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ORIGINAL ARTICLE

In vitro antibacterial properties of essential oil and organic extracts of *Premna integrifolia* Linn

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Abstract The aims of this study were to examine the chemical composition of the essential oil of *Premna integrifolia* Linn (Lamiaceae), and to test the efficacy of the oil and various organic extracts as an antibacterial potential. The chemical compositions of the essential oil were analyzed by GC–MS. Twenty-nine compounds representing 94.81% of the total leaves oil were identified, of which phytol (27.25%), α-humulene (14.21%), spathulenol (12.12%), 1-octen-3-ol (8.21%), eugenol (6.69%) and phenylethyl alcohol (5.81%) were the major compounds. The oil (15 μL disk^{-1}) and extracts (300 μg disk^{-1}) of *P. integrifolia* displayed a great potential of antibacterial activity against *Sarcina lutea* IFO 3232, *Bacillus subtilis* IFO 3026, *Escherichia coli* IFO 3007, *Pseudomonas* sp. ATCC 13867, *Klebsiella pneumoniae* ATCC 10031 and *Xanthomonas campestries* IAM 1671 with their respective zones of inhibition of 12.0 ± 1.2 to 22.1 ± 1.2 mm and MIC values of 62.5–250 μg mL^{-1}. The results of this study suggest that the natural products derived from *P. integrifolia* may have potential use in food, pharmaceutical and/or agro industries for preservatives or antimicrobial agents.

1. Introduction

Many pathogenic microorganisms such as *Sarcina lutea*, *Bacillus subtilis*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas* sp. and *Xanthomonas campestries* have been reported as the causal agents of some diseases in human and plants (McCabe-Sellers and Samuel, 2004). A variety of different chemical and synthetic compounds have been used as antimicrobial agents to inhibit pathogenic bacteria. The demands for more natural antimicrobials have driven scientists to investigate the effectiveness of inhibitory compounds such as essential oils, and extracts from plants (Demirci et al., 2008; Rahman and Kang, 2009). Essential oils are a complex mixture of compounds, mainly monoterpenes, sesquiterpenes, and their corresponding oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols, and oxides) from plants, which are widely known for their scents and flavors. Plant-derived essential oils have been long used as flavoring agents or preser-

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vatives and confectionary products and also have a broad spectrum of *in vitro* antimicrobial activities (Conner, 1993). Thus essential oils and plant extracts are promising natural antimicrobial agents with potential applications in food, pharmaceutical and/or agro industries for controlling of various pathogens and spoiling bacteria.

The pharmaceutical and therapeutic potentials of essential oils and their individual constituents have been evaluated. The essential oil can be used alone, or as a part of therapeutic pharmaceutical composition, which includes at least one antimicrobial compound and a pharmaceutically acceptable carrier. The essential oils and their volatile constituents can be used via inhalation or massage therapy. In the field of complementary and alternative respiratory medicine, inhalation of peppermint essential oil vapors has been suggested as an adjunct in combined multi-drug therapy in patients with disseminated and infiltrative pulmonary tuberculosis (Shkurupii et al., 2002). Massage therapy using essential oil can be useful in the treatment for people suffering from dementia (Ballard et al., 2002). Inflammatory diseases, such as allergy, rheumatism and arthritis are often alleviated using essential oil massage therapy (Maruyama et al., 2005). The use of essential oils may improve food safety and protect our body against bacteria causing food poisoning. If essential oils were to be more widely applied as antibacterials in foods, the organoleptic impact would be important. In addition, it is recommended to apply essential oils or their compounds as part of a hurdle system and to use it as an antimicrobial component along with other preservation techniques, e.g. in combination with reduced temperature and reduced pH or using a synergistic combination of essential oils and their compounds (Ultee et al., 2000).

Premna integrifolia Linn. (Lamiaceae) is a garden shrub, 40 species of this genus are widely naturalized in tropical and subtropical regions throughout the world, including Australia, China and the United States, India and Bangladesh. In Bangladesh, it is found mostly in Chittagong and Sunderbans. It is also been seen cultivated in home yards and gardens. In the ayurvedic system of medicine, *P. integrifolia* is known as Ganikarica while its conventional name is Ganiari. There are many species belonging with this genus but only two species namely *P. latifolia* and *P. integrifolia* were recorded as medicinal species. *P. latifolia* is grown as a large tree but *P. integrifolia* is comparatively smaller known as shrub i.e., a woody plant smaller than a tree with branches lower than the ground and it is generally grown to 5–6 m tall. Normally most species of *Premna e*specially *P. integrifolia* is used for the remedies of different ailments as in the traditional system in the indigenous part of the country. The plant is very effective in reducing blood cholesterol (Dey, 2006). If 12–18 g of leaf and bark is mixed with four cups of water and after boiling a person suffering from Jaundice drinks it, the Jaundice will be reduced and he will come round. It is also used for the correction of menstrual problems. It is very effective for the treatment of kidney disease, liver problems and constipation (Husain et al., 1992).

