Chemical constituents and antibacterial activities of essential oils from aerial parts of three *Limnophila species* (Scrophulariaceae) in Southern Vietnam

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Abstract
In this study, essential oils from aerial parts of three Limnophila species in certain southern regions of Vietnam, including L. aromatica, L. chinensis and L. rugosa were extracted using steam distillation and the chemical compositions were analyzed by GC-MS. The chemical compositions of essential oils from L. aromatica and L. rugosa were compared with those in previous studies, in which samples were collected in other areas of Vietnam and other Asian countries.

As a result, the essential oil yields of L. rugosa, L. chinensis and L. aromatica were 0.12%, 0.10% and 0.06% respectively. The chemical composition of the essential oil from L. aromatica had 13 substances, majority of which were limonene (46.1%), trans-bicyclo [5.4.0] undecane (21.8%) and (+)-trans-isolimonene (18.6%). Essential oil of L. rugosa had 9 substances, in which methyl chavicol (73.5%) and trans-anethole (25.6%) were predominant while β-ocimene (40.1%), 3-carene (27.5%), 2-carene (12.9%), β-terpinene (7.1%) and α-ocimene (5.8%), β-caryophyllene (2.6%) were 6 main substances out of 14 compounds finding L. chinensis species. In addition, antibacterial activity assays in this study demonstrated that essential oils from aerial parts of three Limnophila species were resistant to five bacterial pathogens including Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhimurium.

Keyword: *Limnophila*, essential oils, gas chromatography-mass spectrometry.

Introduction

Limnophila R.Br. is a plant genus of Scrophulariaceae with around 40 species found worldwide. They are mainly located in tropical and subtropical regions of Southeast Asia, South Asia, Pacific islands as well as North America. They are herbaceous habitats which are aquatic or semi-aquatic environments such as marshes, riversides, forest paths or similar wet places[1-2]. Several species in this genus have been widely used in food and medicine by indigenous people in many Asian countries, majority of which are *L. aromatica, L. micrantha, L. rugosa, L. indica, L. conferta andL. gratissima*[3]). Some biological activities of *Limnophila* species have recently been reported such as antimicrobial, anti-inflammatory, antioxidant, antitubercular, cytotoxic, anthelmintic and wound healing activities[3].

Recently, Vietnam has been known to be home to 17 *Limnophila* species, some of which have been used as vegetables and spices in cuisine, such as *L. aromatica* and *L. rugosa*[4]. However, researches on chemical compositions and biological activities of those species in Vietnam are still limited. Of those, *L. aromatica* and *L. rugosa* are the only two that have been studied in terms of chemical composition and biological activity in Vietnam and some other Asian countries. For example, the chemical composition of essential oils from *L. aromatica* collected in Northern Vietnam has been reported in some studies in Vietnam[5], Bangladesh[6] and Thailand[7].

Furthermore, the whole *L. aromatica* tree has been known for its many medicinal uses, such as antibacterial, diuretic, stomach pain, viral jaundice, fever andeye pain[8], anti-inflammatory[9], anti-tumor[10] and antioxidants[11]. Similarly, *L. rugosa* has also been studied for the chemical composition of essential oils with samples collected in the south[12] and the Northern Vietnam[13], China[14] and India[15]. *L. rugosa* is also used in traditional medicine to treat a number of diseases such as diarrhea, indigestion, abdominal pain[3].

In this work, we studied on chemical composition and antibacterial ability of essential oils extracted from aerial parts of three *Limnophila* species collected in southern Vietnam including *L. aromatica, L. rugosa.* and *L. chinensis*. The chemical composition of essential oils from *L. aromatica* and *L. rugosa* was compared with those in previous studies, in which samples were collected in different areas in Vietnam and some other Asian countries while that from *L. chinensis* was first conducted. Particularly, antibacterial activity of essential oils from *L. chinensis* and of *L. aromatica* was analyzed.

