Malays. Appl. Biol. (2018) 47(2): 15–23

CHEMICAL EVALUATION AND ANTIOXIDANT PROPERTIES OF EXTRACTS AND ESSENTIAL OIL FROM *Stevia rebaudiana* LEAVES

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Accepted 3 April 2018, Published online 25 May 2018

ABSTRACT

In spite of its prominence as a natural non-calorific sweetener used to replace table sugar, *Stevia rebaudiana* contains a variety of valuable phytoconstituents functioning as natural food-derived antioxidants with a protective effect against oxidative damage. In this study, leave extracts were screened for their phytochemical compounds and quantified for their total phenolic content (TPC) and flavonoid content (TFC). Qualitative phytochemicals screening shows the extracts were rich in tannin, steroid, alkaloids, coumarin, glycosides, and flavonoids. Interestingly, the highest TFC recovery was found in water extract (84.42 mg QE/g dry weight) while TPC detected showed no significant difference between acetone extract (170.0 mg GAE/g dry weight) and water extract (168.86 mg GAE/g dry weight). The essential oil (EO) was analysed by gas chromatography-mass spectrometry (GC-MS) and revealed thirty one compounds representing 92.85% of the total oils were detected. Some new compounds such as γ-sitosterol (1.15%), globulol (2.56%), epiputranjivol (4.69%), betulinic aldehyde (8.30%), and ionone (2.54%) were firstly identified from this plant. Among crude extracts, water extract possesses the highest percentage of radical scavenging activity (% RSA) with IC_{50} of 3.37 µg/mL lower than standard trolox (5.83 µg/mL). The IC_{50} of EO was significantly found to be 0.91 µg/mL comparable with standard ascorbic acid (0.51 µg/mL), thus reflecting water extract and essential oil as an excellent antioxidant sources.

Key words: *Stevia rebaudiana,* chemical evaluation, antioxidant activity, essential oil, bioactive compounds

INTRODUCTION

Stevia rebaudiana or also known as a 'sweet herb' is a small herbaceous perennial shrub of Asteraceae family growing wild in sandy soil native to region of Paraguay and Brazil (Sharma *et al.,* 2009). Among the 230 species in the genus *Stevia*, only the species *S. rebaudiana* and *S. phlebophylla* produce sweet principle known as steviol glycosides (Brandle & Telmer, 2007). Stevioside, one of the stevia glycosides, is about 300 times sweeter than saccharose and it has been applied as non-calorific substitute to conventional sucrose for treatment of diabetes mellitus, obesity, and hypertension (Pou *et al.,* 2007). Besides stevioside, *S. rebaudiana* also contains substantial amount of nutrients such as protein, fibres, carbohydrates, fatty acids, vitamins, volatile oils and micronutrients which adds nutritive value as food products (Chatsudthipong & Muanprasat, 2009).

Many plants have recently gained scientific interest for their extracts and essential oil (EO) in many different purposes, and one of greatest interest is antioxidant compound with possible use in food industry, cosmetology and medicine (Gawel-Beben *et al.,* 2015). Significant antioxidant activity in

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various essential oils such as clove, cinnamon, rosemary and spices is commonly reported. Hence, considerable attention on antioxidant activity of EO has been paid among researchers (Kapoor *et al.,* 2009; Basappa *et al.,* 2015; Boligon *et al.,* 2013). Various studies found that *S. rebaudiana* offer therapeutics values as they have antihypertensive, anti-inflammatory, anti-tumour, and anti-inflammatory properties (Gupta *et al.,* 2013). The protective effects of plant products are linked with naturally occurring antioxidant compounds such as phenolic acids, flavonoids, anthocyanins, vitamin C, magnesium, carotenoids, volatile oils and other phytochemical constituents predominantly present in *S. rebaudiana* leaves (Naomi, 2012). Foods containing rich in phenols or their potential antioxidant properties contribute to healthy effects and important to consumers. In addition, the antioxidant compounds present in edible plants have recently promoted as food additives due to nontoxic effects.

The uses of synthetic antioxidants have high antioxidant activity such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) but may cause adverse reactions which have possible activity as promoters of carcinogenesis (Tadhani *et al.,* 2007). The wide array of biologically active phytochemicals makes *S. rebaudiana* a valuable safer ingredient for pharmaceuticals and cosmeceuticals, other than food products (Gawel-Beben *et al.,* 2015). Thus, knowledge of the phytochemical profile of *S. rebaudiana* is important in understanding potential bioactivities of the compounds responsible for health effects as already demonstrated in human clinical trials (Lemus-Mondaca *et al.,* 2012).

However, for the extracts and essential oil to be safely used by human, least toxic solvent and safe extraction method were essential for optimised phytochemical isolation of natural sources of antioxidants with high activity to replace the harmful synthetic antioxidants. To the best of our knowledge, the chemical evaluation and antioxidative properties of leave extracts and EO from *S. rebaudiana* are remain insufficiently explored in Malaysia. Hence, the objectives of this study were to investigate phytochemical compound qualitatively and quantitatively, to characterize the chemical composition of EO by GCMS, and to evaluate the antioxidant properties of the extracts and EO. In addition, the correlation profile among TPC, TFC and antioxidant activity of *S. rebaudiana* extracts and EO were also investigated. Positive outcomes from the study could enhance the utilization of *S. rebaudiana* aqueous extract and EO for many therapeutic purposes.

