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COMPARISON OF HYDRODISTILLATION METHODS FOR EXTRACTION OF ESSENTIAL OILS FROM *BEACKEA FRUTESCENS* AND EVALUATION FOR THE ANTIBACTERIAL ACTIVITY

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Abstract: Essential oils from the leaves of *Beackea frutescens* were extracted by using three hydrodistillation methods, the Dean-Stark (DS), Clavenger (C) and simultaneous steam distillation extraction (SSDE). The yield of essential oils for each method was 0.32 %, 0.40% and 0.83%, respectively. The essential oils were examined using gas chromatogram and gas chromatography-mass spectrometry. Twenty constituent of the essential oils were identified. The major compounds were terpinolene (22.33%), t-α-bisabolene (10.12%), ρ-cymene (9.85%), linalool (7.32%) and *t*-caryophyllene (6.06%), α-bergamotene (2.42%), nerolidol (1.35%). The pure essential oils obtained and four emulsion samples which contained 0.2-0.5% of the essential oils shown antibacterial activity against *Staphylococcus aureus.* The inhibition zones were 10.5 -17.0 mm. The inhibition zone exhibited by the emulsion samples varied dependent on the percentage of the essential oil used.

KEYWORDS: Essential oil, *Baeckea frutescens*, Emulsion, Antibacterial, ρ-cymene.

Introduction

Beackea frutescens (cucur atap) is a member of Myrtaceae family, and is a well known aromatic and medicinal herb (Satake *et al.*, 1998). This species has an attractive weeping shrub with white flowers. Its height can achieve 3 meters and it can be easily found on sandy coast of the east coast of Peninsular Malaysia. The Chinese traditionally use the plant to treat rheumatism, snake-bites, scald, enteritis, urination, fever and eczema (Tsui *et al.*, 1996). In Indonesia, *B. frutescens* is one of the raw materials ingredients in "jamu" preparation (Mardisiswojo, 1985). Meanwhile, in Peninsular Malaysia, the leaves are used for post-partum medicines (Nor Azah *et al.*, 2000).

This plant contains about 2.0% of essential oils with sweet odor, which is viable for essential oil production and suitable as a natural source for fragrance (Nor Azah *et al.*, 2000). *B. frutescence* essential oils have potential as constituent of a skin care product. Previous study by Nor Azah and co-workers (2000) displays antifungal activity against dermatophytic fungi, *Microsporum canis* and *Trichophyton mentagrophytes*. It also posses antioxidant property in autooxidation of linoleic acid in water–alcohol system assays (Nor Azah *et al.*, 2000).

The major components of non-volatile constituent of this plant are phloroglucinols and flavanones (Fujimoto *et al.*, 1996, Makino & Fujimoto, 1999), chromones and chromanones (Tsui & Brown, 1996a, 1996b), chromone C-glycosides (Satake *et al.*, 1999). Methanolic extract of the leaf have also exhibited potential antimicrobial activity against cariogenic bacterium *Streptococcus mutant*, known to cause dental cavity (Hwang *et al.*, 2004).

Several techniques has been adopted in the extraction of the essential oil from plant which include hydrodistillation, steam distillation, maceration and the latest is supercritical fluid extraction (SFE) using carbon dioxide and ultrasonic-assisted extraction (US) (Da Poto *et al.*, 2009). The method of extraction was chosen based on the nature of the chemical compounds of the essential oils and cost effective of the production in industrial scale. The SFE and US methods is preferred when mild extraction condition is required. The steam distillation is the most suitable to be adopted to a small-medium industry, because it only involved the lowest cost for setting up the extraction plant. In hydrodistillation of the essential oil, the type of apparatus used is important to ensure all of the components in the essential oils were extracted with a good yield.

In this study, the effectiveness of the three different methods of essential oil extraction of *B. frutescens* essential oil were compared. In addition, the antibacterial property of the essential oils and emulsions were investigated.

Materials and Methods

Plant Material

The fresh *B. frutescens* leaves and flowers (3.0 Kg) of wild growing population were collected from Setiu, Terengganu. The voucher specimens were deposited into Biodiversity Laboratory, Institute of Oceanography. The voucher specimen number of the sample is still under construction.