Material and Methods

Plant materials: Specimens of the three studied species (Figure 1) collected in southern Vietnam are mentioned in table 1. The specimens were stored in herbarium of Binh

Chau-Phuoc Buu Nature Reserve, Xuyen Moc District, Ba Ria-Vung Tau Province.

Bacterial strains: Bacterial strains used for the antimicrobial activity assay in this study were *Bacillus subtilis* (ATCC 11774), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13076) and *Salmonella typhimurium* (ATCC 13311). The strains were preserved in 20% glycerol at -20° C in Microbiology Laborotary, Department of Biotechnology, Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City. They were transferred to Luria-Bertani broth and incubated at 37^0C for 24 hours prior to the analysis.

Distillation of the essential oils: Classical steam distillation using Clevenger apparatus was used to extract the essential oils from aerial parts of the specimens. In this process, 500 g of washed and drained sample was placed in a distillation flask and filled with 1500 ml of distilled water. The flask was then subjected to Clevenger apparatus to extract the essential oils for 4 hours. Essential oils were attracted by evaporated water to form the mixture of steam and essential oils, which is then condensed by refrigerant into liquid. The yields of essential oil were calculated using the following formula RO = M/B_m x 100% (M: Mass of essential oils; B_m: Mass of sample)[16].

Gas chromatography/mass spectrometry (GC/MS) analysis: The chemical composition of the extracted essential oils was determined using gas chromatography coupled with mass spectrometry (GC / MS) on Aligent GC 7890B-MS 5975C with HP-5MS column (30m × 250µm, 0.25µm). In the process, helium was used as carrier gas at a pressure of 13,209psi; volume of 0.2 µl. The thermal program was set to start at 50 degrees Celsius and increase linearly to 320 ° C with a heating rate of 8°C min^{-1}. The retention indices of compounds were calculated using C8-C30 Alkanes Calibration Standard obtained from Sigma Co.

Table 1
Collection sites and voucher numbers for samples of three *Limnophila species* in this paper

Scientific names	Voucher numbers	Collection site	Location
L. aromatica	Do 01	Hoc Mon District, Ho Chi Minh City	10°50'40.5"N; 106°35'18.6"E
L. rugosa	Do 02	Cu Chi District, Ho Chi Minh City	10°56'10.2"N; 06°37'28.9"E
L. chinensis	Do 03	Binh Chau-Phuoc Buu Nature Reserve, Xuyen Moc District, Ba Ria-Vung Tau Province	$10^0$32'47"N; $107^0$28'57"E

