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Comparison of Chemical constituents and antibacterial activities and antioxidant activities of the essential oil from leaves and fruits of *Bridelia retusa* (L.) A. Juss.

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The essential oils from the leaves and fruits of *Bridelia retusa* (L.) A.Juss. were isolated by hydrodistillation. The essential oils were obtained in 0.0013% yield as a pale yellow liquid and 0.0026% yield as a violet-light brown liquid for the leaf oil and fruit oil respectively. The composition of each essential oil was analysed by means of GC-(FID) and GC-MS. Eleven constituents accounting for 48.77% of total leaves oil were identified. The most abundant compound was phytol (33.4%), followed by phthalic acid (5.2%), 6, 13-dimethoxy-2, 3, 9, 10-tetramethylpentacene-1, 4, 8, 11-tetrone (3.4%), heptacosane (2.3%) and nonacosane (1.2%). Sixteen constituents accounting for 51.8% of total fruits oil were identified. The major components were dibutyl sebacate (25.6%), phytol isomer (4.8%), diacetin (4.3%), tricosane (3.9%), isophytol (2.7%), erucylamide (2.5%), phthalic acid (1.9%), hexadecanoic acid (1.5%) and eicosane (1.2%). The essential oils exhibited strong antioxidant activities with the IC_{50} values of 1.12±0.0010 mg/mL and 1.79±0.0005 mg/mL for the leaf and fruit essential oils respectively, by using the ABTS radical cation scavenging assay. The antibacterial activity of the essential oils was performed by using the standard disc diffusion method. The results revealed that the leaf and fruit essential oils of *B. retusa* were active against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* with the minimum inhibitory concentrations (MICs) between 20-50 mg/mL.

Keywords: GC-MS, essential oil, *Bridelia retusa* (L.) A. Juss., antibacterial and antioxidant activities.

Introduction

Bridelia retusa (L.) A.Juss. belongs to Euphorbiaceae family, it is a tree up to 10-20 m high. It is found in dry evergreen and deciduous forest and open land, e.g. India, Sikkim, Bhutan, Sri Lanka, Myanmar, Indochina, China, Thailand, Malay Peninsula and Sumatra [1]. It is used in folk medicine to treat diabetes, rheumatism dysentery and diarrhea. Paste of leaf mixed with the leaves of *Curculig orchiodes* and the oils of castor, coconut and gingerly are applied externally to treatment of skin disease [2] and applied externally to cure wound[3]. In India, its bark extract was used as treatment of dysentery[4] and used as contraceptive to develop sterility[5]. A flavonoid isolated from benzene fraction of ethanol extract of the leaves showed antimicrobial activity against human pathogenic bacteria[6]. Eight compounds were isolated from the stem bark of *B. retusa.* They showed antifungal activity against *Cladosporium cladosporioides*[7]. The chloroform extract of fruits showed inhibition zones against *S. aureus*, *E. coli* and *P. aeruginosa* but the methanolic extract showed antibacterial activity against *S. aureus* and *P. aeruginosa*. The methanolic extracts of leaves and stems exhibited antibacterial activity against *S. aureus*, and only the methanolic extract of the leaves showed inhibition zone against *P. aeruginosa*[8]. The methanolic extract of *B. retusa* significantly potentiated the cellular immunity by facilitating the foot pad thickness responses to the sheep RBCs in sensitized rats with a dose of 200 mg/kg. The study stated that *B. retusa* showed a significant stimulation of the cell mediated immunity and no effect

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on the humoral immunity[9]. There is no previous published research on the chemical constituents and antibacterial activity and antioxidant properties of the leaf and fruit essential oils from *B. retusa*. This study reports on the chemical constituents of the leaf and fruit essential oils, obtained by hydrodistillation and the antibacterial and antioxidant activities.