Essential Oil Extraction Method

A 0.5 kg leaves and flowers of *Beackea frutescens* were placed into 5 L round bottom flasks and filled with distilled water into three quarter volume of the flask. Three flasks were used for different distillation methods. The flasks were assembled into three different hydrodistillation apparatus; Clavenger, Dean-Stark and Simultaneous Steam Distillation Extraction (SSDE). The extraction of the essential was carried-out for six hours at 100ºC. The floral water containing essential oils were entrapped in 5 ml hexane. The hexane layer was dried over anhydrous Na_2SO4. Excess hexane was removed from the essential oil by using a rotary evaporator and further purging with N_2 to obtain the pure essential oil. The essential oils obtained were directly analyzed by GC and GCMS.

Determination of Percentage of Essential Oil Obtained

The yield were averaged over two replicate and calculated based on the dry weight of plant material. First, fresh samples were grinded into smaller pieces and dried in oven at 100ºC. After 5 hours drying, the weight of the samples was measured. Below are the formulas for the calculation of the percentage of the essential oil produced from the three-hydrodistillation method.

Weight of a beaker:	M1
Weight of a beaker with samples:	M2
Weight of a beaker with dry samples:	M3
Weight of samples:	M4 = M2-M1
Percentage of vapors:	M = (M4-M5) x 100/M4
Calculation factor of oven drying:	F=100-M/100
Percentage of essential oil produced:	Y=Ex100/WxF

Where:
E = essential oil produced
W = Weight of samples
F = Calculation factors of oven's drying

Gas Chromatography Analysis

Analysis was carried out using TRACE GC 2000 series equipped with FID detector and a Heliflex AT-1 fused silica capillary column (30 mm x 0.32 mm i.d., 0.25 µm film thickness). Prior to injection, the essential oil was diluted in hexane (50 µl/10 ml). The GC set up is under the following conditions: Initial oven temperature 350ºC for 5 min, then to 250ºC at 3ºC/min and held for 5 min: injector and detector temperature, 250ºC: carrier gas was helium at a flow rate of 1.2 ml/min: sample size 0.4 µl: split ratio, 1:50. Peak areas obtained with Shimadzu C-R6A Chromatopac data processor were used for indication of relative amounts of individual components without response factor correction.

Gas Chromatography-Mass Spectrometry Analysis

Analysis of chemical component in the essential oil was carried out by using Shimadzu GC-17A (Model QP5050) GCMS system equipped with Wiley library software. Condition: HP 5 column (30m x 0.25mm i.d., 0.25 µm film thickness). Temperature was programmed about 50°C as initial temperature and 290°C, final temperature at 5°C/min. Helium was used as a carrier gas at 1ml/min flow rate. Significant MS operating parameters: ionization voltage, 70 eV; ion source temp. 200ºC; scan mass range, 40-350 u. Identification of chemical components was based on comparison of their mass spectral data with existing Wiley library.

Preparation of emulsion

Five emulsion samples were prepared by mixing the Tween 20, Brij 35, mineral oil, bees wax, water and a various proportion of the essential oil (A1-A5) .The concentration of the essential oil in emulsion system were as follows; A1 is the formulation of emulsion containing 0.1% EO, A2 is the formulation of emulsion containing 0.2% EO, A3 is the formulation of emulsion containing 0.3% EO, A4 is the formulation of emulsion containing 0.4% EO, A5 is the formulation of emulsion containing 0.5% EO. Briefly, in the emulsification process, the formulation is separated into two portions, the oil phase and the water phase. In the oil phase, the surfactants (Tween 20 and Brij 35) and the bees wax are heated to 70°C and mixed. After the beeswax dissolved in the two surfactants, the mixture of hexane and essential oil is added in after the solution's temperature has dropped to 50°C. When the mixture has reached homogeneity, the water that has been heated to 50°C is added. When the mixture reached homogeneity, the mixture is stirred slowly with the stirrer until the temperature of the mixture drops to 35°C. Then the samples are stored in dark and cool places. The samples are under daily observation in order to detect any changes. Stability test is taken 3 weeks after the process.