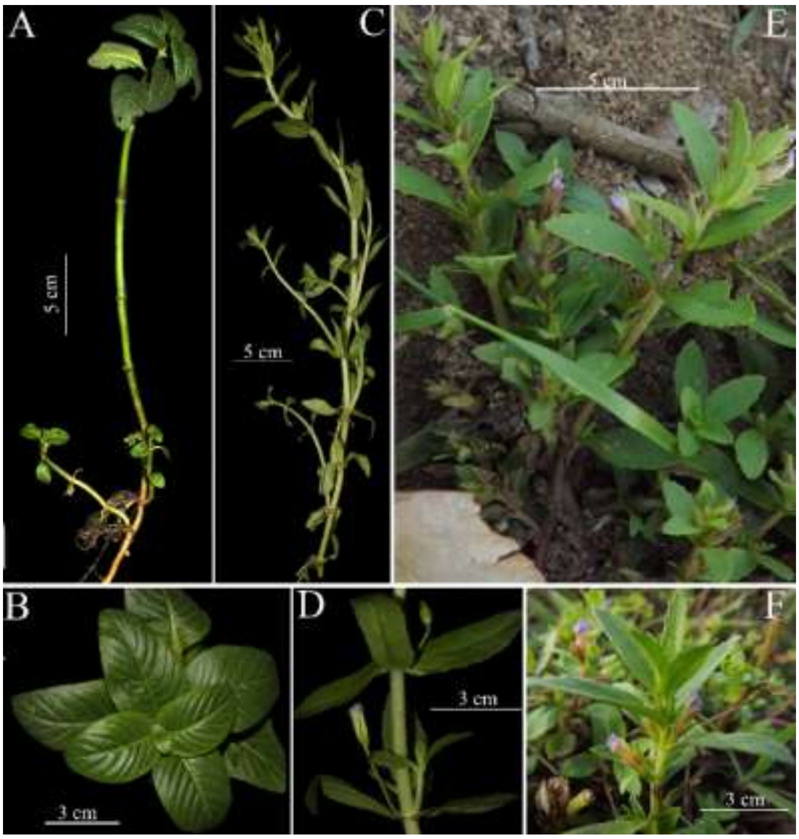

Figure 1: Three specimens in this study. A-B: *L. rugosa.* C-D: *L. aromatica*. E-F: *L. chinensis*

Antibacterial activity assay: The antibacterial activity of essential oils was analyzed according to Bauer et al.[17] The aforesaid bacterial strains were cultured in Luria-Bertani Broth until 0.5 McFarland turbidity standard was reached. This bacterial culture was used to test the antibacterial activity of essential oils in the process called disc diffusion test, in which 0.1 ml of bacterial culture was spread on Petri plate containing Mueller Hinton Agar medium. The sterile paper discs containing 10ul of the essential oil solution were placed on the surface of the Petri dish spread with bacteria.

The plate was left at 4^0C for 2 hours to let essential oils to be absorbed completely into agar medium. The plate was then incubated at 37^0C for 16-18 hours. Gentamycin antibiotic disc (Nam Khoa, Vietnam) was used as a positive control for the experiments. Zone of inhibition was measured after 16-18 hours of incubation to evaluate the resistance of essential oils against the bacterial strains. The experiment was conducted in triplicate and the results were expressed as mean ± standard deviation. Data were statistically interpreted and calculated using Statgraphics Centurion 15.2 and Excel 2010 software.

Results and Discussion

Chemical compositions: The yields of essential oil from *L. rugosa*, *L. chinensis* and *L. aromatica* were 0.12%, 0.10% and 0.06%, respectively. The essential oil compositions from *L. rugosa*, *L. aromatica* and *L. chinensis* were 9, 13 and 14 respectively (Table 2, 3 and 4). Although the three species belong to the same genus *Limnophila*, their essential oil compositions were greatly variable. We noticed that humunele were found both in *L. aromatica* and *L. chinensis* with the contents of 2.85% and 1.61% respectively.

As aforesaid, *L. rugosa* is a common species, essential oil composition of which has been studied by several previous studies. The essential oil composition from aerial parts of *L. rugosa* in this study was consistent with those of previous studies. Noticeably, the two major components found were methyl chavicol and trans-anethole, whose content varied greatly with different geographical areas. For instance, the contents of methyl chavicol and trans-anethole in this study were 73.5% and 25.6% respectively (Table 4) which was equivalent to 70.8% and 24.9% found in a study by Nguyen and Le[12].

This was likely due to that fact that the specimens of the two studies were collected at two geographically-close regions in Southern Vietnam, namely Ho Chi Minh City and Tay Ninh Province. Meanwhile, the essential oil of *L. rugosa* collected from Northern Vietnam and China had considerably high amounts of trans-anethole (89.4% and 76.4% respectively) and low contents of methyl chavicol of 0.4 and 21.9%[13,14]. Therefore, this result re-confirms that the essential oil composition in *L. rugosa* species varies depending on different geographical areas, which have been proved by a number of previous studies.

