 mm with furrows 100 mm apart. Nursery management included watering and weed control. Seedlings were transplanted at 4 weeks of age.

Layout of the experiment

Three different seed brands were used as blocking factor (Prime seeds, Avanos and Starke Aryes). There were 3 large blocks; each measured 19 m length x 10.75 m width. The blocks were separated by 2 m pathway. Within each block were smaller blocks of 19 m length x 1.35 m width. Each plot measured 3 m length x 1.35 m width. Each plot was separated from the other by 1 m between plots and between blocks by 2 m. Transplants were spaced at an in-row spacing of 15 cm and inter-row of 45 cm.

Lantana leaf extract preparation

Young lantana leaves were pounded to a paste and then ratios of pounded lantana: distilled water (3:1, 2:1 and 1.1) were used to come up with concentrations. The final concentrations were therefore 3, 2 and 1 kg l^{-1} which were the LLE treatments used in the experiment (Table 1). The extract concentrations were first filtered through a clean sterilized muslin cloth and then through an

Table 1. Lantana leaf extract (LLE) treatment codes and concentrations.

Treatment code	Name of treatment and concentration
T1	Dimethoate 7.5 ml 10 l^{-1} of distilled water (positive control)
T2	LLE 3 kg l^{-1}
T3	LLE 2 kg l^{-1}
T4	LLE 1 kg l^{-1}
T5	No LLE (distilled water)

ordinary filter paper. After the filtering process, each concentration (obtained from 1 l distilled water which had been mixed with each of the pounded amounts of moringa leaves (3, 2 and 1 kg) was then mixed with 5 g of sugar, and stored in air tight glass bottles at 4°C before it was used. Sugar was integrated with all treatments to act as a sticking agent and as an attractant. Sugar was used in horticultural crops (Cao et al., 2016). Sugar has sweetness characteristic which attracts pests to a host, or retains a pest on a host, implying that it has a strong impact on aphid-host choice (Powell et al., 2006), but there is no evidence of adverse interference with any treatments' effect, nor showing that contact synthetic pesticides were improved by addition of sugars (Shelly et al., 2014).

Source of aphids and introduction on rape

Live adult aphids were collected in mid-October 2016 on a white paper from an infested rape field. The adult aphids (*Brevicoryne brassicae*) were identified by Zimbabwe Open University Horticultural Crops Section, Entomomlogy Clinic.

Infestation

After a thorough check of the experimental plants that were clean of aphid infestation, 10 adult aphids (Schwartzberg and Tumlinson, 2013) were immediately introduced on each plant on the same day and about the same time, 6 weeks after transplanting and physically checking that they were healthy and active. The infestation period was 10 min after introducing the adult aphids to the plants, before application of treatments. This implies that there were 10 aphids per plant; infestation time before treatment was 10 min. Each of the rape plants were also physically re-examined *in situ* to determine the consistence of the number of adult aphids previously introduced (10) per plant (Muzemu et al., 2011). No aphid was observed to escape as the number per plant remained 10; the shortness of the infestation period before applying the treatments allowed no aphid to escape before treatments were applied. The sugar in the treatments also aided in retaining the aphids on the plants.

Experimental design, treatments and application for contact mode assessment

Each of the seed brands (Prime seeds, Avanos and Starke Aryes) had 80 plants $plot^{-1}$ and each plot had four lines; each cropline had 20 plants. Two rows at each plot centre were the net rows from where 5 plants were randomly chosen from each row for adult aphid count. Treatments [3, 2 and 1 kg l^{-1} pounded lantana leaves mixed with distilled water, including dimethoate 40% emulsifiable concentrate (EC) and distilled water, as positive and negative controls, respectively] were applied on different seed brands shortly (10 min) after introducing the adult aphids to the plants, before the aphids changed hosts or reproduced. A knapsack was used to apply the treatments at a rate of 1 l 5 m^{2-1} (Muzemu et al., 2011; Pahla et al., 2014). Plastic spray-shields were set up to prevent spray drift of the different treatments from affecting neighboring plots (Pahla et al., 2014). The shields also provided barrier to any aphid flights which might take place. The treatments were arranged in a randomized complete block design (RCBD), 3 x 5 factorial arrangement. Each treatment was replicated 5 times. Average mortality was recorded at 6, 12, 18 and 24 h (after application of treatments to the seed brands) by physically examining the entire rape plants *in situ* to assess the number of aphids left per plant and calculating the eliminated ones which were then subjected to statistical analysis.

Data analysis

Two-way analysis of variance (ANOVA) was used for the two factors: seed brand and treatment, using GenStat statistical package 14th Edition to analyze the data. Separation of treatment means of all treatments (including positive and negative controls) was then employed using the Bonferroni test at $p = 0.05$. The Bonferroni test was used in the current study because the number of contrasts to be estimated (or comparisons to be tested) was small. The Bonferroni test also allows equal and even unequal sample sizes to be tested if they inevitably occur in an experiment.

RESULTS

The lowest lantana concentration of adult aphid mortality at 6, 12, 18 and 24 h after application was significantly higher ($p = 0.05$) than the negative control. T1 was found to have all the 10 live aphids on the sampled plants.

Mortality of adult aphids 6 h after treatment

Mortality was affected by the LLE treatments ($F_{4,28} = 23.97$; $p < 0.001$); there were also significant differences among seed brands ($F_{2,28} = 3.47$; $p = 0.045$). However, there was no interaction between treatment and seed brand ($F_{8,28} = 0.37$; $p = 0.927$). Although, T1 had significantly higher mortality than lantana treatment T2, T2 gave the highest mortality in all the other lantana

Table 2. Means for 6, 12, 18 and 24 h of adult aphid (*Brevicoryne brassicae*) mortality after application of treatments. Values are the mean ± standard error of the mean (SEM).

Treatment code	Means of adult aphid mortality (hours) after treatment application			
	6	12	18	24
T1	1.67±0.4^{d}	4.67±0.6^{d}	6.67±0.4^{d}	9.89±0.5^{d}
T2	1.22±0.2^{cd}	4.67±0.6^{d}	6.33±0.6^{d}	9.67±0.6^{d}
T3	0.67±0.4^{bc}	3.00±0.2^{c}	4.78±0.2^{c}	7.56±0.4^{c}
T4	0.22±0.3^{ab}	2.33±0.4^{b}	3.56±0.3^{b}	6.56±0.2^{b}
T5	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

treatments; it showed highly significant difference ($p < 0.001$) (Table 2). Avanos and Prime seed showed the same adult aphid mortality (at T2), but higher than Starke Aryes at T3.

Results of Bonferroni test showed that adult aphid mortality of T5 was significantly higher ($p < 0.05$) as compared to the control at 6, 12, 18 and 24 h after application.

Mortality of adult aphids 12 h after treatment

There was significant effect ($F_{4,28} = 176.93$; $p < 0.001$) of LLE treatments on mortality but there were no significant differences among seed brands ($F_{2,28} = 3.15$; $p = 0.058$). There was also no interaction between treatment and seed brand ($F_{8,28} = 1.40$; $p = 0.240$). T1 and T2 were not significantly different; T2 was highly significantly different ($p < 0.001$) from the other LLE treatments. T2 also showed significantly larger mortality effect than T2 at 6 h after treatment (Table 2).

