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ESSENTIAL OIL CONSTITUENTS ASSESSMENT AND ANTIOXIDANT PROFILES OF *Stevia rebaudiana* BERTONI LEAVES FROM MALAYSIA

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ABSTRACT

Since the 15th century, essential oils (EO) were having a strong interest as therapeutic agents and recently EO has been widely used in pharmaceutical, cosmetic, food and agricultural industries. The previous study showed that EOs from *Stevia rebaudiana* Bertoni (SrB) possess high antioxidant, anti-inflammatory, and antimicrobial properties. In this study, the chemical constituents of EO from SrB leaves which grown in Selangor, Malaysia were obtained by ethanol Soxhlet extraction. Total polyphenolic content (TPC), total flavonoid content (TFC), and chemical composition using a gas chromatography-mass selective detector (GC-MSD) analyses of *Stevia rebaudiana* Bertoni essential oil (SrB EO) were carried out. The TPC and TFC of SrB EO were 14.44±0.63 mg gallic acid equivalent (GAE) /g sample extract and 32.19±1.38 mg quercetin equivalent (QE)/g sample extract, respectively. Based on GC-MSD analyses, one hundred peaks were obtained and 28 compounds were identified where lupeyl acetate occupied the highest percentage with 14.25% of the chemical constituents. The antioxidant activities of SrB EO were studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and ferric reducing antioxidant power (FRAP) assays. The IC_{50} of SrB EO using DPPH and ABTS were 71.36±1.57 µg/mL Trolox equivalent (TE) and 8.88×10^{2}±0.16 µg/mL TE, respectively while the FRAP activity was 9.55±0.26 mg/g TE. These results show the promising antioxidant properties of SrB EO.

Key words: Essential oils; *Stevia rebaudiana* Bertoni; antioxidant activities; GC-MS

INTRODUCTION

Stevia rebaudiana Bertoni (SrB) is a perennial shrub which belongs to the Asteraceae or Compositae family. This shrub is native to Paraguay and Brazil, and botanically identified in 1889. Presently, its cultivation has spread to Canada and some parts of Europe and Asia. The leaves of this plant have an incredible sweetness index compared to other natural sweeteners. Other than being used as food additive and sugar substitute, SrB has also been reported to possess antimicrobial, antioxidant, anticancer, antidiabetic and may improve gastrointestinal function such as in relieving heartburn or stomach pain (Singh, 2011; Smith, 2012; Zaidan *et al.*, 2019). In fact, these beneficial effects sourced from the bioactive compound of SrB which is its EO.

EO is volatile, transparent, and rarely colored liquid, lipid soluble, and a soluble organic solvent with generally lower density than water (Bilia *et al.*, 2014). Mostly EO from a plant used in food, drugs, and perfumery purposes. Since the 15th century, EO has been used as therapeutic agents, such as peppermint oil, rosemary oil and coriander oil used for digestives problems while chamomile oil, celery oil, juniper oil, and coriander oil can be used as an anti-inflammatory effect. On the other hand, researchers are interested in bioactive compounds isolated from a plant for antimicrobial purposes because of the resistance that microbes have built

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against antibiotics (Muanda *et al.*, 2011). EO can be derived from all plant organs, which are buds, flowers, leaves, stems, twigs, seeds, fruits, roots, woods, and barks (Bilia *et al.*, 2014). However, the research on the chemical constituents of solvent SrB EO extract and its antioxidants capacities are lacking. Therefore, the aim of this study was to determine the chemical composition and antioxidant potential for further application and development of solvent SrB EO extract.

MATERIALS AND METHODS

Chemical

All the chemicals used in this study were of analytical grade unless otherwise specified. Acetate buffer (pH 3.6), ethanol and methanol were obtained from R&M Chemicals. Folin-Ciocalteu's phenol reagent, quercetin, sodium carbonate (Na_2CO_3) and hydrochloric acid (HCl) were purchased from Merck. DPPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ABTS and iron (III) chloride ($FeCl_3$) were acquired from Sigma-Aldrich. Gallic acid was obtained from Cayman Chemical, aluminum chloride ($AlCl_3$) and potassium acetate ($C_2H_3O_2K$) were purchased from Friendemann Schmidt. Potassium persulfate ($K_2S_2O_8$) was purchased from Acros Organics and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) was purchased from Affymetrix.