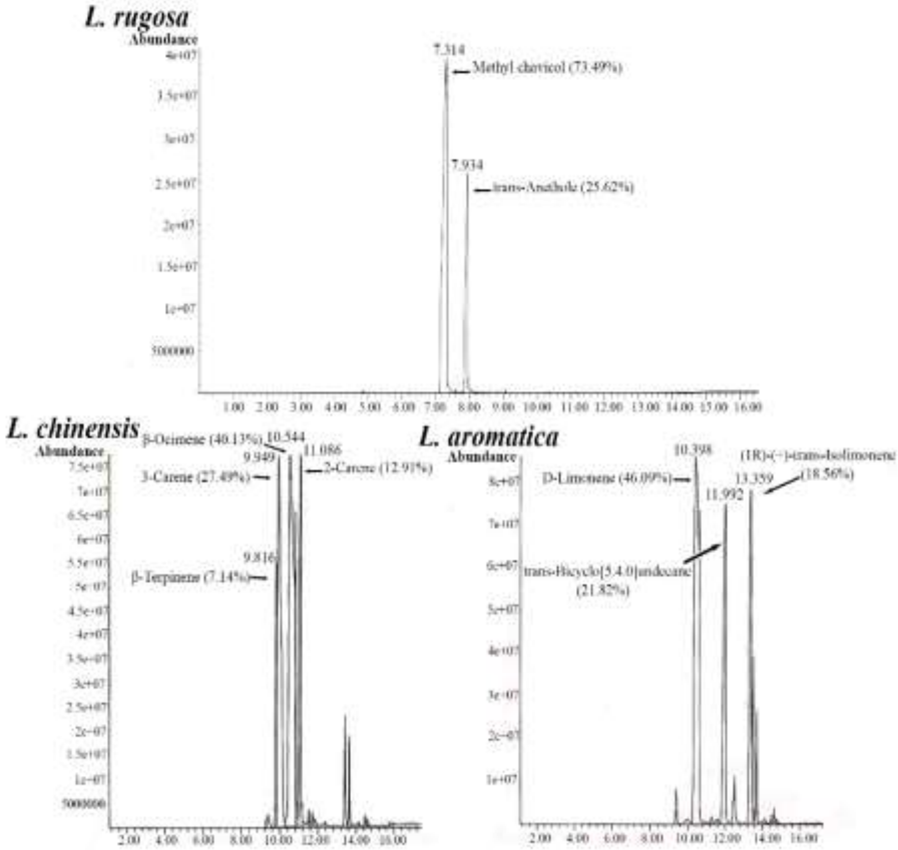

Figure 2: Chromatograms of essential oil components from the three studied species.

Table 2
Chemical constituents of essential oils from aerial parts of *L. aromatica*

Compounds	RI	%
α-pinene	1293	1.1
t-butylglyoxal	1328	0.6
Limonene	1349	46.1
5,10-unecadineoic aci, 2-,ethylene-, methyl ester	1398	0.3
7-methylenebicyclo [4.2.0] octane	1419	0.4
trans-bicyclo[5.4.0]undecane	1445	21.8
Cyclohptene, 5-ethylidene-1-methyl-	1481	2.4
(+)-trans-isolimonene	1535	18.6
p-mentha-1,8,ien-7-yl acetate	1545	4.8
Humunele	1560	2.8
1,6,10-Doecatrien-3-ol, 3,7,11-trimethyl-	1590	0.2
1,7-octaiene, 2,7-imethyl-3,6-bis(methylene)-	1616	0.2
δ-cadinene	1637	0.2
Total		99.5

Table 3
Chemical constituents of essential oils from aerial parts of *L. chinensis*

Compounds	RI	%
trans-thujene	1288	0.2
γ-terpinene	1292	0.6
β-terpinene	1316	7.1
3-carene	1324	27.5
β-ocimene	1357	40.1
α-ocimene	1374	5.8
2-carene	1389	12.9
(S)-camphor	1415	0.3
Butanoic acid, but-3-yn-2-yl este	1426	0.4
1-(1-adamatyl)-1-phenylethaol	1435	0.3
β-caryophyllene	1540	2.6
Humunele	1560	1.6
3-undecen-5-yne, (E)-	1615	0.2
Cycclohexan	1627	0.1
Total		99.7

Table 4
Chemical constituents of essential oils from aerial parts of *L. rugose*

Compounds	RI	%
1-hexen-3-ol	1045	0.1
3,7-dimethyl-4,6-octadien-3-ol	1128	0.1
Methyl chavicol	1186	73.5
trans-anethole	1218	25.6
Benzofuran-3(2H)-one, 6-(3-chloropropoxy)-	1248	0.2
trans-α-Bergamotene	1264	0.1
2H-1-benzopyran-2-one	1271	0.1
α-caryophyllene	1276	0.1
Oxalic acid, allyl pentadecyl este	1284	0.1
Total		99.9