Antibacterial Assay

Antibacterial property against Staphylococcus aureus of the pure essential oil (EO) and the emulsion containing the essential oil were determined by using an agar disc diffusion method (Vandepitte *et al.*, 1991, Habsah *et al.*, 2000). Briefly, the essential oils and the emulsion (0.2 mg/ disc) were loaded onto filter paper discs (6mm Ø) and dried in laminar flow to remove the solvent of stock solution. Then, the discs were located on the surface of the previous inoculated agar. The plates were inverted and incubated for 24 hours at 30ºC. Clear inhibition zones around the disc were measured after the incubation period edge of paper disc. The clear inhibition zones around the discs indicated the presence of antimicrobial activity. Antibiotic disc Erythromycin (1 mg/disc) was used as a positive control.

Results and Discussion

The essential oil obtained from the leaves and flowers of *B. frutescens* were dependent on the hydrodistillation methods used (Table 1). SSDE was found to be the most effective which yielded 0.83% essential oils followed by Dean-Stark and Clavenger with 0.32% and 0.40%, respectively. SSDE is an efficient method because it allowed continuous extraction of the essential oils by the hexane, and it is occurred in a gas phase. Besides, the volume of hexane will not be decreased throughout the hydrodistillation process, as compared to the Clavenger and Dean-Stark method. The GC profile of the essential oils produced by the three different methods are shown in Figure 1 and the comparison of the essential constituent produced from Dean-Stark (EODS), Clevenger (EOC) and Simultaneous Steam Distillation Extraction (EOSSDE) apparatus is given in Table 2. However, the essential oils constituent profile was the same (Figure 1), and only minor differences in percentage of individual essential component was found. Only a trace of compound n, which was suspected as α-cedrol, was found in EODS, but the percentage of α-cedrol in EOSSDE and EOC was higher i.e. 0.45% and 0.57% respectively . The percentage of ρ-cymene (peak c) was higher than γ-terpinene (peak d), both in EOC and EOSSDE, but in contrary the percentage of γ-terpinene was higher than ρ-cymene in EODS (Figure 1).

Twenty compounds were identified as components of the essential oil of *B. frutescens* (Table 3). The compounds were identified based on library search and comparison with at least one literature data. The analysis of essential oil produced using SSDE apparatus showed major constituents of the essential oil were found to be monoterpene (41.86%), alcohols (13.86%), sesquiterpenes (20.57%), benzene derivatives (9.87) (Table 4). The major monoterpenes components were found to be terpinolene (22.33%), γ-terpinene (13.13%) limonene oxide (3.65 %) and 2-β-pinene (2.38%). The main alcohols components detected were linalool (7.32%), α-terpineol (1.88%), epoxylinalool (1.65%) and nerolidol (1.35%). *T*-α-bisabolene (10.12%) and *t*-caryophyllene (6.32%) and α-bergamotene (2.42%), identified as sesquiterpenes hydrocarbons group. A major benzene derivative components was ρ-cymene (9.85%). However, 1,8-cineole which was proven to be toxic to the insect and was reported to be a minor constituent of *B. frutescen* (Ibrahim *et al.*, 1998), was not identified in our findings although it was reported that six out of 42 Myrtaceae family essential oil extracts are rich in 1,8-cineole (Lee *et al.*, 2004).

Preliminary evaluation for the antibacterial property against *S. aureus* of the pure essential oils and four emulsion samples which contained 0.1%-0.5% of the essential oil was performed qualitatively using a disc diffusion assay. Results showed that all the six samples showed a high antibacterial activity with inhibition zone ranging from 10.5-17 mm (Table 5). It is evidence that the pure essential oil showed high antibacterial activity against *S.aureus,* with inhibition zone 17 mm. Furthermore, the inhibition zone increased with the increasing amount of essential oil present in the emulsion system. The emulsion system, which contains 0.1%, did not show any antibacterial activity. The antibacterial activity could be contributed by ρ-cymene, a bactericidal compound which can be found in the essential oil of fennel and other several spices (Burt, 2004). This compound was not found in the essential oil of *Alpinia choncigera*, which only showed a weak inhibition against bacteria and fungi (Halijah *et al.*, 2009). Our result further supported that both volatile and non-volatile constituent of the leaf exhibited potential antibacterial activity, as reported by Nor Azah *et al.* (2000) and Hwang *et al.*(2004).