Extraction of SrB EO

Dried leaves of SrB were obtained from Koperasi Usahawan Warisan Niaga Selangor (KOWARIS) Berhad. EO from SrB leaves was prepared using Soxhlet apparatus. A sample of 15.0 g dried SrB leaves were put into a thimble and transferred into a Soxhlet extractor, and the oil was extracted with 200 mL of ethanol at 80ºC and above for 10 cycles. Then, the products were purified using a rotary evaporator at 78ºC and left under the fume hood for one hour. A greenish oil crude with the sharp sweet smell (25.8% w/w) was obtained. The oil crude was stored at -20ºC until used. For further assay, 1.0% w/v of EO in methanol was used.

Analysis of SrB EO using GC-MS

The GC–MS analysis of the essential oil samples of *Stevia rebaudiana Bertoni* was performed using an Agilent GC–MS (Agilent Technologies 7890A model, USA) coupled with 5975 mass selective detector (MSD) and equipped with an HP-5MS fused silica capillary column (30 m × 0.25 i.d. mm. film thickness 0.25 ìm, Agilent, USA). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. Injector and mass transfer line temperature were set at 250°C and 300°C, respectively. The oven temperature was programmed from 50°C to 200°C at 8°C min^{-1} and then held isothermally for 20 min and finally raised to 300°C at 10°C min^{-1}. Then, a 1 µL of methanolic diluted sample (1.0% w/v) was injected in the splitless mode using an autosampler injector (Agilent 7693). Identification of compounds of the essential oil was performed using an Agilent MSD Chemstation software version E.02.02.1431.

Analysis of phenolic compounds

Total polyphenolic compounds (TPC)

TPC of SrB EO was determined using the Folin Ciocalteu reaction method according to Cao *et al.* (2009) and a modified method of Muanda *et al.* (2011). A 0.1 mL of samples (1.0 mg/mL) was mixed with 1 mL of 10-fold-diluted Folin Ciocalteu's phenol reagent. After 3 min, 3 mL of 7.5% w/v Na_2CO_3 was added and vortexed. Then, the samples were allowed to stand for 30 min at room temperature (25°C). The absorbances of the sample were measured using a Spectro UV-Vis RS Model UV-2502 (LaboMed. Inc., USA) at 765 nm. The results were expressed in gallic acid equivalents (GAE/g).

Total flavonoid compounds

The TFC contents of SrB EO was measured by colorimetric assay according to the method of Hossain *et al.* (2013). A 0.5 mL of sample (1 mg/mL) was added with 0.1 mL 10% w/v $AlCl_3$ and 0.1 mL 1 M $C_2H_3O_2K$. Then, 4.3 mL of distilled water was added. Next, the sample was vortexed and incubated for 30 min at room temperature (25°C). The absorbances of the sample were measured using a Spectro UV-Vis RS Model UV-2502 (LaboMed. Inc., USA) at 415 nm. Quercetin was used as a standard solution.

Analysis of antioxidant activities

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging activity of SrB EO was measured according to the method adapted from Kutti Gounder and Lingamallu (2012). In this assay, the antioxidant activity was evaluated by assessing its free radical inhibitory activities. Different concentration of samples and standard solution (Trolox) were prepared (1.56-200 µg/mL). A sample (1 mL) was mixed with 1 mL of 0.2 mM of DPPH solution in methanol. The mixture was vortexed for 1 min and incubated at 25°C for 30 min. These procedures were done in a dark due to the sensitiveness of DPPH solution to light. After

incubated, the samples were measured using a Spectro UV-Vis RS Model UV-2502 (LaboMed. Inc., USA) at 517 nm. The percentage of free radical scavenging activity was calculated as follows,

$$\% \text{ DPPH Scavenging activity} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

where *Ac* is the absorbance of the control and *As* is the absorbance of the sample. The antioxidant activities of the compounds are expressed in terms of IC_{50}, which indicated the inhibitory concentration at 50% of the initial DPPH.

$$y = \frac{100}{(1 + 10^{((logIC50-x)*Hillslope))})}$$

where *y* is the percentage scavenging activity and *hillslope* indicates the steepness of the family of curves.