Material and methods

Plant material

Fresh leaves and fruits of *Bridelia retusa* (L.) A. Juss. were collected from Huay Kaew Arboretum, Huay Kaew Road, Chiang Mai, Thailand in June 2010, and identified by J. F. Maxwell, Chiang Mai University, Thailand. A voucher specimen (No. 3) was deposited in the herbarium of Department of Biology, Faculty of Science, Chiang Mai University.

Isolation of the essential oil

Fresh plant materials (1 kg) was chopped into small pieces and subjected to hydrodistillation for 8 hours, in a modified Clevenger-type apparatus, with a water-cooled oil receiver to reduce formation of artifacts due to overheating during hydrodistillation to yield a pale yellow oil (0.0013% of the fresh weight of leaves) and a violet-light brown oil (0.0026% of the fresh weight of fruits). The oil was collected over water, separated and dried over anhydrous sodium sulphate. After that the oil was transferred to a glass vial and kept at a temperature of 4-7°C for further analysis by Gas Chromatography-Mass Spectroscopy (GC-MS).

GC-MS Analysis

The essential oil was analysed on a Hewlett-Packard GC-MSD 6850 series 2 mass spectrometer fitted with a HP-5 (Hewlett-Packard 19091S-433E) cross-linked fused silica capillary column (30 m, 0.25 mm i.d.), coated with 5% phenyl methyl siloxane (0.25 µm film thickness). The analytical conditions were: the oven temperature was programmed from 75°C for 0.50 min, isothermal, then heating to 270°C and isothermally for 35 min at 260°C. Injector temperature was 270°C. Samples were injected automatically by splitting and the split ratio was 1:100. The mass spectrometer had a delay of 2.40 min to avoid the solvent peak and then scanned from m/z 35 to m/z 550. Ionization energy was set at 70 eV. The carrier gas was He at a flow rate of 1.0 mL/min. The identification of volatile components were accomplished by comparison of their GC retention indices (RI) as well as their mass spectra (NIST and NISTREP) with corresponding data of authentic compounds or published spectra.

Antibacterial activity

The antbacterial activity of the essential oil was evaluated by the standard disc diffusion method[10]. The microorganisms used were *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27553), *Bacillus subtilis*, *Salmonella enteritidis* and *Enterococcus sp.* The strains were maintained in agar conservation at room temperature. The strains inoculum were diluted in sterile 0.85% Saline to obtain turbidity visually comparable to a McFarland Nº 0.5 standard (10^{7-8} CFU/mL). Sterile nutrient agar plates were prepared and incubated at 37oC for 24 h to check for any contamination. Sterile filter paper discs (Whatman No.1) of 6 mm diameter were placed in appropriate position on the surface of the plate with quadrants marked at the back of the petri dishes. Ten µL of the oil was carefully added into the sterile filter paper discs by means of steriled dropping automatic pipette. The *in vitro* antibacterial activity of different concentration of essential oil at 10, 20, 30, 40 and 50 mg/mL (in ethanol) was studied by disc diffusion method against *S. aureus*, *E. coli* (ATCC25922), *P. aeruginosa*, *B. subtilis*, *S..enteritidis* and ,*Enterococcus sp* .. The Petri dishes were incubated at 37oC for 24 h and the diameter of the zone of inhibition measured in mm. The zone of inhibition was calculated by measuring the minimum dimensions of the zone of no microbial growth around the disc and minimum inhibitory concentrations were determined. An average of three independent determinations was recorded (Table 3).