Mortality of adult aphids 18 h after treatment

Effect of LLE on aphid mortality after 18 h of application was significant ($F_{4,28} = 204.53$; $p < 0.001$), but seed brand effect was not significant ($F_{2,28} = 2.27$; $p = 0.084$). Seed brand-treatment had no interaction ($F_{8,28} = 0.54$; $p = 0.816$) while T2 was not significantly different from the T1, and had higher significance ($p < 0.001$) than all the lower LLE treatment concentrations (T3 and 4) and the negative control (Table 2).

Mortality of adult aphids 24 h after treatment

Lantana leaf extract significantly affected ($F_{4,28} = 436.10$; $p < 0.001$) mortality but seed brand effect was not significant ($F_{2,28} = 1.80$; $p = 0.184$). There was no interaction between seed brand and treatment factors ($F_{8,28} = 0.55$; $p = 0.809$). Table 2 shows that T2 was not significantly different ($p = 0.005$) from T1 but highly significant ($p < 0.001$) with regards to the other treatments.

At 24 h after application of LLE treatments, T3 and T4 had comparably higher lethality than the same treatments in shorter periods of time.

DISCUSSION

The study shows that lantana has effect on aphids. Biopesticides' effectiveness against aphids have been reported. Extracts of *Lippia javanica* leaf powder and *Solanum delagoense* ripe fruit pulp were found to possess pest control properties against rape aphids and tomato red spider mites (*Tetranychus evansi*), respectively (Muzemu et al., 2011). Plant extracts of *Artmesia vulgaris*, *Algeratum conyzoids*, *Vitex trifolia*, *Corcus calamus* and *Azadirachta indica* all exhibited insecticidal activity against cotton aphid (*Aphis gossypii*) (Devi et al., 2003). The current study is in agreement with the studies done by Rajashekar et al. (2014). Methanol extract from lantana leaf powder was found to be efficacious against test storage pests, *Sitophilus oryzae*, *Callosobruchus chinesis* and *Tribolium castaneum.*

High concentration of *L. javanica* leaf powder and *S. delagoense* ripe fruit pulp extracts used in previous studies showed that highest concentrations had the highest efficacy (Muzemu et al., 2011). The higher the concentration, the more the efficacy and the lower the concentration, the lower the efficacy, which results in only a repellent effect. In a study by Ogendo et al. (2003), 50% storage pests' mortality varied from five to six days after treatment with the highest concentration of 7.5 to 10.0% w/w lantana powder was used. In this study, the greatest kill was at 24 h (9.667) when 3 kg l^{-1} was used. It implies that the lantana treatments used were generally highly concentrated in order to effectively kill a large number of aphids. The degree of efficacy was greatly influenced by dosage or concentration of the extract used and the length of time after application; 24 h had the greatest kill. Higher concentrations over a longer duration are more likely to produce greater lethal effects. Lower concentrations result in only a repellent effect. The 10 live adult aphids found in the plots of T5 unlike T4 plots

implied that lantanta at the lowest concentration (T4) was effective, probably as a repellant and that distilled water had no lethal effect. There was an increasing number of aphids which included nymphs weeks later. Toxicity is a function of time after application, and time after application is a function of dose and length of the time (Rozman et al., 2009). The positive control (dimethoate 40% EC) in the current study which caused a high mortality to aphids agrees with study of Bezerra-Silva et al. (2012) who assessed the contact effect of synthetic pesticide, and found that it is equally highly deleterious to *Bucephalogonia xanthophis.*

Most biopesticides used against aphids reported in literature showed deleterious effect. In the current study, lantana was effective in controlling aphids. For example, among the three seed brands, Avanos and Prime seed showed high aphid mortality record with no difference between the two seed brands in aphid mortality at T2. However, Avanos showed higher mortality at T3. The results suggest that different seed brands respond differently to lantana application when they are infested by aphids. In the current study, lantana was most effective on Avanos when tested at 6 h.

This means aphids can be controlled effectively by lantana plant derivatives. Seeds and leaf extract of flowering lantana (Baidoo and Adam, 2012) have also proved efficacious against cabbage aphid (Mekuaninte et al., 2011).

The use of natural products and their analogues have been considered for the management of agricultural insect pests. This is due to the fact that they are less detrimental to the environment than synthetic chemical insecticides. In the current study, mortality could have been due to the properties of the extract. *Lantanine* plant metabolite from lantana has been characterized as having defensive mechanisms against insect pests (Ghisalberti, 2000).

Conclusion

Lantana leaf extract used in the current study showed potential to control aphids in rape. The highest concentration (3 kg l^{-1}) showed the most efficacious effect on *B. brassicae* in *B. napus*. Lower concentrations showed lower efficacy. The least was recorded when no extract treatment (distilled water) was applied. The higher the concentration, the higher the efficacy, and the lower the concentration, the lower the efficacy, thus, resulting in only a repellent effect. The study has shown that the degree of efficacy of lantana leaf extract is greatly influenced by the dosage or concentration of the extract applied and the exposure time. The study also suggests that different seed brands respond differently to lantana application in terms of mortality of aphids infesting them.

Lantana can be of importance to the resource-poor farmers in many areas of developing countries. The mortality recorded for the treated plants was an indication that they can be used as alternatives to chemical insecticides. The findings of the present study therefore indicate that leaves of lantana have some toxic properties and therefore could be considered as potential source of biopesticide for economical and environmentally friendly pest control strategies against aphids in rape.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Baidoo PK, Adam JI (2012). The effects of extracts of *Lantana camara* (L.) and *Azadirachta indica* (*A. Juss*) on the population dynamics of *Plutella xylostella*, *Brevicoryne brassicae* and *Hellula undalis* on cabbage. Sustain. Agric. Res. 1:229-234.

Belmain SR, Haggar J, Holt J, Stevenson PC (2013). Managing legume pests in sub-Saharan Africa: Challenges and prospects for improving food security and nutrition through agro-ecological intensification. Chatham Maritime (United Kingdom): Natural Resources Institute, University of Greenwich. P 34.

Bezerra-Silva GCD, Silva MA, De Miranda MP, Lopes JRS (2012). Effect of contact and systemic insecticides on the sharpshooter *Bucephalogonia xanthophis* (Hemiptera: Cicadellidae), a vector of *Xylella fastidiosa* in citrus. Florida Entomol. 95(4):854-861.

Canhilal RG, Carner GR, Griffin RP (2006). Life history of the squash vine borer, *Mellitia cucurbitae* (Harris) (Lepidoptera: Sessiidae) in South Carolina. J. Agric. Urban Entomol. 23:1-16.

Cao He-He, Liu Hui-Ru Zhang, Zhan-Feng, and Liu Tong-Xian (2016). The green peach aphid *Myzus persicae* perform better on pre-infested Chinese cabbage *Brassica pekinensis* by enhancing host plant nutritional quality. Sci. Rep. 6:21954.

Devi N, Singh TK, Chitra DL (2003). Efficacy of certain botanical insecticides against aphids (*Aphis gossypii* Glover) on Brinjal. Pestology 27(3):6-10.