2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay

The ABTS performed according to the method of Pellegrini *et al.* (2001). The ABTS radical solution was prepared by mixing 7.0 mM ABTS and 2.45 mM of $K_2S_2O_8$ at 37°C for 16 hr in a dark. Then, the ABTS radical stock solution was diluted with methanol until an absorbance of 0.7±0.2 absorbance unit (AU) at 734 nm was achieved. Then, 1 mL of samples and standard solution at different concentrations (0.05-3.2 mg/mL) were added with 1 mL of ABTS stock solution. After incubated at room temperature (25°C) for 10 min, the absorbances of the sample were measured using a Spectro UV-Vis RS Model UV-2502 (LaboMed. Inc., USA) at 734 nm. The percentage of free radical scavenging activity was calculated as follows,

$$\% \text{ ABTS Scavenging activity} = \frac{\text{Ac} - \text{As}}{Ac} \times 100$$

where *Ac* is the absorbance of the control and *As* is the absorbance of the sample. The antioxidant activities of the compounds are expressed in terms of IC_{50}, which indicated the inhibitory concentration to scavenge 50% of the initial ABTS.

$$y = \frac{100}{(1 + 10^{((logIC50-x)*Hillslope))})}$$

Where *y* is the percentage scavenging activity and *hillslope* indicates the steepness of the family of curves.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted according to the method of Chizzola *et al.* (2008). Working FRAP reagent was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of 10.0 mM TPTZ in 40 mM HCl, and freshly prepared 20 mM $FeCl_3.6H_2O$. Then, 1.8 mL of the FRAP reagent was mixed with 60.0 µL of the sample (0.2 mg/mL) and 180.0 µL of distilled water. After 4 min resting time, the absorbances of the sample were measured using Spectro UV-Vis RS Model UV-2502 (LaboMed. Inc., USA) at 593 nm. The FRAP of SrB EO was expressed as Trolox equivalent per gram (TE/g).

RESULTS AND DISCUSSION

The chemical composition of SrB EO

A yield of 25.8% (w/w) of essential oil from SrB was obtained from Soxhlet extraction in 10 cycles using ethanol. The identified compounds and GC-MS chromatogram SrB EO are shown in Table 1 and Figure 1, respectively. There are 28 different compounds identified with over 80% similarities, representing 89.42% of the SrB EO composition. According to GCMS analysis, the SrB EO consists of oxygenated monoterpenes (3.08%), oxygenated diterpenes (10.52%), oxygenated triterpenes (21.62%), hydrocarbon sesquiterpenes (0.47%), oxygenated sesquiterpenes 0.57%), phenols (8.27%), fatty acids and derivatives (20.29%) and carbohydrates (21.68%). Terpene is one of the phytochemical which commonly found in EO; which plays many pharmacological effects like antimicrobial, antioxidant, and anti-inflammatory (Braun and Cohen, 2010). Appealingly, some new terpenes were found in SrB EO such as chavibetol, 3-methyldiadamantane, phytol acetate, and 3,7,11,15-tetramethyl-2-hexadecen-1-ol which have not been reported previously.

According to Table 1, lupeyl acetate was the most abundant component of SrB EO (14.25%), followed by levoglucosan (12.64%), D-Allose (9.04%), Lupeol (7.37%), and hexadecanoic acid methyl ester (6.09%). Zaidan *et al*. (2018) reported that SrB EO extracted by subcritical fluid carbon dioxide ($SC\text{-}CO_2$) also contained lupeol acetate (lupeyl acetate) as the major constituent of EO, followed by epiputranjivol, α-linoleic acid, hexadecanoic acid, and spathulenol. Previous studies showed that the constituents of SrB EO were slightly different due to the different method of extractions (Siddique *et al.*, 2010; Muanda *et al*., 2010). It has been reported the major compounds identified from steam and hydrodistillation of SrB leaves were α-cadinol, spathulenol, caryophyllene oxide, and β-guaiene (Siddique *et al.*, 2010). However, Muanda *et al*. (2010) reported the