Conclusion

Qualitatively, the individual component of *B. frutescens* essential oils were not affected by the type of apparatus using in the hydrodistillation method, which are Dean-Stark, Clavenger and Simultaneous Steam Distillation Extraction (SSDE). In contrary, the yield of essential oils obtained was depending upon the type of apparatus used. Simultaneous Steam Distillation Extraction (SSDE) was the most effective method, which gave 0.83% of the essential oil. The emulsion prepared containing 0.2-0.5% of *B. frutescens* essential oils did possessed antibacterial activity against *S. aureus,* a dermatophytic fungi, thus the essential oil of *B. frutescens* is suitable to be developed as a skin-care product.

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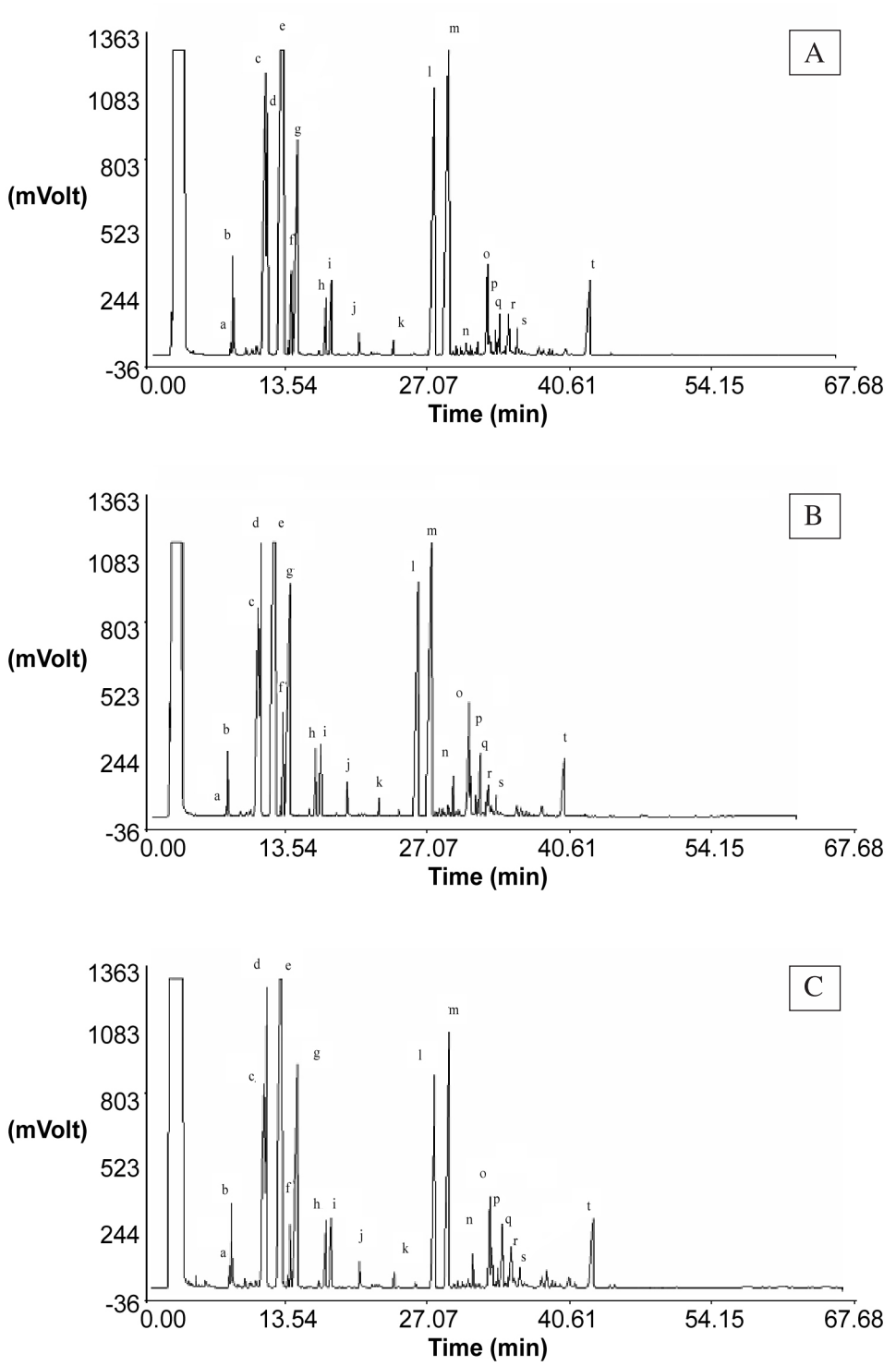