Dayan FE, Cantrell CL, Duke SO (2009). Natural products in crop protection. Bioorg. Med. Chem. 17:4022-4034.

Georghiou G (2000). The magnitude of the resistance problems. In Pesticide resistance strategies and tactics for management. National Academy Press, Washington, D.C. pp. 11-44

Ghisalberti EL (2000). *Lantara camara* L. (Verbenaceae). Fitoterapia. 71:467-486.

Ijarotimi OS, Eleyinmi AF, Ifesan BOT (2003). Evaluation of the nutritional status of adolescents in institutionalised secondary schools in Akure, Nigeria. Food Agric. Environ. 1(3, 4):64-68.

Khan IA, Ahmad M, Akbar R, Hussain S, Saeed M, Farid A, Shah RA, Fayaz W, Shah B, Din MMU (2015). A study on Losses due to *Brevicoryne brassicae* in different *Brassica* genotypes under screen house conditions. J. Entomol. Zool. Stud. 3(6):16-19.

Kuntashula E, Mafongoya PL, Sileshi G, Lungu S (2004). Potential of biomass transfer technologies in sustaining vegetable production in the wetlands (dambos) of eastern Zambia. Exp. Agric. 40:37-51.

Mekuaninte B, Yemataw A, Alemseged T, Nagappan R. 2011. Efficacy of *Melia azadarach* and *Mentha piperata* plant extracts against cabbage aphid, *Brevicoryne brassicae* (Homoptera: Aphididae). World Appl. Sci. J. 12:2150-2154.

Muzemu S, Mvumi BM, Nyirenda SOM., Sileshi GW, Sola P, Chikukura I, Kamunula JF, Belmain SR, Stevenson PC (2011). Pesticidal effects of indigenous plant extracts against rape aphids and tomato red spider mites. Afr. Crop Sci. Confer. Proc. 10:169-171.

Nyakudya IW, Jimu L, Marashe M, Katsvanga CAT (2010). Comparative growth and yield responses of rape (*Brassica napus* L.) to different soil fertility management amendments. EJEAFChe.

9(1):207-214.
Oldham CH (1999). Brassica crops and allied Cruciferous crops. Biotech Books, Delhi, India.
Ogendo JO, Belmain SR, Deng AL, Walker DJ (2003). Comparision of toxic and repellent effects of *Lantana camara* L. with *Tephrosia vogelii* Hook and a synthetic pesticide against *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) in stored maize grain. Insect Sci. Appl. 23(2):127-135.
Pahla I, Moyo M, Muzemu S, Muziri T. (2014). Evaluating the effectiveness of botanical sprays in controlling aphids *Brevicoryne brassicae* on rape *Brassica napus* L. IJAAR 5(1):1-6.
Powell G, Tosh CR and Hardie J (2006).Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. Annu. Rev. Entomol. 51: 309-330.
Rajashekar Y, Ravindra KV, Bakthavatsalam N (2014). Leaves of *Lantana camara* Linn. (Verbenaceae) as a potential insecticide for the management of three species of stored grain insect pests. J. Food Sci. Technol. 51(11):3494-3499.
Rozman KK, Doull J, Wayland JH (Jr) (2009). Dose and time determining, and other factors influencing, toxicity. Hayes' Handbook of Pesticide Toxicology. pp. 3-101.
Schwartzberg EG, Tumlinson JH (2013). Aphid honeydew alters plant defense responses. Funct. Ecol. 28:386-394.
Shelly T, Epsky N, Jang EB, Reyes-Flores J, Vagas R (2014). Trapping and the detection,control, and regulation of Tephritid fruit fries. Springer Dordrecht Heidelberg, New York, London.
Shiberu T, Negeri M (2016) Effects of synthetic insecticides and crude botanicals extracts on cabbage aphid (*Brevicoryne brassicae* L.) (Hemiptera: Aphididae) on Cabbage. J. Fertil. Pestic. 7:162.
Singh B, Bakhetia DRC (1987). Screening and breeding techniques for aphid resistance in *Oleiferous brassica*: A review. Oilcrops Network, International Development Research Centre, Canada. P 50.
Stevenson PC, Nyirenda SP, Mvumi BM, Sola P, Kamanula, JF, Sileshi GW, Belmain SR (2012). Pesticidal plants: A viable alternative insect pest management approach for resource-poor farming in Africa. In: O, Koul, GS, Dhaliwal S, Khokhar and Singh R (Eds.), botanicals in environment and food security, Jodhpur: Scientific Publishers, pp. 212-238.
Ulusoy MR, Olmez BS (2006). Effect of certain *Brassica* plants on biology of the cabbage aphid *Brevicoryne brassicae* under laboratory conditions. Phytoparasitica 34(2):133-138.

academicJournals

Vol. 17(8), pp. 255-262, 21 February, 2018
DOI: 10.5897/AJB2017.16349
Article Number: F666DDD56055
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

The antibacterial activity of bacterial endophytes isolated from *Combretum molle*

Mamonokane O. Diale[1*], Eunice Ubomba-Jaswa[1,2] and Mahloro H. Serepa-Dlamini[1]

[1]Department of Biotechnology and Food Technology, Faculty of Science, University of Johannesburg, Doornfontein Campus, South Africa.
[2]Water Research Commission, Lynnwood Bridge Office Park, Bloukrans Building, 4 Daventry Street, Lynnwood Manor, Pretoria, South Africa.

Received 30 November, 2017; Accepted 26 January, 2018

Although intense research has gone into the exploration of various Combretaceae species towards the discovery of therapeutic relevant compounds, their endophytes have never been explored as potential repositories of alternative sources of novel and medically beneficial equivalents. In the present study, five bacterial endophytes (*Lysinibacillus*, *Staphylococcus*, *Enterobacter*, *Pseudomonas* and *Bacillus* species) were isolated from different parts (hard stem, leaves and soft stem) of *Combretum molle* and identified to species level using morphological data and sequencing of the 16S rRNA. Four of the five endophytes showed varying degrees of antimicrobial characteristics against *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.*

Key words: Bacterial endophytes, bioactive compounds, *Combretum molle*, phylogenetic analysis, medicinal plant.

INTRODUCTION

Antibacterial resistance of microbial pathogens remains a threat to public health worldwide (Costelloe et al., 2010). Infections are increasingly becoming a challenge and established antibiotics have become less effective against some common bacterial infections (Bhalodia and Shukla, 2011). Such challenges are often due to inappropriate use of antibiotics, large and increasing numbers of immunocompromised patients, delays in diagnosis of infection and poor hygiene (Santos et al., 2015). As a result, there is need for the search of new, diverse and efficacious antimicrobial compounds. Until this is accomplished, naturally derived products remain an essential source for novel pharmaceuticals. A range of microorganisms termed endophytes have been shown to be a rich source of bioactive compounds that can be used in therapeutics (Ravnikar et al., 2015).