Table 1. GC-MS analysis of SrB EO

Peak	R. Time	Library/ID	CAS No.	Area %	Match value %
1	9.9623	Cyclopentasiloxane, decamethyl-[i]	000541-02-6	2.9237	83
2	10.7383	Catechol[f]	000120-80-9	0.5738	93
3	11.2433	Ethanol, 2-phenoxy-[f]	000122-99-6	2.1944	80
4	12.803	Decanoic acid, methyl ester[g]	000110-42-9	1.5275	98
5	12.9914	Decanoic acid, methyl ester[g]	000110-42-9	1.7111	98
6	13.4661	Chavibetol[a]	000501-19-9	0.3004	97
7	13.6093	n-Decanoic acid[g]	000334-48-5	0.474	98
8	13.6394	Eugenol[a]	000097-53-0	2.2638	96
9	14.0689, 14.1744	Decanoic acid, ethyl ester[g]	000110-38-3	1.7375	98
10	14.6567	Tyrosol[f]	000501-94-0	0.1439	80
11	15.6363	Levoglucosan[h]	000498-07-7	12.6416	80
12	15.7041	D-Allose[h]	002595-97-3	9.0418	86
13	16.0055, 16.0507	Phenol, 2,4-bis(1,1-dimethylethyl)-[f]	000096-76-4	0.8762	96
14	16.0507	Phenol, 2,5-bis(1,1-dimethylethyl)-[f]	005875-45-6	4.4866	95
15	16.4802, 16.548	Dihydroactinidiolide[a]	015356-74-8	0.5195	98
16	18.5524	3-methyldiadamantane[d]	028375-86-2	0.4681	87
17	20.7151	Phytol, acetate[b]	076337-16-1	4.9621	80
18	20.8206	2-Pentadecanone, 6,10,14-trimethyl-[e]	000502-69-2	0.569	80
19	21.4309	3,7,11,15-Tetramethyl-2-hexadecen-1-ol[b]	102608-53-7	0.9068	83
20	22.2296	Hexadecanoic acid, methyl ester[g]	000112-39-0	6.0854	99
21	23.7216	Hexadecanoic acid, ethyl ester[g]	000628-97-7	0.6748	98
22	26.6529	9,12-Octadecadienoic acid, methyl ester, (E,E)-[g]	002566-97-4	1.9591	99
23	26.8789	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-[g]	000301-00-8	5.2419	99
24	27.2708	Phytol[b]	000150-86-7	4.4628	90
25	27.7681	Octadecanoic acid, methyl ester[g]	000112-61-8	0.8758	99
26	44.8356	Lupeol[c]	000545-47-1	7.3715	90
27	46.2221	Steviol[b]	000471-80-7	0.1852	91
28	49.2512	Lupeyl acetate[c]	001617-68-1	14.2497	83

Compound	Percentages
Oxygenated monoterpenes[a]	3.08
Oxygenated diterpenes[b]	10.52
Oxygenated triterpenes[c]	21.62
Hydrocarbon sesquiterpenes[d]	0.47
Oxygenated sesquiterpenes[e]	0.57
Phenol[f]	8.27
Fatty acid and derivatives[g]	20.29
Carbohydrate[h]	21.68
Other[i]	2.92
Unknown compound	10.58

major constituents of SrB EO were carvacrol, caryophyllene oxide, and spathulenol.

Total phenolic and flavonoid compounds of SrB EO

Phenolic compounds are antioxidants, thus they are able to scavenge free radicals. It can be classified into two major classes; flavonoids and non-flavonoids. The phenolic compounds can donate hydrogen to peroxy radicals and produce quinone radicals. The quinones formed are unstable and may undergo further reactions. Furthermore, the formed quinones can oxidize other phenolic compounds and they are being reduced at the same time (Bartosz, 2014).