Previous reports showed that the root of *P. integrifolia* contained some bioactive compounds such as alkaloids, flavonoids, glycosides, tannins, phenolic compounds and diterpenoids (Mali and Bhadane, 2010; Yadav et al., 2010, 2011). In this study, we examined the chemical composition of the essential oil from leaves of *P. integrifolia* by GC–MS, and tested the antibacterial efficacy of the essential oil and various organic extracts with emphasis for the possible future use of the extracts as an alternative to chemical bactericides.

2. Materials and methods

2.1. Plant materials

The leaves of *P. integrifolia* were collected from the Boro Parulia, Gopalgonj; Arappur, Jhenaidah and Ambaria, Kushtia of Bangladesh, in January 2009 and identified by Dr. Oliur Rahman, Bangladesh National Herbarium, Dhaka, where a voucher specimen (DACB 35170) has been deposited.

2.2. Isolation of the essential oil

The air-dried leaves (200 g) of *P. integrifolia* were subjected to hydro distillation for 3 h using a Clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4 °C for further analysis.

2.3. Preparation of organic extracts

The air-dried leaves *P. integrifolia* were first pulverized into powdered form. The dried powder (50 g) was then extracted with hexane, chloroform, ethyl acetate and methanol separately at room temperature for 7 days and the solvents were evaporated by vacuum rotary evaporator temperature at 50 °C. The extraction process yielded hexane (7.3 g), chloroform (6.2 g), ethyl acetate (7.4 g) and methanol (6.5 g) extracts respectively. Solvents (analytical grade) for extraction were obtained from commercial sources (Sigma Aldrich, St. Louis, MO, USA).

2.4. Gas chromatography–mass spectrometry (GC–MS) analysis

The GC–MS was carried out using total ion monitoring mode on a Varian 3800 gas chromatograph interfaced to a Varian Saturn ion trap 2200 GC–MS spectrometer. The temperatures of transfer line and ion source were 280 °C and 275°C respectively. Ions were obtained by electron ionization mode. The VF-5 capillary column (30 m length, 0.25 mm I.D. and 0.25 μm film thickness) was used. A 20% split injection mode was selected with a solvent delay time of 3 min with injection volume 0.2 μL. The initial column temperature was started at 50 °C for 1 min, programed at 8 °C min^{-1} to 200 °C and heated until 280 °C at 10 °C min^{-1}. Injection port was set at 250 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. Molecular ions (mass range: 40–500 m z^{-1}) were monitored for identification. The relative percentage of the oil constituents was expressed as percentage by peak area normalization.

Identification of components of the essential oil was based on their retention indices, relative to a homologous series of *n*-alkane (C_8–C_{20}) on the VF-5 capillary column under the same operating conditions and computer matching with the GC–MS spectra from the Wiley 6.0 MS data and literature data (Adams, 2001).

2.5. Microorganisms

The following microorganisms were used in the antibacterial test: *Sarcina lutea* IFO 3232, *Bacillus subtilis* IFO 3026, *Escherichia coli* IFO 3007, *Pseudomonas* sp. ATCC 13867, *Klebsiella pneumoniae* ATCC 10031 and *Xanthomonas campestries* IAM 1671. The strains were kindly provided by Prof. Yong Se Lee, Department of Bioresource Technology, College of Agriculture and Environmental Science, Daegu University, Korea. Cultures of each bacterial strain were maintained on Luria-Bertani (LB) agar medium at 4 °C.

2.6. Determination of antibacterial activity of essential oil and organic extracts

The dried extracts were dissolved in the same solvent used for their extraction and sterilized by filtration using 0.22 μm sterile Millipore filter (Millipore Corp., Billerica, MA, USA). Then the antibacterial test was carried out by the agar disk diffusion method (Murray et al., 1999) using 100 μL of standardized inoculum suspension containing 10^7 CFU mL^{-1} of bacteria. The essential oil was diluted 1:5 (v/v) with methanol and aliquots of 15 μL were spotted onto the sterile Whatman No. 1 filter paper disks (6 mm diameter); while 10 μL of 30 mg mL^{-1} of each organic extract (300 μg $disk^{-1}$) was applied on the filter paper disks and placed on the inoculated LB agar. Negative controls were prepared using the same solvents employed to dissolve the samples. Standard antibiotic, streptomycin (20 μg $disk^{-1}$ from Sigma–Aldrich Co., St. Louis, MO, USA) was used as positive control for the tested bacteria. The plates were incubated micro aerobically at 37 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition against the tested bacteria. Each assay in this experiment was replicated three times.