Endophytes are microorganisms that reside within plant tissues without causing any substantive harm (Kumar et al., 2015). Endophytes can either be fungal or bacterial in nature and are capable of producing biologically active compounds, some of which are used by the plant as part of its arsenal in its defence against pathogens, while some promote plant growth (Gonzalez-Teuber et al., 2014; Strobel and Daisy, 2013). Most of the bioactive

*Corresponding author. E-mail: diale.olga@gmail.com. Tel: 0813949982.

compounds extracted from endophytes have shown a plethora of bioactivities including but not limited to antimicrobial, immunosuppressant and anticancer (Nair and Padmavathy, 2014).

Combretum molle is used as a remedy throughout Africa to cure various diseases such as infertility in women, malaria and microbial infections (Ademola and Eloff, 2010). *C. molle* leaves have been reported to possess analgesic, anti-inflammatory cardiovascular, antibacterial, antifungal, anti-malarial, antitrypanosomal and anthelmintic effects (Morais-Lima et al., 2012; Ojewole, 2009). To date, no endophytes studies have been carried out from *C. molle,* thus the aim of the present study was to isolate and identify endophytic bacteria from *C. molle* and further test their crude extracts on pathogenic microorganisms.

MATERIALS AND METHODS

Plant sample collection

The plant material was harvested from Lwamondo village in Venda (23°02'37.7"S 30°24'00.2"E), Limpopo province, South Africa. Healthy, disease free plant parts (stem and leaves) of *C. molle* were collected and placed in sterile polyethylene bags and transported to the laboratory at 4°C.

Identification of the plant

Plant material was identified at the University of Johannesburg herbarium (JRAU). The sample specimen was deposited in the herbarium and assigned voucher number Diale-Serepa-Dlamini 1 and species name *C. molle*.

Isolation of bacterial endophytes

Immediately after collection of the plant material in the laboratory, the endophytes were isolated from the plant (soft, hard stems and leaves) using a method described by Jasim et al. (2014). In brief, plant parts were thoroughly washed with tap water to remove dust and cut into small segments (1 to 3 cm long). Soil debris-free plant parts were subsequently treated with Tween 80 for 10 minutes with vigorous shaking followed by rinse with distilled water. The plant samples were further immersed in 70% ethanol for 1 min and then treated with 1% sodium hypochlorite (NaOCl) for 10 min. The samples were then rinsed five times with sterile distilled water and the final wash was spread on nutrient agar plates as controls.

For isolation of bacterial endophytes, the outer surface of the sterile plant parts was trimmed; the pieces were then macerated in phosphate buffered saline (PBS). Serial dilutions of up to 10^{-3} were prepared and 0.1 mL of the dilution was spread on nutrient agar plates. Plates (including the controls) were incubated at 30°C for 2 days. The plates were observed daily for bacterial colony growth. Isolated colonies were re-cultured on sterile Nutrient agar plates until pure colonies were obtained. Glycerol (30%, glycerol diluted in sterile distilled water) stocks of each bacterial isolate were prepared and stored at -80°C for future use.

Morphological identification of endophytic bacteria

Gram staining

Pure colonies were subjected to Gram staining as described by Collins et al. (2004) to establish morphological characteristics such as shape and Gram stain reaction. Gram stain slides were observed using a compound bright-field microscope (OLYMPUS CH20BIMF200) with 100× magnification (Gupta et al., 2015).

Scanning electron microscope

Sample preparations for the Scanning Electron Microscope (SEM) were prepared using Golding et al. (2016) and Schadler et al. (2008) methods. In brief, bacterial strains were grown in 5 mL Luria broth overnight at 30°C, shaking at 150 rpm. The bacterial cultures were centrifuged at 1100 × *g* for 10 min and the supernatant discarded. This was followed by brief rinse with distilled water and fixing the pellet with (1:1v/v) 1% formaldehyde and 2% glutaraldehyde for 1 h at room temperature (25°C). Samples were centrifuged at 1100 × *g* for 10 min; the supernatant was removed and the pellet washed with 1000 µL of sterile distilled water. For dehydration, bacterial cells were treated with different concentrations of ethanol (30, 50, 70, 90, 95 and 100%) with 10 min intervals. Samples were stored open at 4°C overnight. The dehydrated samples were mounted on SEM stubs, and coated with gold using emscope SC 500 and viewed with TESCAN VEGA SEM (VEGA 3 LHM, AVG9731276ZA) connected to a monitor.

Molecular identification of the bacterial endophytes using the 16S rRNA

Extraction of genomic DNA

Pure colonies of each bacterial isolate obtained from nutrient agar were inoculated into nutrient broth and grown overnight at 30°C. Cultures were centrifuged at 13000 × *g* for 5 min and supernatants discarded. The DNA was extracted using ZR fungal or Bacterial DNA kit (Zymo Research, catalog No R2014) following manufacturer's protocol. The extracted DNA was quantified using the NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher scientific, USA).

Polymerase chain reaction (PCR) amplification and sequencing

The 16S rRNA gene of each bacterial isolate was amplified by PCR following protocol and primers described by Yeates et al. (1997). The PCR products were cleaned with ExoSAP-it™ following manufactures recommendations and sequenced at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

Phylogenetic analysis

The obtained sequences were screened for chimeras using DECIPHER (Wright et al., 2012) and subjected to BLAST (v.2.6.0) analysis at NCBI against rRNA sequence database of bacteria and archaea to identify closest bacterial species. Only bacterial species with 99 to 100% similarity were selected for phylogenetic analysis. Alignments of nucleotide sequences (isolate and species obtained through BLAST) were performed using MUSCLE with default options (Liu et al., 2016). Phylogenetic trees were constructed using a Neighbor-Joining (NJ) method based on the Tamura-Nei model (Tamura and Nei, 1993). The positions with gaps and missing nucleotide data were eliminated. All evolutionary analyses were conducted in MEGA 7 (Kumar et al., 2016). The 16S rRNA gene sequences of bacterial isolates identified in the study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the following accession numbers MF105747 *Enterobacter* species SSRP1, MF105748 *Lysinibacillus* species HSRN, MF105749

Table 1. Bacterial endophytes isolated from different part of *Combretum molle*.

Bacterial colony code	Plant part	Gram staining	Shape
HSRN	Hard stem	Gram positive	Rod
LCP	Leaves	Gram positive	Cocci
SSRP1	Soft stem	Gram negative	Rod
SSRN1	Soft stem	Gram negative	Rod
LRP	Leaves	Gram positive	Rod

Pseudomonas species SSRN1, MF105750 *Bacillus* species LRP, and MF105751 *Staphylococcus* species LCP. The assigned names of the bacterial isolates were based on the BLAST homology percentages as well as phylogenetic results.

Phytochemical analysis

Sample preparation

C. molle plant parts (stem, leaves and bulk) were dried at 27°C for 7 days and then they were blended into a fine powder.

Qualitative analysis of phytochemicals on C. molle

Phytochemical screening was conducted using Trease and Evans (1983) and Harbourne (1983) methods.

Phytochemicals analysis of endophytes crude extract

Phytochemical screening of endophytes crude extracts was conducted using the same methods (Trease and Evans, 1983; Harbourne, 1983) with some modifications.