L. aromatica is another common species in genus *Limnophila*, whose chemical composition has been investigated by several previous studies. However, the specimens from those studies were mainly collected in areas, which are geographically far away from our sampling area. For example, Do et al[5] identified some major components in essential oils from *L. aromatica* collected in Northern Vietnam, such as methyl benzoate (27.7%), pulegone

(23.4%), limonene (20.2%) while in this study were mainly limonene (46.1%), trans-bicyclo [5.4.0] undecane (21.8%) and(+)-trans-isolimonene (18.6%) (Table 2). Thus, *L. aromatica* specimens collected in the North[5] and the South (from this study) Vietnam were similar in limonene, while the remaining substances were different.

Another study has reported the essential oil composition of *L. aromatica* species in Thailand, mainly containing limonene (15.1%), (+)-trans-isolimonene (14.5%) and α-humulene (6.0%)[7], which was in agreement with our results with limonene, (+)-trans-isolimonene and humulene contents of 46.1%, 18.56% and 2.8% respectively.

Meanwhile, essential oil composition of *L. aromatica* in Bangladesh contained mainly (Z)-ocimene (39.21%), terpinolene (17.2%), camphor (12.8%) and β-myrcene (9.4%)[6] which was significantly different from that of this species specimens collected in the south of this study as well as in the north of Vietnam[5] and Thailand[7].

L. chinensis, also known as *L. synalieri* or *L. hirsuta*, is another member of genus *Limnophila* in this study, which has a wide distribution in many countries including China, Cambodia, India, Indonesia, Laos, Malaysia, Thailand, Australia and Vietnam[2,4]. Despite its wide distribution, *L. chinensis* is less common and has never been studied for its chemical composition and biological activity[13,18].

This work studied the chemical composition of essential oils from *L. chinensis*. As a result, the essential oil of *L. chinensis* in this study had a total of 14 substances, majority of which included 6 substances including β-ocimene (40.1%), 3-carene (27.4%), 2-carene (12.9%), β-terpinene (7.1%) and α-ocimene (5.8%), β-caryophyllene (2.6%) (Table 3).

Bacterial activities: The antibacterial ability of essential oils in some species of genus *Limnophila* has also been recorded in previous studies. For example, the essential oils of *L. conferta* and *L. gratissima* were proven to be resistant to four bacterial strains including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa*[19,20]. Similarly, Nguyen and Le[12] have reported that the essential oil from *L. rugosa* was resistant to 4 bacterial strains (*S. aureus, B. subtilis, E. coli, P. aeruginosa*) and 1 yeast strain (*Candida albicans*).

In this study, essential oil extracted from aerial parts of *L. rugosa* was greatly resistant to two *Samonela* strains (*S. enteritidis and S. typhimurium*) with average inhibition zone of 17.2 ± 0.8mm and 18.3 ± 1.5mm followed by *E. coli* (12.7 ± 1.5mm), *B. cereus* (11.3 ± 0.6mm) and *P. aeruginosa* (9.7 ± 0.6mm) (Table V, Figure III). Meanwhile, the antibacterial ability of essential oils from *L. aromatica* and *L. chinesis* has never been studied before and this study reported the antibacterial ability of essential oil extracted from aerial parts of these two species.

As a result, the essential oils of *L. rugosa* was resistant against 4 bacterial strains including *S. enteritidis, P. aeruginosa, B. cereus* and *E. coli* with average inhibition zone of 14.3 ± 0.6mm, 14.2 ± 0.8mm, 13.8 ± 0.8mm and 11.6 ± 0.6mm respectively while that of *L. chinesis* was resistant to *S. enteritidis* (16.3 ± 0.8mm), *E. coli* (15.3 ± 1.0) and *B. cereus* (13.2 ± 1.0mm) (Table 5, Figure 3).