MATERIALS AND METHODS

Sample collection and preparation

S. rebaudiana dried leaves were obtained from Koperasi Warisan Munsyi, Selangor Berhad (KOWARIS), Malaysia. The dried stevia leaves were grounded into powdered form by using dry blender. The powdered leaves were kept in opaque sealed container, at room temperature for further analysis.

Crude extracts preparation

The plant extracts were prepared using maceration technique (Kamal, 2016). Accurately, 50 g of powdered leaves was added with 500 mL of solvent. The mixtures were macerated in orbital incubator shaker at 200 rpm, for 90 minutes, at 40ºC. The mixtures were then strained and the marc (damp solid material) was pressed by using vacuum pump. Solvent extraction method was employed using 60% ethanol, 60% acetone, hexane and water.

Extraction of essential oil

The essential oil was extracted using modified method described by Wong *et al.* (2014). Dried leaves of *S. rebaudiana* were subjected to Soxhlet extraction using an absolute ethanol as an extraction solvent. The solvent was heated to reflux at 100°C for 10 hours. After the extraction, the product was further concentrated using a rotary evaporator at 60–65°C for 3 hours to obtain a greenish volatile oil with characteristic odour.

Qualitative phytochemical screening

Standard screening tests of water extract, 60% ethanol extract, 60% acetone extract, and hexane extracts were subjected to qualitative phytochemical screening for chemical constituents' identification (Auwal *et al.,* 2013; Sawant & Godghate, 2013; Zohra, 2015). For working solution, 0.5 g of crude extracts were dissolved in 100 mL distilled water, to have 5 µg/µL stock solutions.

Quantitative phytochemical screening

Total phenolic content (TPC)

Estimation of total phenol was conducted using Vongsak method with minor modification, using Folin-Ciocalteu reagent (FCR) (Vongsak *et al.,* 2013). The assay is based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolic compounds to a blue reaction product (Chaovanalikit & Wrolstad, 2004). Sample solution was measured at absorbance 750 nm. The standard curve was obtained using gallic acid standard (0-100 µg/mL) and the results were expressed as mg gallic acid equivalent (GAE/g dry weight).

Total flavonoid compounds (TFC)

Total flavonoid content of the extracts was performed by aluminium chloride ($AlCl_3$) colorimetric method (Woisky & Salatino, 1998) with slight modification. Absorbance at 415 nm was measured for sample solution against a reagent blank using UV-Vis spectrophotometer. The standard curve was attained using quercetin solution in the range of 0-100 µg/mL. Referring to quercetin standard curve, the results were expressed as mg quercetin/g dry weight, which was prepared under the same conditions. The total flavonoid were expressed as mg quercetin equivalents (QE/g dry weight).

Analysis of essential oil by GC-MS

Chemical analysis of *S. rebaudiana* essential oil was implemented by GC-MS according to Siddique *et al.* (2012) method, GC-MS (Model QP5050A, Shimadzu Japan) equipped with a fused silica capillary column (30 m × 0.25 i.d. mm. film thickness 0.25 µm). Helium (He) 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. 0.2 µL of diluted samples (1:100 w/v, in methanol) were manually injected in split less mode.

Antioxidant assay

Antioxidant activity of the extracts and essential oil were analysed using DPPH radical scavenging activity by using modified microplate protocol that has been reported by Lee *et al.* (2014). The assay was conducted in the 96-well plate using 10 mg/mL of stocks samples dissolved in dimethyl sulfoxide (DMSO). Various samples concentration was prepared by 1:1 serial dilution with DMSO before adding DPPH reagent. The plates were incubated for 30 minutes at room temperature in the dark. Absorbance reading at 515 nm of the plate were taken by using a Synergy 2 with UV-Vis microplate reader (Biotek, USA). Calculation formula of radical scavenging activity is as follows:

$$\text{Radical scavenging activity, RSA (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

Ac = absorbance of control
As = absorbance of sample

RESULTS AND DISCUSSION

Yield of crude extracts and essential oil

In the present study, the effect of different solvent extracts on extraction yield of *S. rebaudiana* leaves were evaluated by using binary solvents of 60% ethanol and 60% acetone. Referring to Table 1, the yields of extraction by various solvents decreased in the following order: 60% ethanol extract > 60% acetone extract > water extract > hexane extract. The result showed that the extraction yield increases with increasing polarity of the solvent due to the presence of polyphenols or intermediate polar compounds in the leaves which are more preferable towards aqueous mixtures containing ethanol and acetone. However, the most polar solvent of water showed lower extraction yield compared to 60% ethanol and 60% acetone, which might be due to loss of water content during freeze-drying process. Therefore, the binary solvent system of water and organic solvent may enhance the extraction of compounds that are soluble in both water and organic solvent as observed in 60% ethanol and 60% acetone extracts. Study by Do *et al.* (2014) showed that increasing water concentration in the organic solvent enhances extraction yield as compared to pure solvent, which was also observed by other studies (Arnorld, 2014; Downey & Hanlin, 2010; Kamal, 2016).