Production of secondary metabolites from bacterial endophytes

Nutrient broth (8 L) was prepared in 2 L Erlenmeyer flasks and autoclaved at 121°C for 15 min. Each 2 L flask was inoculated with one of each endophytic bacteria and incubated at 30°C for 7 days (Sandhu et al., 2014). After 7 days of cultivation, sterilized XAD-7-HP resin (20 g/L) (SIGMA, South Africa, BCBR6696V) was added to the culture for 2 h shaking at 200 rpm. The resin was filtered using cheese cloth and eluted with acetone three times. Acetone was removed using a Rota evaporator. The remaining water was extracted with ethyl acetate three times and concentrated using a rotary evaporator (Maloney et al., 2009).

Antibacterial activity of the crude extracts from bacterial endophytes

Antibacterial tests were carried out by using a modified disc diffusion method described by Bauer et al. (1966). All pathogenic strains (Gram-negative strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella oxytoca* ATCC 13182; Gram-positive: *Staphylococcus aureus* NCTC 6571 and *Bacillus cereus* ATCC 10876) were grown overnight at 37°C in Muller-Hinton broth and adjusted using 0.5 McFarland standards such that the concentration was 10^7 to 10^8 colony forming unit (CFU/mL). Under sterile conditions, 0.1 mL of each pathogenic strain was spread on Muller-Hinton agar. Sterile circular paper discs with a diameter of 6 mm were soaked in 10 µL of each bacterial endophyte crude. 10 µL of 1 mg/L streptomycin (SIGMA-ALDRICH, Switzerland, BCBP5897V) was used as positive control and loaded on the discs as described earlier. Six discs of different crude extracts including the control were placed on each spread plate inoculated with different pathogenic strains and incubated at 30°C for 72 h. Antimicrobial activity was observed daily by measuring the zone of inhibition (in mm). The antibacterial test was performed in triplicates.

RESULTS AND DISCUSSION

Isolation and identification of bacterial endophytes

Morphological identification

The surface sterilization of plant material is important for isolation and studying endophytes. The stems and leaves of *C. molle* were surface sterilized for isolation of bacterial endophytes. The surface sterilization method was satisfactory as no growth emerged on control plates. Thus, the isolated bacterial colonies can be considered true endophytes. Five bacterial colonies were isolated (Table 1). The colonies were differentiated based on their Gram reaction, colony colour and morphology. The Gram stain reaction results showed three of the bacterial isolates to be Gram-positive and two Gram-negative bacterium. The morphological shapes observed from Gram stain reaction were further confirmed using SEM results (Figure 1A to E) which had uniform cells indicating that the bacterial cultures were pure.

Phylogenetic relationship

The BLAST search results of the 16S rRNA gene sequences resulted in varying bacterial genera. Bacterial endophyte HSRN had maximum identity to *Lysinibacillus fusiformis* (100%), LCP had maximum identity to *Staphylococcus epidermis* (100%), SSRP1 had maximum identity to *Enterobacter cloacae* (99%), SSRN1 had maximum identity to *Pseudomonas fulva* (100%) and LRP had maximum identity *Bacillus subtilis* (99%), thus the isolated putative bacterial endophytes can be considered bacterial strains of *Lysinibacillus*, *Staphylococcus*, *Enterobacter*, *Pseudomonas* and

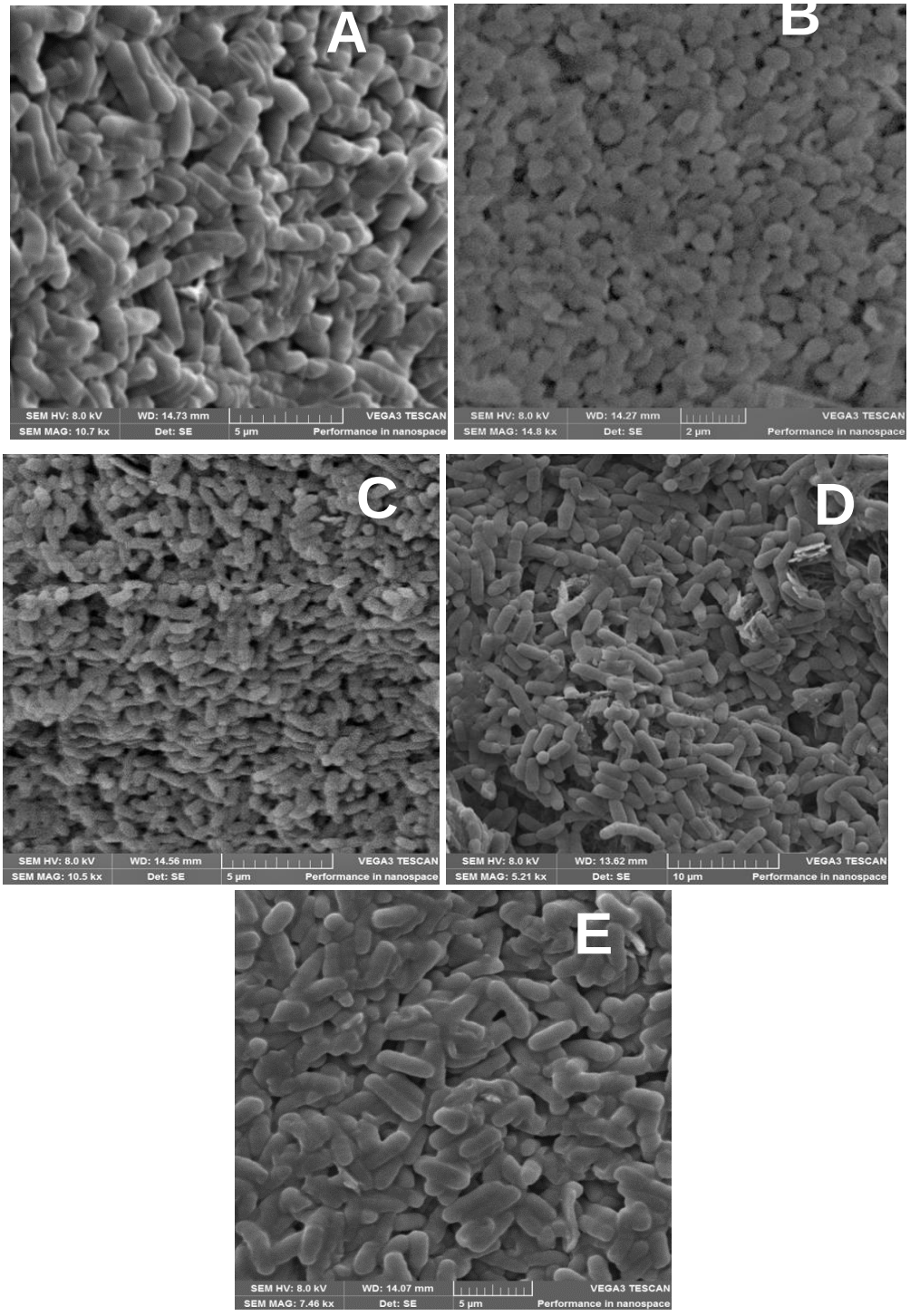


Figure 1. Scanning electron micrographs showing cell morphology of five endophytic bacteria isolated from *Combretum molle*. (a) *Lysinibacillus* spp. HSRN; **(b)** *Staphylococcus* spp. LCP; **(c)** *Enterobacter* spp. SSRP1; **(d)** *Pseudomonas* spp. SSRN1; **(e)** *Bacillus* spp. LRP.