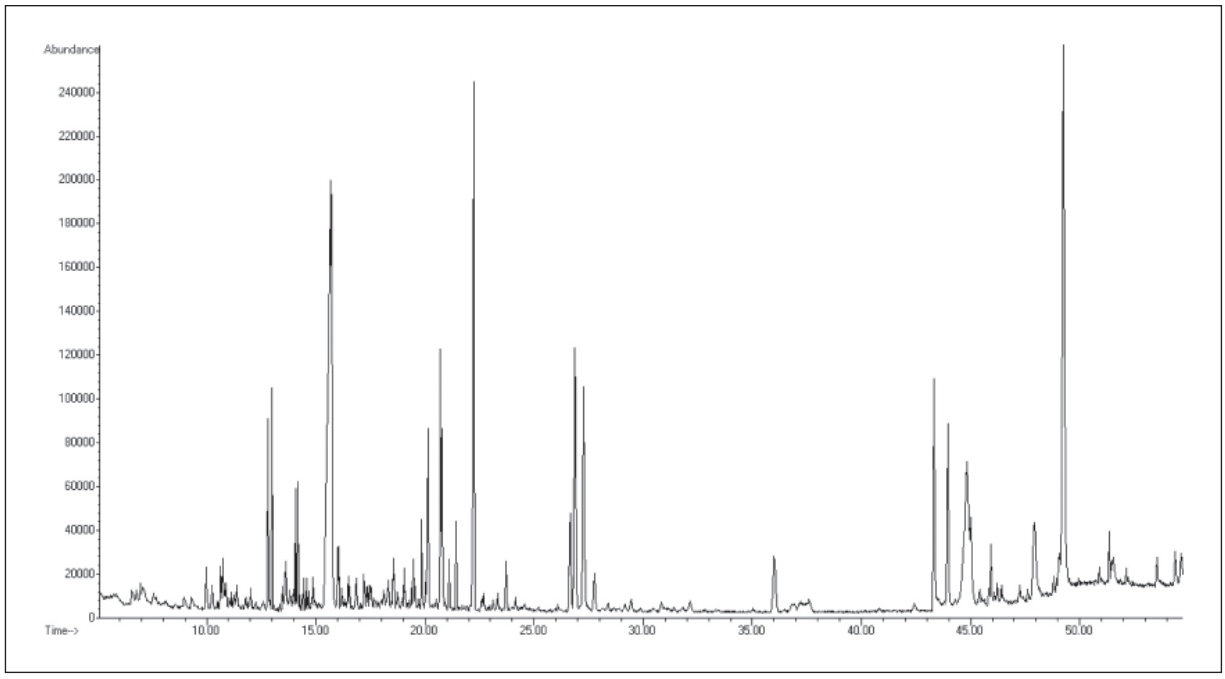

Fig. 1. A typical chromatogram of the constituents of essential oil from the leaves of *Stevia rebaudiana* Bertoni.

Table 2. The TPC and TFC activity of SrB EO (1.0 mg/mL)

Extract	TPC mg GAE/g	TFC mg QE/g
SrB EO	14.44±0.63	32.18±1.38

The TPC and TFC of SrB EO are shown in Table 2. The TPC value was obtained from the calibration curve y=0.0081x with R^2=0.9923, where x is the absorbance and y is the concentration of gallic acid which expressed as mg GAE/g. The TFC value was obtained from the calibration curve y=0.0048x with R^2=0.9987, where x is the absorbance and y is the concentration of quercetin which expressed as mg QE/g. The TPC of SrB EO was 14.44±0.63 mg GAE/g which was higher than that of the value (1.15±0.57 mg GAE/g) reported by Muanda *et al*. (2011). At the same time, the TFC value of SrB EO was 32.18±1.38 mg QE/g which was also higher compared to that of the value (2.23±0.8 mg QE/g) reported by them. The solvent extraction is believed to affect the composition and quantity of phenolic compounds. In this study, absolute ethanol was used while the hydrodistillation method was commonly used in the previous study. As ethanol (log P = -0.18) has lower polarity than water, the more nonpolar compounds were expected to be extracted. The lower polarity of a solvent also showed remarkably effect on phenolic contents and antioxidant activity (Oldoni *et al.*, 2015). It was also reported that increased water content in the solvent would decrease the TPC and TFC of the extracts. In the previous study, the extraction of phenolic compounds from *Limnophila aromatica* also showed that absolute ethanol gave the highest TPC and TFC values (Do *et al.*, 2014).