Figure 1: GC profiles of *B. frutescens* essential oil extracted by using different hydrodistillation method, Dean-Stark (A), Clevenger (B) and SSDE (C)

Table 1: Percentage of the essential oils obtained from the three hydrodistillation methods

Apparatus	Percentage of essential oil obtained (%)
Dean-Stark (DS)	0.32
Clevenger (C)	0.40
Simultaneous steam distillation (SSDE)	0.83

Table 2: Comparison of peak areas in GC analysis of the essential oil constituent of *B. frutescens* extracted using three different of hydrodistillation methods.

Peak no.	Compounds	Time (Min)	Dean-Stark (%)	Clevenger (%)	SSDE (%)
1	α-pinene	7.64	0.25	0.21	0.27
2	2-β-pinene	7.88	2.40	1.10	2.38
3	ρ-cymene	11.14	10.32	9.00	9.87
4	γ-terpinene	11.33	8.91	13.00	13.13
5	Terpinolene	12.03	22.00	20.00	22.43
6	Epoxylinalool	13.69	2.21	1.89	1.65
7	Linalool	14.33	7.77	7.56	7.34
8	Terpinen-4-ol	17.10	1.19	1.10	0.98
9	α-terpineol	17.65	2.00	2.03	1.85
10	Geraniol	20.38	0.29	0.18	0.27
11	α-longipinene	23.78	0.32	0.25	0.45
12	*t*-caryophyllene	27.84	9.00	6.70	6.32
13	*t*-α-bisabolene	29.09	11.17	9.92	10.12
14	α-cedrol	31.46	0.08	0.57	0.45
15	α-bergomotene	33.13	2.60	6.87	2.47
16	δ-cadinene	33.33	0.25	0.42	0.72
17	Nerolidol B (*cis or trans*)	34.31	0.56	1.12	1.32
18	Caryophyllene oxide	35.23	0.57	0.43	0.23
19	Humulene oxide	36.04	0.32	0.28	0.26
20	Limonene oxide	43.27	4.73	2.76	3.65

Table 3: Comparison of type of compound obtain from different techniques of extraction

Type of Compound	Percentage (%)		
	Dean-Stark	Clevenger	SSDE
Monoterpene	38.29	37.07	41.86
Sesquiterpene	24.23	24.87	20.57
Monoterpene and Sesquiterpene Alcohol	14.10	14.45	13.86
Benzene derivative	10.32	9.00	9.87

Table 4: Retention times indices and identification of the essential oils component from *B. frutescens.*