Bacillus spp. (Table 2). To our knowledge, this is the first study to report these bacterial endophytes from *C. molle*.

The phylogenetic analysis showed that the endophytic bacterial isolates are grouped with various closely related bacterial species (Figure 2). From Figure 2, *Enterobacter* spp. SSRP1 MF105747 had a sister relationship with *Kosakonia cowanni* KP236256 a species isolated from a sea grass *Thalassia hemprichii* with a bootstrap of 100%.

Table 2. BLAST analysis of the 16S rRNA genes of bacterial endophytes from *C. molle*.

Bacterial colony code	Plant part	GenBank accession number	Assigned bacterial name	Closest NBCI related bacterial species with accession number
HSRN	Hard stem	MF105748	*Lysinibacillus* spp.	*Lysinibacillus fusiformis* KX867805
LCP	Leaves	MF105751	*Staphylococcus* spp.	*Staphylococcus epidermis* MG027640
SSRP1	Soft stem	MF105747	*Enterobacter* spp.	*Enterobacter cloacae* CP022532
SSRN1	Soft stem	MF105749	*Pseudomonas* spp.	*Pseudomonas fulva* MF421780
LRP	Leaves	MF105750	*Bacillus* spp.	*Bacillus subtilis* MF187644

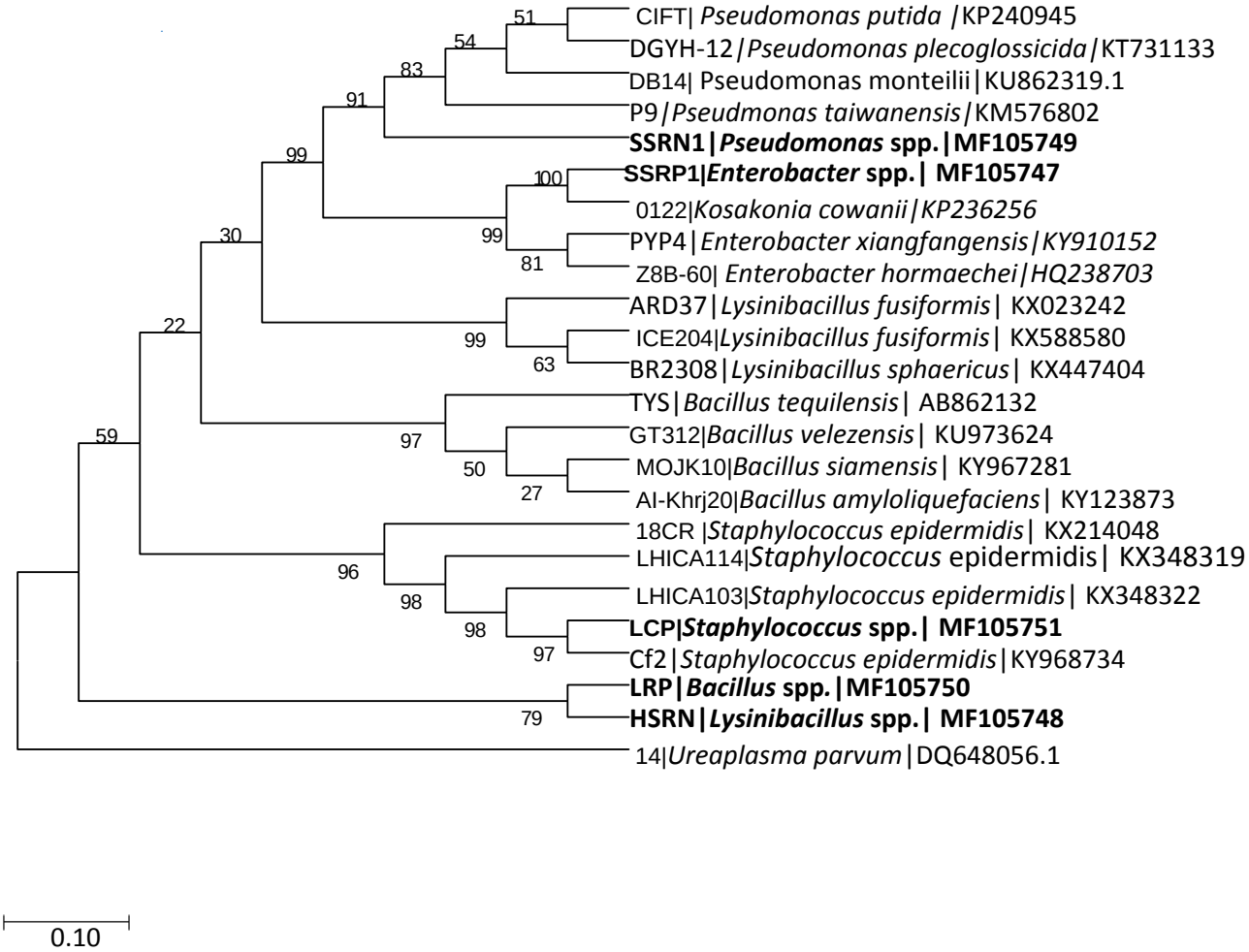


Figure 2. Neighbour joining tree based on 16S rRNA gene sequence of five endophytic bacteria isolated from *C. molle* and other similar species selected from GenBank.

K. cowanni belongs to the Enterobacteriaceae family. *Lysinibacillus* spp. HSRN MF105748 and *Bacillus* spp. LRP MF105750 also had a sister relationship with 75% bootstrap value. These two species are from the same family name Bacillaceae. *Staphylococcus* spp. LCP MF105751 had a sister relationship with *Staphylococcus epidermidis* KY968734 a species isolated from sphenoid sinus biopsy with a 97% bootstrap value. *Pseudomonas* spp. SSRN1 MF105749 shared a common ancestor with *Pseudomonas taiwanensis* KM576802 isolated from rhizosphere soil with 91% bootstrap value.

All current bacterial endophytes strains were reported as endophytes from various plant species (Chaudhry and Patil, 2013; Christina et al., 2013; Mahummad et al., 2014; Zhao et al., 2015). Similar studies on isolation of endophytes have been reported by other researchers,

Table 3. Qualitative analysis of phytochemicals in leaf, stem, and bark of *Combretum molle*.

Test	Leaves	Stem	Bark
Alkaloids	-	-	-
Flavonoids	+	+	+
Steroids	-	+	+
Tannins	+	+	+
Saponins	-	-	-

Table 4. Qualitative analysis of phytochemicals of crude extracts of endophytes isolated from *Combretum molle*.