Antioxidative assay

ABTS Assay

The antioxidant activity of the essential oil was investigated using the ABTS radical cation scavenging assay[11] compared with the Trolox standard. For the ABTS assay, 20 µL of essential oil (50 mg mL^{-1}) was mixed with 2.0 mL of diluted ABTS solution (A_{734nm} = 0.700 ± 0.020) and the absorbance was determined at 734 nm after 5 min incubation at room temperature. Appropriate solvent blank was run in each assay. All determinations were carried out at least three times, and in triplicate. Inhibition of free radical by $ABTS^{\bullet+}$ in

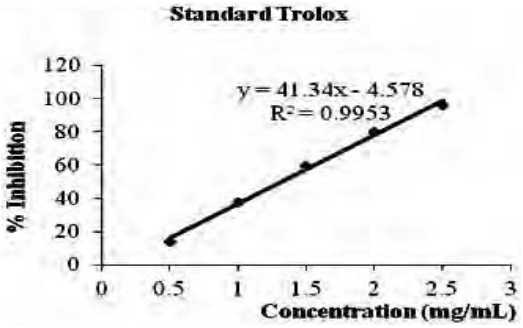

Figure 1—A linear regression (R^2 = 0.9953) of Trolox.

Table 1—Chemical constituents of the leaf essential oil of *Bridelia retusa* (L.) A. Juss.

Peak	Compounds	RA(%)	RI_{exp}	RI_{lit}	Identification	References
1	Tetradecanoic acid	0.4	1772	1767	RI, MS	20
2	Isophytol	0.3	1948	1944	RI, MS	21
3	Dibutyl phthalate	0.4	1965	-	RI, MS	-
4	Phytol	33.4	2117	1949	RI, MS	21
5	2-Propenoic acid	0.9	2173	-	RI, MS	-
6	Pentacosane (CAS)	0.5	2498	2500	RI, MS	22
7	Phthalic acid	5.2	2544	-	RI, MS	-
8	Heptacosane	2.3	2698	2700	RI, MS	23
9	Pentamethoxyflavone	0.6	2814	-	RI, MS	-
10	Nonacosane	1.2	2897	2900	RI, MS	23
11	6, 13-dimethoxy-2, 3, 9, 10-tetramethylpentacene-1, 4, 8, 11- tetrone	3.4	-	-		-
	Total	48.6				
	Acyclic diterpene alcohol	33.4				
	Acyclic terpenoid	0.3				
	Carboxylic acids	6.1				
	n-Alkanes	4.0				
	Saturated fatty acid	0.4				
	Flavonoid	0.6				
	Polyacenepolyquinone	3.4				
	other	0.4				

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.
RI, MS: comparison of the mass spectrum with MS libraries and RI of literature.

percent (% Inhibition) was calculated by the following formula (Equation 1):

$$\% \text{ Inhibition} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100 \quad ...(1)$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. The percentage inhibition of the absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data (Figure 1).

DPPH Assay

The antioxidant activity of the essential oil was also measured in the term of hydrogen donating or radical scavenging ability using the stable DPPH method as modified by Thongchai *et al.*[20]

Results and discussion

The essential oils of fresh leaves and fruits from *B. retusa* were obtained by hydrodistillation, afforded pale yellow oil, the yield being 0.0013% (w/w) in leaves and violet-light brown oil, the yield being 0.0026% (w/w) in fruits. The essential oils were separately subjected to GC and GC-MS analysis. Identification of the essential

Table 2—Chemical constituents of the fruit essential oil of Bridelia retusa (L.) A. Juss

Peak	Compounds	RA(%)	RI_{exp}	RI_{lit}	Identification	References
1	Diacetin	4.3	1353	-	RI, MS	-
2	γ-Eudesmol	0.7	1642	1632	RI, MS	22
3	a-Eudesmol	0.4	1665	1654	RI, MS	22
4	Hexahydrofarnesyl acetone	0.5	1844	1835	RI, MS	24
5	Isobultyl phthalate	0.4	1869	-	RI, MS	-
6	Isophytol	2.7	1948	1944	RI, MS	21
7	Hexadecanoic acid	1.5	1972	1966	RI, MS	20
8	Phytol Isomer	4.8	2114	1974	RI, MS	25
9	Dibutyl sebacate	25.6	2175	-	RI, MS	-
10	Citric acid	0.4	2261	-	RI, MS	-
11	2-Propenoic acid	0.6	2331	-	RI, MS	-
12	Phthalic acid	1.9	2544	-	RI, MS	-
13	Erucylamide	2.5	2791	-	RI, MS	-
14	Eicosane	1.2	2797	2000	RI, MS	21
15	Tricosane	3.9	2897	2300	RI, MS	21
16	Bis-(octylphenyl)-amine	0.3	2959	-	RI, MS	-
	Total	51.7				
	Dibutyl ester of sebacic	25.6				
	acid	4.8				
	Diterpene isomer	2.7				
	Acyclic terpenoid	1.1				
	Sesquiterpenoids	1.5				
	Saturated fatty acid	2.9				
	Carboxylic acid	2.5				
	Unsaturated long					
	Chain carboxylic					
	Acid amide	5.1				
	n-Alkanes	5.5				
	other					