2.7. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) of the samples were tested by the standard NCCL method (NCCLS, 2008). Active cultures for MIC determination were prepared by transforming a loopful of cells from stock cultures to flasks and inoculated in LB medium and incubated at 37 °C for 24 h. The cultures were diluted with fresh LB broth to achieve optical density of 10^7 CFU mL^{-1} for the test organisms at 600 nm by UV/Vis Spectrophotometer Optizen 2120UV and Optizen III (Shin et al., 2007). Dilutions, to get the final concentration ranging from 0 to 1000 μg mL^{-1} of essential oil and various organic extracts in Luria-Bertani broth medium were prepared in 96 well microplates. Finally 20 μL inoculum of each bacterial strain (10^7 CFU mL^{-1}) was inoculated onto the microplates and the tests were performed in a volume of 200 μL. The plates were incubated at 37 °C for 24 h. The lowest concentrations of the test samples, which did not show any visual growth of test organisms after macroscopic evaluation, were determined as MICs, which were expressed in μg/mL^{-1}. The MICs were measured for the oil and various extracts, as well as for standards of pure α-humulene, spathuleno and eugenol, which were tested on the same cultures under identical conditions to compare their activity with those of the investigated oil and extracts.

2.8. Statistical analysis

The essential oil and different organic extracts were assayed for antibacterial activity. Each experiment was run in triplicate, and mean values were calculated. The statistical analysis was carried out employing one way ANOVA ($p < 0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

3. Results

3.1. Chemical composition of the essential oil

GC–MS analyses of the oil led to the identification of 29 different compounds, representing 94.81% of the total oil from leaves. The identified compounds are listed in Table 1, according to their elution order on a VF-5 capillary column. The oil contains a complex mixture consisting of mainly oxygenated mono- and sesquiterpenes, and mono- and sesquiterpene hydrocarbons. The major compounds detected in the leaves oil were phytol (27.25%), α-humulene (14.21%), spathulenol (12.12%), 1-octen-3-ol (8.21%), eugenol (6.69%), phenylethyl alcohol (5.81%) and caryophyllene oxide (2.6%), as shown in Table 1. Hexadecanoic acid (1.06%), 1,8-cineole (0.93%), α-pinene (0.86%), β-pinene (1.11%), β-carryophyllene (0.92%)

Table 1 Chemical composition of the essential oil of *Premna integrifolia* Linn.

RI[a]	Compound	Composition (%)
719	Cyclohexane	1.03
856	Hexan-1-ol	0.65
932	α-Pinene	0.86
961	1-Octen-3-ol	8.21
963	β-Pinene	1.11
966	3-Octanol	0.97
1005	1,8-Cineole	0.93
1028	*cis* -2-Octenal	1.13
1136	Phenylethyl alcohol	5.81
1174	Indole	0.91
1182	Decanal	0.87
1200	Dodecane	0.49
1366	Damascenone	0.18
1380	Eugenol	6.69
1386	Azulene	0.61
1410	Isoeugenol	1.83
1414	β-Carryophyllene	0.92
1426	Benzofuranone	0.98
1447	α-Humulene	14.21
1550	Spathulenol	12.12
1561	Caryophyllene oxide	2.60
1580	Cubenol	1.67
1609	Tetradecanal	0.32
1616	Tumerone	0.83
1755	Pentadecanol	0.64
1869	Pentadecanoic acid	0.23
1968	Hexadecanoic acid	1.06
2009	Eicosane	0.62
2045	Phytol	27.25
	Total	94.81

[a] Retention index relative to *n*-alkanes on VF-5 capillary column.

Table 2 Antibacterial activity of essential leaf oil and various extracts of *Premna integrifolia* Linn.