Many compounds in essential oil extracted from the aerial parts of the three *Limnophila* species have been proved to be resistant to many bacterial strains in previous studies. Among those, limonene has been known as the main component of essential oils extracted from some plant species, which is resistant to pathogenic bacteria. For example, limonene at a concentration of 1000ul l^{-1} was able to resist against several pathogenic bacteria such as *Salmonella senftenberg, Escherichia coli, Staphylococcus aureus* and *Pseudomonas*[21].

In another study, limonene accounting for 46% in essential oil of *Pimpinella flabellifolia* was resistant to *Klebsiella pneumoniae*[22]. Many previous studies have demonstrated that all essential oils containing methyl chavicol as a major component were resistant to many bacterial strains[23].

For instance, methyl chavicol accounting for 87.63% of essential oil extracted from *Ocimum ciliatum* was resistant to many bacterial strains such as *Ralstonia solanacearum, Pseudomonas syringae pv. lachrymans, P. syringae* pv. *syringae, P. tolaasii, Xanthomonas oryzae* pv. *oryzae, X. citri, Brenneria nigrifluens, Pantoea stewartii* subsp. *indologenes, Rhodococcus fascians* and *Agrobacterium vitis*[24].

Table 5
Inhibition zone of essential oils extracted from aerial parts of three *Limnophila* species against five bacterial strains

Tested bacteria	Growth inhibition zone (mm)		
	L. aromatica	*L. chinensis*	*L. rugosa*
B. cereus	11.3±0.6	13.2±1.0	13.8±0.8
E. coli	12.7±1.5	15.3±1.0	11.6±0.6
P. aeruginosa	9.7±0.6	-	14.2±0.8
S. enteritidis	17.2±0.8	16.3±0.8	14.3±0.6
S. typhimurium	18.3±1.5	-	-

In addition, several studies have shown that trans-anethole had an inhibitory mechanism on the growth of *Arthrobacter* sp. or *Staphylococcus aureus*[25,26]. In 2009, Shafaghat[27] demonstrated that β-ocimene, a main compound found in three essential oils extracted from flowers (41.0%), leaves (24.9%) and stem (54.2%) of *Chaerophyllum macropodum*, was able to inhibit the growth of *S. typhimurium* and *E. coli*[27].

Recently, Shu et al[28] showed that 3-careen had the ability to inhibit the activity of two food-borne germs including *Brochothrix thermosphacta* and *Pseudomonas fluorescens* through mechanisms effecting on cell wall permeability, release of potassium ion, cell membrane, enzyme activity.

Conclusion

In this study, the extraction efficiency of essential oils from *L. rugosa*, *L. chinensis* and *L. aromatica* was 0.12%, 0.10% and 0.06% respectively. The chemical composition of the essential oil of *L. aromatica* had 13 substances with four main components including limonene (46.1%), trans-bicyclo [5.4.0] undecane (21.8%) and (+)-trans-isolimonene (18.6%). *L. rugose* essential oil had 9 substances with 2 main substances: Methyl chavicol (73.5%) and trans-anethole (25.6%). Meanwhile, β-ocimene (40.1%), 3-carene (27.5%), 2-carene (12.9%), β-terpinene (7.1%) and α-ocimene (5.8%), β-caryophyllene (2.6%) were the 6 major substances in 14 compounds found in essential oils of *L. chinensis*.