RA: relative area (peak area relative to total peak area).
RI_{exp}: retention indices relative to n-alkanes (C_7-C_{30}) on HP-5MS column.
RI_{lit} : retention indices from literature data.
RI, MS: comparison of the mass spectrum with MS libraries and RI of literature.

oil constituents was performed by comparison of their mass spectra with literature data (NIST and NISTREP) and by a comparison of their programmed-temperature Kovats retention indices (RI) with those in the literature. In the leaf oil of *B. retusa*, eleven compounds were identified accounting for 48.6% of the total oil, and sixteen constituents in the fruit oil which represent 51.7% of its total composition. The components, their retention indices and percentage composition, in order of their elution on the HP-5MS capillary column, are summarized in Table 1 and Table 2 with peak area expressed as a percentage of the total chromatographable components of the essential oil assuming equal relative FID response for each component. The total ion chromatograms of *B. retusa* leaf and fruit essential oils are presented in Figure 2 and Figure 3.

The major constituents of the leaf essential oil were identified as phytol (33.4%), phthalic acid (5.2%), 6, 13-dimethoxy-2, 3, 9, 10-tetramethylpentacene-1, 4, 8, 11-tetrone (3.4%), heptacosane (2.3%) and nonacosane (1.2%). The major components of the fruit essential oil were identified as dibutyl sebacate (25.6%), phytol isomer (4.8%), diacetin (4.3%), tricosane (3.9%), isophytol (2.7%), phthalic acid (1.9%), hexadecanoic acid (1.5%), and eicosane (1.2%). The minor components of the leaf essential oil were 2-propenoic acid (0.9%), pentamethoxyflavone (0.6%), pentacosane (0.5%), tetradecanoic acid (0.4%), dibutyl phthalate (0.4%) and isophytol (0.3%). The minor components of the fruit essential oil were γ-eudesmol (0.7%), 2-propenoic acid (0.6%), hexahydrofarnesyl acetone (0.5%), isobultyl phthala (0.4%), α-eudesmol (0.4%), citric acid (0.4%), and bis-(octylphenyl)-amine (0.3%). The most prominent compound found in the leaf essential oil of *B. retusa* was phytol (33.4%). It is an acyclic diterpene alcohol, which has been reported to have anticancer activity[12] and also possessed antibacterial activity[13]. Phytol can also be used as a precursor for the production