Microorganism	Zones of inhibition (mm)					
	Essential oil	Various extracts				SM
		MeOH	Hexane	$CHCl_3$	EtOAc	
Sarcina lutea IFO 3232	22.1 ± 1.2[a]	20.1 ± 0.5[a]	14.1 ± 0.7[a]	18.2 ± 1.1[a]	20.1 ± 1.2[a]	20.2 ± 0.5[a]
Bacillus subtilis IFO 3026	20.2 ± 1.3[b]	18.2 ± 1.1[b]	14.2 ± 0.5[a]	16.1 ± 1.5[b]	19.3 ± 1.2[a]	16.1 ± 0.7[c]
Escherichia coli IFO 3007	15.3 ± 1.2[c]	15.1 ± 1.0[c]	12.2 ± 1.1[b]	13.1 ± 0.5[c]	15.2 ± 1.1[b]	17.4 ± 1.2[b]
Pseudomonas sp. ATCC 13867	14.2 ± 0.7[cd]	13.1 ± 0.5[d]	12.2 ± 1.1[b]	12.1 ± 1.2[c]	13.1 ± 1.2[c]	16.2 ± 1.3[c]
Klebsiella pneumoniae ATCC 10031	13.1 ± 1.5[d]	13.2 ± 1.1[d]	12.3 ± 1.2[b]	12.0 ± 1.2[c]	13.3 ± 1.6[c]	17.3 ± 0.6[b]
Xanthomonas campestries IAM 1671	14.2 ± 0.5[cd]	14.1 ± 0.5[cd]	12.1 ± 1.2[b]	12.1 ± 1.2[c]	15.2 ± 0.6[b]	16.3 ± 0.6[c]

Diameter of inhibition zones of essential oil including diameter of disk 6 mm (tested at a volume of 15 μL $disk^{-1}$).
Various extracts (300 μg $disk^{-1}$).
Standard antibiotic: SM, streptomycin (20 μg $disk^{-1}$).
Values are given as mean ± S.D. ($n = 3$).
Values in the same column with different superscripts are significantly different ($p < 0.05$).

and 3-octanol (0.97%) were also found to be the minor components of *P. integrifolia* oil in the present study.

3.2. Antibacterial activity of essential oil and organic extracts

The *in vitro* antibacterial activity of essential oil, various organic extracts of *P. integrifolia* against the employed bacteria were qualitatively assessed by the presence or absence of inhibition zones. The oil exhibited antibacterial activity against all two Gram-positive and four Gram-negative bacteria at the concentrations of 15 μL of 1:5 (v/v) dilution with MeOH. The oil exhibited a potent inhibitory effect against *S. lutea* IFO 3232, *B. subtilis* IFO 3026, *E. coli* IFO 3007, *Pseudomonas* sp. ATCC 13867, *K. pneumoniae* ATCC 10031 and *X. campestries* IAM 1671 with diameter of inhibition zones ranging from of 13.1 ± 1.5 to 22.1 ± 1.2 mm, as shown in Table 2. Methanol, chloroform and ethyl acetate extracts of *P. integrifolia* also revealed a great potential of antibacterial activity against all bacteria, at the concentrations of 300 μg $disk^{-1}$ (Table 2). Methanol and ethyl acetate extracts showed the strongest antibacterial effect against *S. lutea* IFO 3232, *B. subtilis* IFO 3026, *E. coli* IFO 3007 and *X. campestries* IAM 1671 (inhibition zones: 20.1 ± 0.5 to 14.1 ± 0.5 mm). On the other hand, chloroform extract showed moderate to high antibacterial effects against most of the bacteria that have being tested (inhibition zones: 12.0 ± 1.2 to 18.2 ± 1.1 mm). Hexane fraction displayed a moderate inhibitory effect against most of the bacteria. In this study, the oil, methanol and ethyl acetate extracts exhibited higher or similar types of antibacterial activity than that of streptomycin against Gram-positive bacteria. The blind control did not inhibit the growth of the bacteria tested. Methanol and ethyl acetate extracts showed higher activity compared with hexane and chloroform extracts.

3.3. Minimum inhibitory concentration (MIC)

As shown in Table 3, the MIC values for the oil were found lower for *S. lutea* IFO 3232, *B. subtilis* IFO 3026, *E. coli* IFO 3007 and *X. campestries* IAM 1671 (62.5–125 μg mL^{-1}) than for *Pseudomonas* sp. ATCC 13867 and *K. pneumoniae* ATCC 10031 (250 μg mL^{-1}). On the other hand, MIC values of the various organic extracts against the tested bacteria were found in the range 62.5–250 μg mL^{-1} (Table 3). In this study, the Gram-positive bacteria were found to be more susceptible to the essential oil and various solvent extractions than Gram-negative bacteria. Standard pure compounds such α-humulene, spathulenol and eugenol in this study were also exhibited potent activity as compared to oil or extracts.

Table 3 MIC of essential oil and various extracts of *Premna integrifolia* Linn and standard pure compounds.