Peak No.	Retention Time	(%)	Identification	Characteristic of fragmentation ion
a	5.703	0.25	α-pinene	105(10.4), 93(100), 77(30.8), 67(8.4), 41(20.8), 39(21.2) M=136
b	6.805	2.36	2-β-pinene	136(9.2), 121(12.0), 93(100), 69(42.0), 67(12.0), 41(56.4) 39(25.2) M=136
c	8.393	9.85	ρ-cymene	134(51.6), 119(100), 91(57.2), 77(20.4), 65(19.2), 39(16.4) M=134
d	9.329	13.10	γ-terpinene	136(46.0), 121(42.8), 105(20.0), 93(100), 77(50.8), 65(15.2), 39(21.6) M=136
e	9.814	22..33	Terpinolene	136(76.4), 121(86.4), 105(25.2), 93(100), 91(50), 79(40.8), 77(34.0), 53(14.8) M=136
f	10.192	1.65	Epoxylinalool	111(20.8), 94(47.6), 93(34.0), 68(32.8), 67(28.4), 59(100), 55(42.4), 43(57.2) 41(43.6), 39(22.4) M=170
g	10.698	7.32	Linalool	121(24.4), 93(80.0), 80(33.6), 71(100), 69(44.0), 67(22.8), 55(63.6), 43(80.4), 41(79.6) M=154
h	12.885	0.97	Terpinen-4-ol	154(13.6), 136(10.4), 111(48.0), 93(46.4), 86(20.8), 71(100), 55(26.0) M=154
i	13.426	1.88	α-terpineol	136(21.6), 121(30.0), 93(47.6), 81(31.2), 59(100), 43(55.2), 41(30.8), 39(24.0) M=154
j	14.871	0.27	Geraniol	123(9.6), 93(16.4), 69(100), 68(20.8), 67(15.2), 41(96.0), 39(23.6) M=154
k	18.405	0.44	α-longipinene	133(39.6), 119(100), 107(30.0), 105(60.4), 93(43.6), 91(41.2), 79(24.0), 77(23.6), 69(22.4), 55(32.0) M=204
l	18.868	6.06	*t*-caryophyllene	133(61.2), 120(31.2), 107(38.8), 105(38.0), 93(77.6), 91(46.0), 81(39.2), 79(59.6), 77(36.0), 69(81.6), 67(38.4), 55(43.2), 53(33.6), 41(100), 39(48.0) M=204

m	19.868	10.12	*t*-α-bisabolene	121(18.8), 11920.8), 109(22.4), 107(23.2), 105(20.4), 93(100), 91(29.2), 79(29.2), 77(22.8), 69(24.8), 67(27.6), 55(20.8) M=204
n	20.242	0.45	α-cedrol	161(60), 119(100), 105(43.2), 93(78.0), 69(79.2), 55(53.2), 41(97.2) M=222
o	20.679	2.42	α-bergomotene	119(86.4), 107(25.6), 105(16.8), 93(100), 91(22.4), 79(22.8), 69(46.0), 55(28.8), 41(50.4) M=204
p	21.406	0.71	δ-cadinene	204(42.4), 189(17.2), 162(23.6), 161(100), 134(60), 119(77.6), 105(69.2), 91(46.0), 81(29.6), 77(22.0), 41(27.6) M=204
q	22.687	1.35	Nerolidol B (*cis or trans*)	161(17.2), 136(19.6), 107(28.0), 93(57.6), 81(23.2), 79(20), 71(33.2), 69(92.4), 67(26.8), 55(37.2), 43(50), 41(100) M=222
r	23.732	0.25	Caryophyllene oxide	121(24.8), 109(36.8), 107(34), 95(43.2), 93(62), 91(54.4), 79(76.8), 69(51.6), 67(38), 55(41.6), 43(98.8), 41(100), 39(38.8) M=220
s	24.624	0.26	Humulene oxide	138(66.8), 109(76.0), 107(40.4), 105(36.8), 96(53.6), 93(38.0), 82(36.8), 81(36.8), 68(45.2), 67(60.8), 53(40.4), 43(100), 41(71.6) M=220
t	25.139	3.65	Limonene oxide	93(30.4), 81(56.4), 79(29.2), 68(33.2), 67(63.2), 55(42.8), 53(31.6), 43(100), 41(84.8), 39(72.8), 29(37.2), 27(61.2) M=152

Table 5: Inhibitory Zone for Tested *Staphylococcus aureus* and Extract of *B. frutescens*.

Sample	A1 (mm)	A2 (mm)	A3 (mm)	A4 (mm)	A5 (mm)	EO (mm)	Std (mm)
Inhibition zone	-	10.5	11	11.5	12.5	17	23

Std = Erythromycin (1 mg/disc)
EO = pure essential oils; A1 = formulation of emulsion containing 0.1% EO; A2 = formulation of emulsion containing 0.2% EO, A3 = formulation of emulsion containing 0.3% EO, A4 = formulation of emulsion containing 0.4% EO, A5 = formulation of emulsion containing 0.5% EO