Antioxidant analysis of SrB EO

In this study, there were three assays used in determining the antioxidant activity of the SrB EO which were DPPH radical scavenging; and inhibition of free radical ABTS capacity to reduce metal iron using FRAP. These methods have been used indirectly in the determination of antioxidant activities where, when a sample containing antioxidants reacts with a stable synthetic radical solution within time; and it is turned to a colorless product which happens in DPPH and ABTS assays, or it will turn into colored form which takes place in FRAP assay (Papadopoulos, 2008).

DPPH radical scavenging assay

The radical DPPH is considered a model of lipophilic radical. This assay is based on the principle that when the stable free radical DPPH (purple) reacted with antioxidants, it will be reduced and the absorbance decreases which results in the formation of hydrazine, the yellow solution (Hashemi *et al.*, 2018). Antioxidants like phenols, phenothiazines, pyridinols, aromatic amines and thiols may scavenge the free radical through hydrogen atom donation. The antioxidant activities of the compounds are expressed in terms of IC_{50}, which indicated the inhibitory concentration at 50%

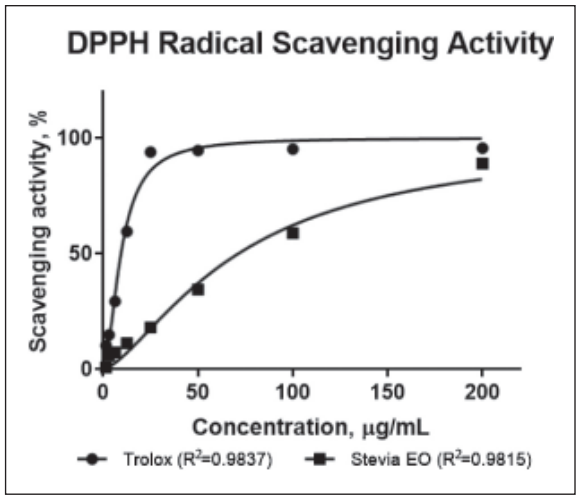

Fig. 2. The radical scavenging activity DPPH of SrB EO.

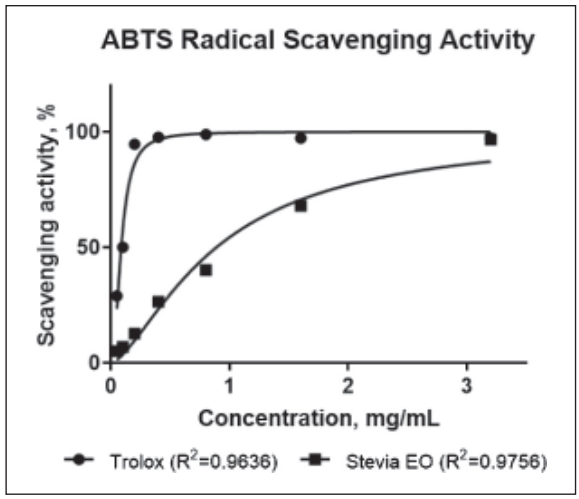

Fig. 3. The radical scavenging activity ABTS of SrB EO.

Table 3. Antioxidant Analysis (AOA) values of the SrB EO

Assay	IC_{50} of Trolox (µg/mL)	IC_{50} of SrB EO (µg/mL)	AOA (mg TE/g)
DPPH	9.395±0.22	71.36±1.57	22.87±0.38
ABTS	85.95±0.01	8.88×10^2±0.16	14.97±0.06
FRAP	NA	NA	9.55±0.26

of the initial DPPH. The compounds with high antiradical potential have low IC_{50}. The IC_{50} of DPPH assay was obtained using the equation y=100/(1+10^((LogIC_{50}-x)*$HillSlope$))) where the hillslopes for Trolox and SrB EO are 1.843 and 1.48 respectively (Figure 2). The IC_{50} of DPPH assay of SrB EO was 71.36 µg (Table 3). When compared with Trolox, the radical scavenging activity of SrB EO was a little bit lower however; increasing the concentration of SrB EO would increase the scavenging activity. According to Muanda *et al.* (2011), the IC_{50} of hydrodistilled SrB EO was 19.26 µg/mL. However, the IC50 of the water extract of SrB leaves was 83.45 µg/mL (Shukla *et al.,* 2012). These results showed that the amount of antioxidant might vary because of different extraction method and standard references used. However, SrB EO has still possessed a significant antioxidant activity.