Test	*Lysinibacillus* spp. HSRN	*Staphylococcus* spp. LCP	*Pseudomonas* spp. SSRN1	*Enterobacter* spp. SSRP1	*Bacillus* spp. LRP
Alkaloids	-	-	-	-	-
Flavonoids	+	+	+	+	+
Steroids	-	-	-	-	-
Tannins	+	+	-	+	+
Saponins	-	-	-	-	-

where *Pseudomonas* and *Bacillus* spp. were isolated from *Echinacea* medicinal plant (Christina et al., 2013). *L. fusiformis* isolated from the medicinal plant *Panicum virgatum* and *S. epidermis* isolated from rice seeds have been reported to be endophytes (Ryan et al., 2008). The aforementioned indicates that these species are present as endophytes within a variety of plant species which makes them more interesting for further studies, such as plant growth promotion and their possible applications in drug discovery and agriculture.

The five-isolated putative bacterial endophytes represent five different genera, which indicate diverse bacterial endophytes present within *C. molle*. Endophytes are known to vary in diversity based on seasonal collection or sampling time, plant age, plant tissue type and environment (Jasim et al., 2013). In this study, it was strongly believed that *C. molle* is likely to be associated with other different types of bacterial endophytes.

Phytochemical analysis

Secondary metabolites studies of the leaves, stem and bark of *C. molle* showed the presence of tannins, flavonoids and steroids (Table 3). These secondary metabolites possess properties which are of great importance in the drug development (Joseph et al., 2013). Flavonoids are known to have antimicrobial, anti-cancer, anti-inflammatory and anti-viral properties (Kabera et al., 2014) and tannins have stringent properties and can be utilized for antibacterial, anti-diarrheal, haemostatic and anti-haemorrhoidal drugs (Ashok and Upadhyaya, 2012). All five endophytes crude extracts revealed the presence of flavonoids and tannins, except *Pseudomonas* spp. SSRN1 showed absence of tannins as indicated in Table 4. Elof et al. (2008) reported that the family Combretaceae contains a wide variety of tannins and flavonoids. Presence of flavonoids was also detected in other plants of the same genera named *Combretum erythrophyllum* (Bhatnagar et al., 2012). Thus, presence of flavonoids and tannins in *C. molle* indicates therapeutic potential of the plant.

Antimicrobial activity

The crude extracts of the putative endophytic bacterial strains were assayed for antimicrobial activity against pathogenic strains (Gram-negative strains: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. oxytoca* ATCC 13182; Gram-positive: *S. aureus*, NCTC 6571 and *B. cereus*, ATCC 10876). Among the five endophytic bacteria, only four except *Staphylococcus* spp. LCP showed antimicrobial activity. *Pseudomonas* spp. SSRN1 and *Enterobacter* spp. SSRP1 were considered as the most active strains as they both had a moderate activity against *S. aureus*. High zone of inhibition was by *Pseudomonas* spp. SSRN1 and *Enterobacter* spp. SSRP1, followed by *Lysinibacillus* spp. HSRN, then lastly *Bacillus* spp. LRP (Table 5). Endophytic bacteria have potential to produce novel natural compounds with antibacterial and antifungal activity (Christina et al., 2013). Bacterial endophytes (*Pseudomonas* spp. and *Bacillus* spp.) isolated from *Plectranthus tenuiflorus* have shown great antimicrobial activity against some human pathogenic strains such as *Salmonella typhi*, *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae* *Proteus mirabilis*, *Candida albicans* (EI-Deeb et al.,

Table 5. Antimicrobial activity of Endophytic bacteria from *C. molle*.

Pathogenic strains	*Staphylococcus* spp. LCP	*Bacillus* spp. LRP	*Pseudomonas* spp. SSRN1	*Enterobacter* spp. SSRP1	*Lysinibacillus* spp. HSRN	*Streptomycin* 1 mg/L
Bacillus cereus ATCC 10876	-	+	+	+	+	+++
Staphylococcus aureus NCTC 6571	-	-	++	++	+	+++
Escherichia coli ATCC 25922	-	+	+	+	+	+++
Pseudomonas aeruginosa ATCC 27853	-	+	+	+	+	+++
Klebsiella oxytoca ATCC 13182	-	-	-	-	-	++

+, Weak activity; ++, moderate activity; +++, Strong activity; -, No zone of inhibition.

2013). Furthermore, *Enterobacter* spp. isolated from *Raphanus sativus* L. also showed antibacterial activity against a few human pathogenic bacteria including *E. coli, Salmonella enteritidis, Shigella sonnei, Salmonella typhimurium, P. aeruginosa, Shigella flexneri* and *B. cereus* (Seo et al., 2010). *Pseudomonas* spp. have proven to possess antimicrobial compounds called ecomycins and pseudomycins (Christina et al., 2013). Secondary metabolites from *C. molle* were also reported to possess antimicrobial activity (Fankam et al., 2015; Kaleab et al., 2006). It is evident from the current study that the isolated bacterial endophytes also have antibacterial activity with a broad antibacterial spectrum. Thus, the bacterial endophytes with antibacterial activity from the current study can play a part in inhibiting plant pathogens growth. In addition, potential applications such as drug discovery and biocontrol use in agriculture can arise from these bacterial endophytes and necessitates further investigations.

Conclusion

This is the first study to report on bacterial endophytes occurrence in *C. molle.* The reported bacterial endophytes isolated have been shown to be a rich source of diverse bioactive compounds with potential applications in drug discovery and agriculture. Studies are currently underway to ascertain if the bacterial endophytes produce the same or similar secondary metabolites as their plant host *C. molle.* Further studies can lead to the development of novel therapeutic drugs from secondary metabolites produced by these bacterial endophytes. Thus, in addition to the well-established photochemistry and bioactivity of *C. molle*, there is now evidence of an extended and potential alternative source of antimicrobials.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was funded by the National Research Foundation of South Africa and University of Johannesburg merit bursary for postgraduate students.

REFERENCES

Ademola IO, Eloff JN (2010). Veterinary parasitology *in vitro* anthelmintic activity of *Combretum molle* (R. Br. ex G. Don) (Combretaceae) against *Haemonchus contortus* ova and larvae. Vet. Parasitol. 169:198-203.

Bauer AW, Kirby WMM, Serris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. 45:493-496.

Ashok PK, Upadhyaya K (2012). Tannins are Astringent. J. Pharmaco. Phytochem. 1:45-50.

Bhalodia NR, Shukla VJ (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* L.: An ethnomedicinal plant. J. Adv. Pharm. Technol. Res. 2:104-109.

Bhatnagar S, Sahoo S, Mohapatra AK, Behera DR (2012). Phytochemical analysis, antioxidant and cytotoxic activity of medicinal plant *Combretum roxburghii* (Family: Combretaceae). Inter. J. Drug. Dev. Res. 4:193-202.

Chaudhry V, Patil PB (2016). Genomic investigation reveals evolution and lifestyle adaptation of endophytic Staphylococcus *epidermidis*. Sci. Rep. 6:1-11.

Christina A, Christopher V, Bhore SJ (2013). Endophytic bacteria as a source of antibiotic: An overview. Pharmacogn. Rev. 7:11-16.

Collins MD, Falsen E, Brownlee K, Lawson PA (2004). *Helcococcus sueciensis* sp. nov., isolated from a human wound. Int. J. Syst. Evol. Microbiol. 54:1557-1560.

Costelloe C, Metcalfe C, Lovering A, Mant D, Hay AD (2010). Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. BMJ 340:1-10.