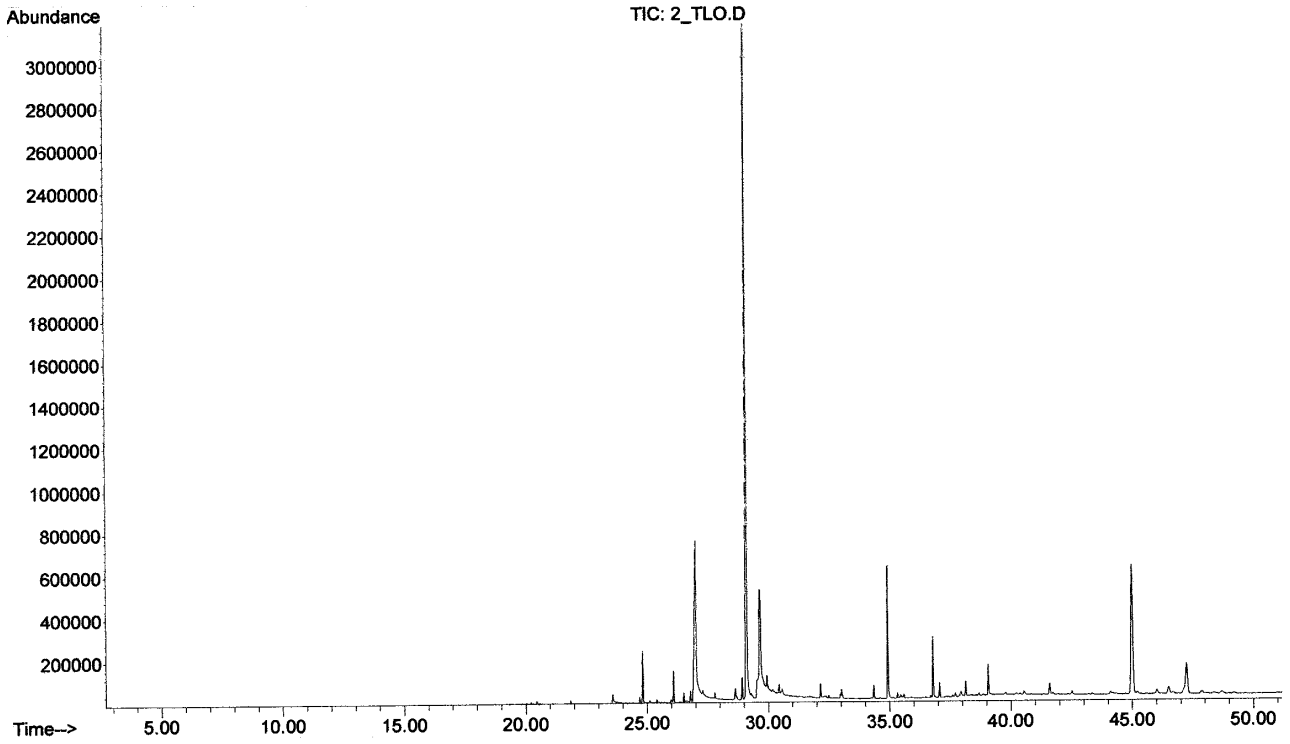

Figure 2—Total ion chromatogram of *B. retusa* leaves essential oil

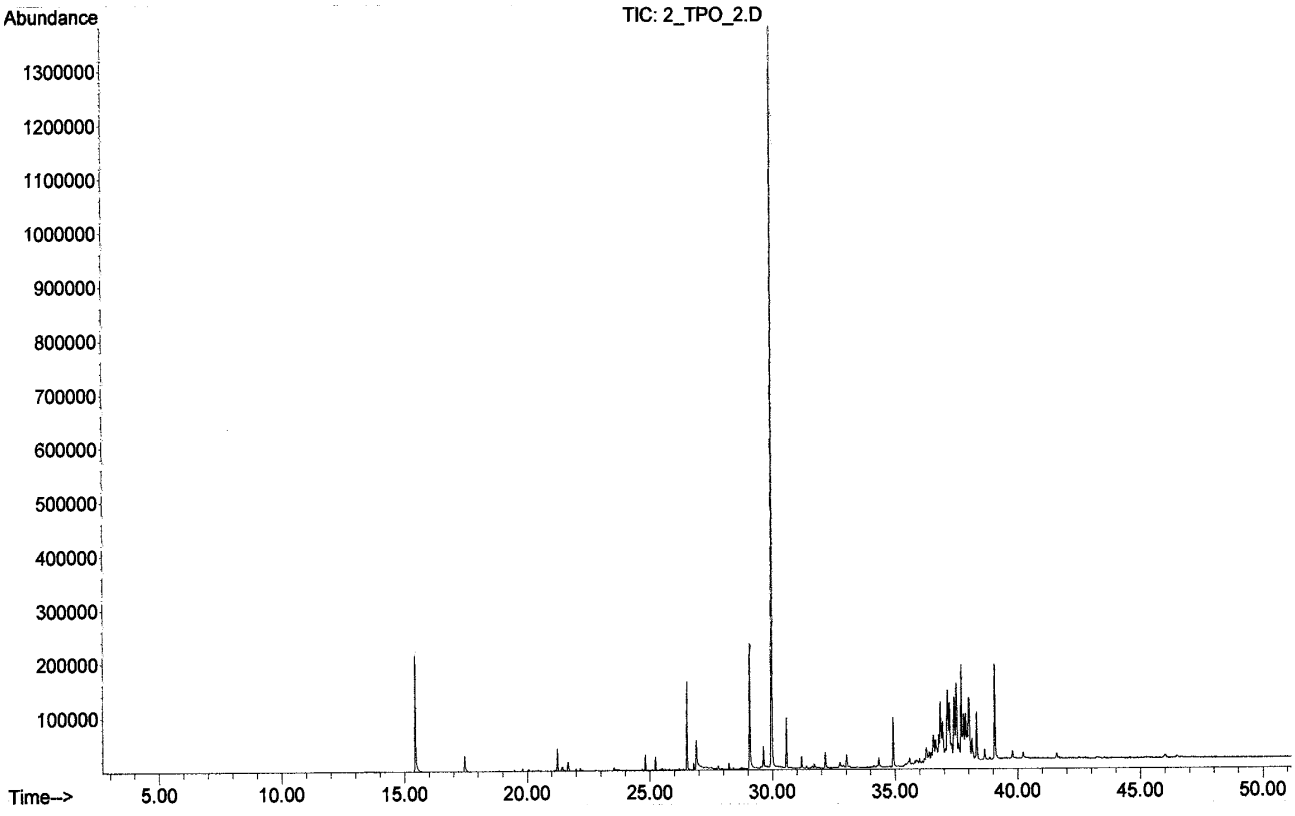

Figure 3—Total ion chromatogram of *B. retusa* fruits essential oil

Table 3—Minimum inhibitory concentrations of the leaf and fruit essential oils of *B. retusa*

Microorganism	Minimum inhibitory concentration (mg /mL)	
	Leaf essential oil	Fruit essential oil
S. aureus	30 (8 mm)[a]	40 (7 mm)[a]
E. coli	40 (8 mm)[a]	20 (7 mm)[a]
P. aeruginosa	40 (8 mm)[a]	50 (7 mm)[a]
B. subtilis	20 (20 mm)[a]	40 (8 mm)[a]
S. enteritidis	50 (7 mm)[a]	40 (8 mm)[a]
Enterococcus sp.	40 (7 mm)[a]	30 (8 mm)[a]

a = Zone of inhibition (mm)

Table 4¾Antioxidant activities of the leaf and fruit essential oils of *B. retusa.*

Test Sample	IC_{50} (mg /mL)*	
	ABTS Method	DPPH Method
Leaf essential oil	1.12 ± 0.0010	0.08 ± 0.0005
Fruit essential oil	1.79 ± 0.0005	0.10 ± 0.0010
Trolox	1.32 ± 0.0010	0.09 ± 0.0005

Trolox was used as positive control
*Values are given as mean ± S.D. of triplicate determinations

of synthetic form of vitamin E[14] and vitamin K1[15]. Pentamethoxyflavone (in the leaf oil) has been found to exhibit anticancer activity[16]. The main compound found in the fruit essential oil was dibutyl sebacate (25.6%). It is a dibutyl ester of sebacic acid. This compound is used as a plasticizer for film coating of tablets, beads, and granules. It is also used as pharmaceutical excipient in both aqueous and solvent based formulation. Alpha-eudesmol is a sesquiterpenoid, which is present in the fruit essential oil. This compound is a potent nonpeptidergic compound which blocks the presynaptic omega-Aga-IVA-sensitive Ca^{2+} channel with relative selectivity[17]. Hexahydrofarnesyl acetone (in fruit oil) has been found to exhibit antibacterial activity against *S. aureus*[18]. The fatty acids, tetradecanoic acid and hexadecanoic acid which are present in the leaf and fruit essential oils have been reported to possess antibacterial activity against *S. aureus*[19].