ABTS radical scavenging assay

In ABTS radical scavenging assay, the antioxidant potential was measured by the capacity of the compound scavenge the ABTS cation radical which observed by the decolorization of a blue-green color of the ABTS radical solution. ABTS is a nitrogen-centered cation radical where scavenging activity happens via electron transfer. Differ from DPPH radical, ABTS is soluble in both organic and aqueous solvent, thus it is applicable for both hydrophilic and lipophilic antioxidants including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants as this radical dissolved in both water and organic solvents (Charles, 2013; Kumar, 2016). The result of the ABTS assay is expressed relative to the Trolox as standard. The IC_{50} of ABTS assay was obtained using the equation y=100/(1+10^((LogIC_{50}-x)*$HillSlope$))) where the hillslopes for Trolox and SrB EO are 2.189 and 1.491 respectively (Figure 3). The IC_{50} of SrB EO in ABTS assay was 0.888 mg/mL (Table 3) which was higher than IC_{50} of DPPH. In contrast, Victoria *et al.* (2012) reported that the antioxidant activity of *Eugenia flora* EO in ABTS assay was higher than the DPPH assay. The same pattern was reported by Ghazghazi *et al.* (2002); which *Rosa canina* also scavenged ABTS radical better than DPPH. The higher DPPH scavenging activity than ABTS due to the higher composition of lipophilic antioxidants compared with hydrophilic antioxidant. As shown in the GC-MS analysis of SrB EO (Table 1), it is also shown that terpenes group possesses higher composition in the essential oil compared to phenols.

FRAP assay

The mechanism of FRAP is based on electron transfer rather than hydrogen atom transfer. This assay is based on the reduction of Fe^{3+} to Fe^{2+} by

phenols. This process conducted in acidic condition (pH 3.6) to maintain the solubility of the iron, thus increases the redox potential rather than hydrogen atom transfer. In the presence of TPTZ, the reduction of Fe^{3+} to Fe^{2+} has occurred with the formation of a blue-colored complex of Fe^{2+}. The reducing potential is related to the degree of hydroxylation and the level of conjugation in phenols (Cerretani and Bendini, 2010). The FRAP values of 0.2 mg/mL SrB EO was 9.55 mg TE/g (Table 3). If compare the AOA of SrB EO (0.2 mg/mL), FRAP value was the lowest compared to other assays. This result was agreeable with the study by Apak *et al.* (2018) because glucose, fructose, and thiols group do not react with working FRAP reagent in the assay. Also, FRAP assay was conducted at low pH, thus decreasing the ionization potential and resulting in the reaction mechanism (Galanakis, 2018). Therefore, in the study of the antioxidant capability of a sample, varies of antioxidant assays required as each method has different condition and utilization of free radicals.

CONCLUSION

In this study, SrB EO consisted of hydrocarbon sesquiterpenes (0.47%), oxygenated sesquiterpenes (0.57%), oxygenated monoterpenes (3.08%), phenols (8.27%), oxygenated diterpenes (10.52%), fatty acids and derivatives (20.29%), oxygenated triterpenes (21.62%), and carbohydrates (21.68%) as major compounds. Also, SrB EO owns significant AOAs which proven in both TPC (14.44±0.63 mg GAE/g) and TFC (32.18±1.38 mg QE/g) assays. As tested in varies of AOAs; DPPH (22.87±0.38 mg TE/g), ABTS (14.97±0.06 mg TE/g) and FRAP (9.55 ±0.26 mg TE/g) assays, which expressed relative to Trolox also showed the SrB EO has high antioxidant capabilities.

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