Microorganism	Minimum inhibitory concentration (MIC)							
	Essential oil	Extracts				Standard pure compounds		
		MeOH	Hexane	$CHCl_3$	EtOAc	α-Humulene	Spathulenol	Eugenol
Sarcina lutea IFO 3232	62.5	62.5	250	125	125	3.90	15.62	7.81
Bacillus subtilis IFO 3026	62.5	62.5	250	125	62.5	3.90	15.62	7.81
Escherichia coli IFO 3007	125	125	250	62.5	62.5	> 20	> 20	15.62
Pseudomonas sp. ATCC 13867	250	250	250	125	125	nt	nt	nt
Klebsiella pneumoniae ATCC 10031	250	250	250	250	125	nt	nt	nt
Xanthomonas campestries IAM 1671	125	125	250	250	250	7.81	15.62	7.81

Minimum inhibitory concentration (values in μg mL^{-1}).
A pure compound is considered inactive when MIC > 20 μg mL^{-1}.
nt: Not tested.

4. Discussion

In this study, the essential oil and various organic extracts of *P. integrifolia* exhibited potential activity against some of the bacterial strains such as *S. lutea* IFO 3232, *B. subtilis* IFO 3026, *E. coli* IFO 3007, *Pseudomonas* sp. ATCC 13867, *K. pneumoniae* ATCC 10031 and *X. campestries* IAM 1671. This activity could be attributed to the presence of oxygenated mono- and sesquiterpene hydrocarbons, and these findings are in agreement with the previous reports (Shunying et al., 2005). In our opinion, major components of the oil, phytol (27.25%), α-humulene (14.21%), spathulenol (12.12%), 1-octen-3-ol (8.21%), eugenol (6.69%), phenylethyl alcohol (5.81%) and caryophyllene oxide (2.6%) have key roles for their antibacterial activities. Antibacterial activities of these compounds have been reported by others (Sartoratto et al., 2004; Inouye et al., 2006; Deba et al., 2008). Pure compounds such as α-humulene, spathulenol and eugenol showed potent antibacterial activity in this study. Also, the antibacterial activity of individual components of essential oils such as α-humulene or spathulenol has been reported previously (Bougatsos et al., 2004; Pichette et al., 2006). On the other hand, the components in lower amount such as hexadecanoic acid, 1,8-cineole, α-pinene, β-pinene and β-carryophyllene also contributed to antibacterial activity of the oils (Salehi et al., 2007; Deba et al., 2008). It is also possible that the minor components might be involved in some type of synergism with the other active compounds (Marino et al., 2001). Further, the antibacterial activity of organic extract could be attributed to the presence of some bioactive phytochemicals (alkaloids, flavonoids, steroids, tannins, phenolic compounds, diterpenoids, etc.) in *P. integrifolia* plant and these findings are in agreement with the previous reports (Gokani et al., 2007; Mali and Bhadane, 2010; Yadav et al., 2010, 2011).

In pharmaceutical industries there is a continuing need to find new and improved antimicrobial agents, especially in view of the increasing incidence of antibiotic resistance. One of the areas which is subjected to considerable interest is plant extracts and in particular their essential oils. Also, the increasing consumer demand for effective and safe natural products means that quantitative data on plant oils and extracts are required. Report on the analyses of essential oils from *P. integrifolia* from South Asian regions and its antimicrobial activity is still scare. Therefore, screening of the medicinal plant, *P. integrifolia* growing in Bangladesh, for antimicrobial activity and phytochemicals is important for finding potential new compounds for medicinal or other uses.

5. Conclusion

The essential oil and organic extracts in our study showed a great potential of antibacterial activity against *S. lutea* IFO 3232, *B. subtilis* IFO 3026, *E. coli* IFO 3007, *Pseudomonas* sp. ATCC 13867, *K. pneumoniae* ATCC 10031 and *X. campestries* IAM 1671. This activity could be attributed to the presence of major components (e.g., α-humulene, spathulenol and eugenol) and/or minor components present in the oil. Results of our study suggest the possibility of using the oil or organic extracts of *P. integrifolia* as natural antimicrobials in food or pharmaceutical industry because they possess strong antibacterial activities. The use of plant extracts and essential oils in consumer goods is expected to increase in the future due to the risk of "green consumerism", which stimulates the use and development of products derived from plants, as both consumers and regulatory agencies are more comfortable with the use of natural antimicrobials. However, further research is needed in order to establish the real application of *P. integrifolia* essential oil or extracts in food or pharmaceuticals.

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