The antibacterial activity of the leaf and fruit essential oils of *B. retusa* against six bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa*, *B.subtilis, S.enteritidis , Enterococcus sp.*) was assessed by determination of the minimum inhibitory concentrations using the disc diffusion assay. The minimum inhibitory concentrations and the inhibition zones are given in Table 3. The oils showed antibacterial activities against both Gram positive and Gram negative bacteria. The leaf and fruit essential oils exhibited potent antibacterial activity against *B. subtilis* and *E. coli* (20 mg /mL) respectively. The leaf essential oil showed antibacterial activity against *S. aureus* with the minimum inhibitory concentration of 30 mg/mL, whereas the fruit essential oil possessed antibacterial activity against *Enterococcus sp.* with the minimum inhibitory concentration of 30 mg/mL. They showed moderate antibacterial activities against *P. aeruginosa* and *S. enteritidis* . The leaf and fruit essential oils of *B. retusa* possessed antibacterial activities, may be due to the presence of phytol, hexahydrofarnesyl acetone, tetradecanoic acid and hexadecanoic acid as described previously.

The antioxidant activity of the leaf and fruit essential oils is evaluated by using the ABTS and DPPH methods. For the ABTS radical cation scavenging assay, the relative capacity of antioxidant to scavenge the $ABTS^{*+}$ radical compared to the antioxidant potency of Trolox (standard) was measured. The IC_{50} values of the leaf and fruit essential oil were calculated by reference to the calibration curve (Figure 1).Free radical scavenging capacities of the oils were also measured by the DPPH assay .In the DPPH assay, the ability of the examined oils act as donor of hydrogen atoms or electrons in the transformation of $DPPH^{*}$ into its reduced form DPPH-H was investigated .The examined oils can reduce the stable purple-colored radical DPPH into yellow-colored DPPH-H. The IC_{50} values of the oils were determined by reference to the calibration curve. Trolox was used as reference standard. The results are summarized in Table 4.The leaf essential oil of *B. retusa* possessed stronger antioxidant activity than that of the fruit essential oil. This may due to the leaf essential oil contained more terpenoids and flavonoids than the fruit essential oil. The leaf essential oil consisted of an acyclic diterpene alcohol, phytol (33.4 %) , an acyclic terpenoid, isophytol (0.3 %) , and flavonoid , pentamethoxyflavone (0.6 %), whereas the fruit essential oil contained isophytol (2.7 %) and sesquiterpenoids (1.1 %).The leaf essential oil possessed more antioxidant activity than that of the standard Trolox.

Conclusions

This is the first report that describes the chemical constituents of the leaf and fruit essential oils of *B. retusa*

and their biological activities. The essential oils were analysed by GC(FID) and GC/MS. The main components found in the leaf and fruit essential oils were phytol and dibutyl sebacate respectively. The essential oils exhibited significant antibacterial activities against *S. aureus, E. coli ,B. subtilis ,Enterococcus sp.,* and *P. aeruginosa* , suggests the oils could also be used for the treatment of bacterial infections and has the potential to be developed as an accessible and relatively inexpensive alternative to synthetic antibiotic drugs. The leaf and fruit essential oils of *B. retusa* also inhibited strong antioxidant activity (ABTS and DPPH methods) because both essential oils contained terpenoids and flavonoids , suggests the oils are rich sources of antioxidants, which indicate their effectiveness in diseases caused by overproduction of radicals.

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