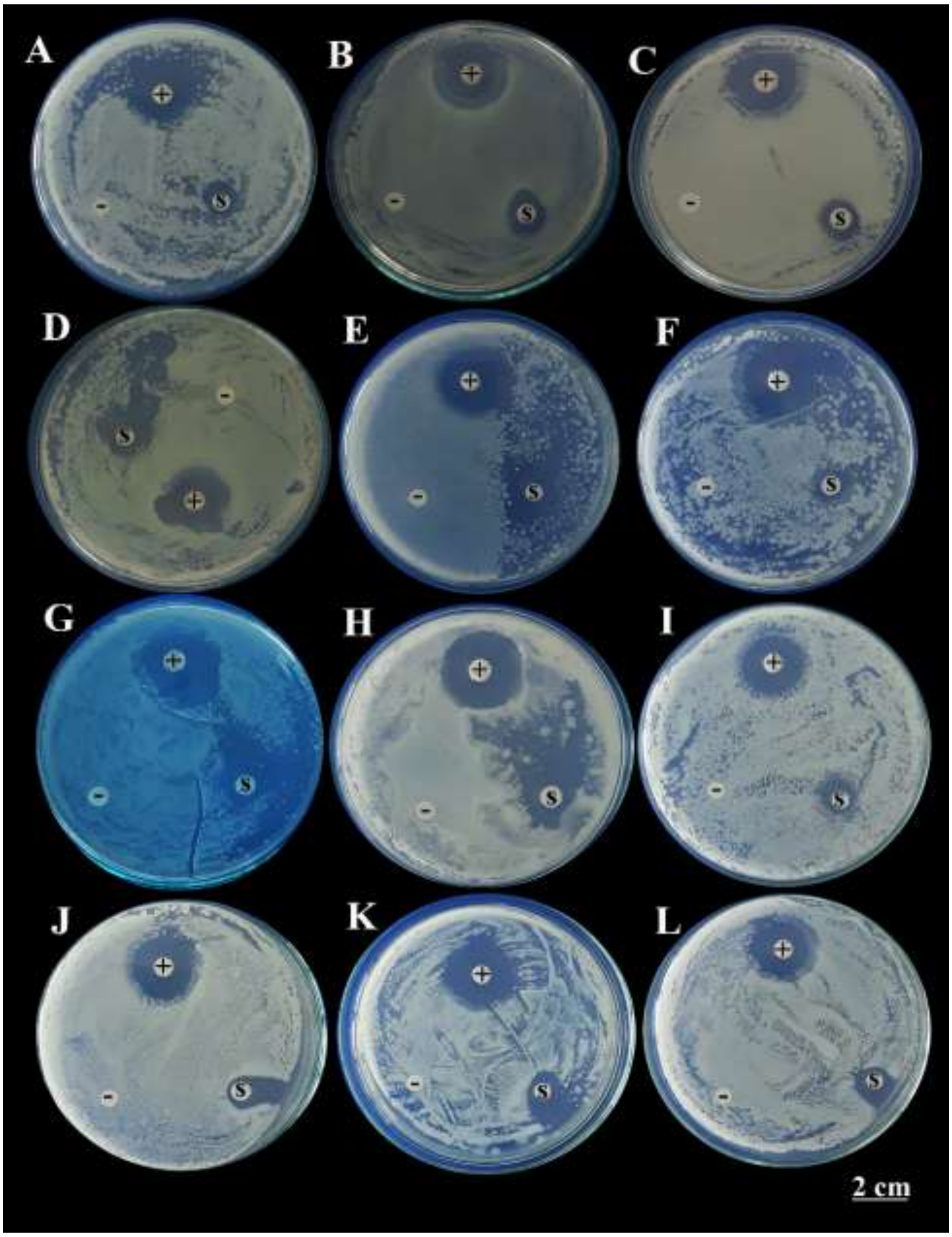

Figure 3: Antibacterial activity of essential oils extracted form aerial parts of three *Limnophila* species against 5 bacterial strains. *A-E: L. aromatica. A- B. cereus*. B- *E. coli,* C-*P. aeruginosa,* D-S. *enteritidis,* E-*S. typhimurium*; F-H. *L. chinensis*. F-*B. cereus;* G- *E. coli,* H-S. *enteritidis; I-L: L. rugosa. I-B. cereus*. J- *E. coli,* K-*P. aeruginosa,* L-S. *enteritidis*. (-) Negative control with sterilized distilled water, (+) Positive control with discs containing gentamicin.

In addition, this study also showed that essential oils from aerial parts of three *Limnophila* species were resistant to five bacterial pathogens including *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Salmonella typhimurium*.

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