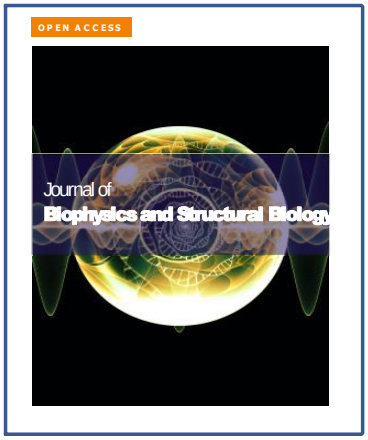
El-Deeb B, Fayez K, Gherbawy Y (2013). Isolation and characterization of endophytic bacteria from *Plectranthus tenuiflorus* medicinal plant in Saudi Arabia desert and their antimicrobial activities. J. Plant. Interact. 8:56-64.

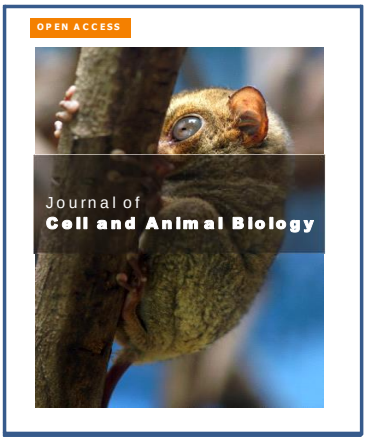
Eloff JN, Katerere DR, Mcgaw LJ (2008). The biological activity and chemistry of the southern African Combretaceae. J. Ethnopharm. 119:686-699.

Fankam AG, Kuiate JR, Kuete V (2015). Antibacterial andantibiotic resistance modifying activity of the extracts

from *Allanblackia gabonensis*, *Combretum molle* and *Gladiolus quartinianus* against Gram-negative bacteria including multi-drug resistant phenotypes. BMC Complement. Altern. Med. 15:1-12.
Golding CG, Lindsey L, Lamboo GC, Beniac DR, Booth TF(2016). The scanning electron microscope in microbiology and diagnosis of infectious disease. Sci. Rep. 4:1-8.
Gonzalez-Teuber M, Jimenez-Aleman GH, Boland W (2014). Foliar endophytic fungi as potential protectors from pathogens in myrmecophytic *Acacia* plants. Commun. Integr. Biol. 7:1-4.
Gupta RM, Kale PS, Rathi ML, Jadhav NN (2015). Isolation, characterization and identification of endophytic bacteria by 16S rRNA partial sequencing technique from roots and leaves of *Prosopis cineraria* plant. Asian. J. Plant. Sci. Res. 5:36-43.
Harbourne JB (1983). Phytochemical screening methods: A guide to modern techniques of plant analysis. Chapman and Hall, London.
Jasim B, Joseph, AA, John C, Mathew J, Radhakrishnan EK (2014). Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zingiberofficinale*. 3 Biotech. 4:197-204.
Joseph BS, Kumbhare PH, Kale MC (2013). Preliminary phytochemical screening of selected Medicinal Plants. Int. Res. J. Sci. Eng. 1:55-62.
Kabera JN, Semana E, Mussa AR, He X (2014). Plant secondary metabolites: Biosynthesis, classification, function and pharmacological properties. J. Pharm. Pharmacol. 2:377-392.
Kaleab A, Avijit M, Franz B (2006). Antibacterial and antifungal activities of extracts of *Combretum molle*. Ethiop. Med. J. 44:269-77.
Kumar GA, Antony RA, Kannan VR (2015). Exploration of endophytic microorganisms from selected medicinal plants and their control potential to multi drug resistant pathogens. J. Med. Plants. Stud. 3:49-57.
Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33:1870-1874
Liu H, He X, Song X, Xu L, Zhang Y, Zhou G, Zhu W, Chang C, Yin Z, Shi Y, Wang C, Chang H (2016). Infection, genetics and evolution isolation and molecular and phylogenetic analyses of encephalomyocarditis virus from wild boar in central China. Int. J. Mol. Epidemiol. Genet. 40:67-72.
Maloney KN, MacMillan JB, Kauffman, CA, Jensen, PR, DiPasquale AG, Rheingold AL, Fenical W (2009). Lodopyridone, a structurally unprecedented alkaloid from a marine actinomycete. Org. Lett. 11:5422-5424.
Morais-Lima GR, Praxedes de Sales IR, Filho MRDC, Taveira de Jesus NZ, Falcao HS, Barbosa-filho JM, Cabral AGS, Souto AL, Tavares JF, Batista, LM (2012). Bioactivities of the genus *Combretum* (Combretaceae): A review. Molecules 17: 9142-9206.
Nair DN, Padmavathy S (2014). Impact of endophytic microorganisms on plants, environment and humans. Sci. World. J. 1-11.
Ojewole JA (2009). Cardiovascular effects of mollic acid glucoside, a1 alpha hydroxycycloartenoid saponin extractive from *Combretum molle* R Br ex G Don (Combretaceae) leaf. J. Nat. Med. 63:117-123.
Ravnikar M, Tercelj M, Janes D, Strukelj B, Kreft S (2015). Antibacterial activity of endophytic fungi isolated from *Conifer needles.* Afr. J. Biotechnol. 14: 867-871.
Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008). Bacterial endophytes: Recent developments and applications. FEMS. Microbiol. Lett. 278:1-9.
Sandhu SS, Kumar S, Aharwal RP (2014). Isolation and identification of endophytic fungi from *Ricinus communis* Linn. and their antibacterial activity. Int. J. Res. Pharm. Chem. 4:611-618.
Santos IP, Silva LCN, Silva MV, Araújo JM, Cavalcanti MS, Lima VLM (2015). Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae). Front. Microbiol. 6:1-7.
Schadler S, Burkhardt C, Kappler A (2008). Evaluation of electron microscopic sample preparation methods and imaging techniques for characterization of cell-mineral aggregates. Geom. J. 25:228-239.
Seo WT, Lim WJ, Kim EJ, Yun HD, Lee YH, Cho KM (2010). Endophytic bacterial diversity in the young Radish and their antimicrobial activity against pathogens. J. Korean. Soc. Appl. Biol. Chem. 53:493-503.
Strobel G, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev. 67:491-502.
Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526.
Trease GE, Evans WC (1983). Pharmacognosy. 14th edtition, Publ. Brown Publications.
Wright ES, Yilmaz LS, Noguera DR (2012). DECIPHER, a Search-based approach to chimera identification for 16S rRNA Sequences. Appl. Environ. Microbiol. 83:717-725.
Yeates C, Gillings MR, Davison AD, Altavilla N, Veal DA (1997). PCR amplification of crude microbial DNA extracted from soil. Lett. Appl. Microbiol. 25:303-307.
Zhao L, Xu Y, Lai XH, Shan C, Deng Z, Ji Y (2015). Screening and characterization of endophytic *Bacillus* and *Paenibacillus* strains from medicinal plant *Lonicera japonica* for use as potential plant growth promoters. Brazil. J. Microbiol. 46:977-989.

Related Journals:





academicJournals

www.academicjournals.org