While yield of hexane extract is much lower compared to water, 60% ethanol and 60% acetone extract as hexane extract is commonly used to remove chlorophylls in plant extract (Sasidharan *et al.,* 2011) or nonpolar compound from plant material. The nonpolar property of hexane solvent allows it to extract fatty materials of plant compounds. This result is in agreement with Doughari (2012) that reported the percentage yield of successive extractive values for leaves of *Symplocos cochinchinensis* using hexane extract has the lowest yield of 2% (w/w). A compositional study by Tadhani & Subhash (2006) revealed that the composition of fatty materials in *S. rebaudiana* is only 4.34% suggesting extraction yield by hexane to be the lowest. As for EO extract, Soxhlet extraction of *S. rebaudiana* dried leaves gave a greenish oil yield of 17%, which was found to be higher than a previous study by Muanda *et al.* (2011) who reported the yield of 3.5 ± 0.15 g using hydro distillation method.

Qualitative phytochemical screening

Qualitative phytochemical screening were conducted to detect the presence of different

Table 1. Extraction yield of *S. rebaudiana* crude extracts and essential oil

Extracts	Extraction yield (% w/w)
Water extract	28.60 ± 0.31
60% ethanol extract	38.60 ± 0.12
60% acetone extract	32.40 ± 0.49
Hexane extract	5.26 ± 0.18
Essential oil	17.0 ± 0.36

Table 2. Qualitative phytochemical screening of *S. rebaudiana* leave extracts

Chemical test	Extracts			
	Water extract	60% Ethanol extract	60% Acetone extract	Hexane extract
Saponin	+	++	++	–
Tannin	+++	+++	+++	+++
Steroid	++	++	+++	++
Alkaloid	++	+++	++	+
Coumarin	+++	+++	+++	++
Cardiac glycosides	–	–	–	–
Flavonoids	+++	+++	+++	+++
Anthocyanin	–	–	–	–

phytochemicals extracted in the *S. rebaudiana* plant leaves extracts. Based on Table 2, the crude extracts subjected to qualitative phytochemicals screening using chemical method shows the leaf extracts were rich in tannin, steroid, alkaloids, coumarin and flavonoids which were found in all crude extracts. Saponin was abundantly present in all extract except hexane with the most intense in 60% ethanol and 60% acetone and slightly abundance in water extract. While cardiac glycosides and anthocyanin were found to be absent in all crude extracts. Tannin and flavonoids were intensely present in all extract and are known to possess antioxidant activity (Arnorld, 2014). Alkaloid was most abundance in 60% ethanol, followed by 60% acetone, water, and least presence in hexane. Alkaloids are the largest group of secondary metabollites which play a metabolic role in the living systems and are involved in the protective function in animals (Doughari, 2012).

Free steroid compound was detected in all of the extracts in this study, where steroid was most abundance in 60% acetone extract, and relatively abundance in water, 60% ethanol, and hexane extract. Coumarin is intensely present in all extract and relative abundance in hexane. It has a sweet odour and recognised as a chemical defences to discourage predation (Borges *et al.,* 2005). Besides, essential oils derived from some plants also contain coumarin derivatives and are used as flavouring in foods (Rosselli *et al.,* 2009). The presence of anthocyanin was negative for all of the extracts in this study however study by Muanda *et al.* (2011) found that water extract and 50% methanol extract of *S. rebaudiana* possess significant amount of anthocyanin. Anthocyanin is present in dermis of the leaves and high exposure to light, osmotic stress, and nutrient deficiency which can lead to its absent (Norkaew *et al.,* 2017).

Quantitative phytochemical assessment

Phenolic is the largest and ubiquitous plant chemicals with flavonoids as the most important natural phenolic (Tsao, 2010). These compounds are the most prevalence for antioxidant activities. As seen in the Figure 1, the amount of TPC obtained decreased in the following order; 60% acetone extract (170.0 mg GAE/g dry weight) > water extract (168.86 mg GAE/g dry weight) > 60% ethanol extract (85.91 mg GAE/g dry weight) > hexane extract (45.23 mg GAE/g dry weight). This study presented that the amount of TPC recovered in water extract was no significant difference compared to 60% acetone. Meanwhile, the amount of TFC recovered in the crude extracts are as followed; water (84.42 mg QE/g dry weight) > 60% acetone (78.33 mg QE/g dry weight) > 60% ethanol (76.46 mg QE/g dry weight) > hexane extract (18.33 mg QE/g dry weight), thus demonstrating significant contribution of phenolic compounds. According to Azmir *et al.* (2013), the polar solvents such as water are frequently used for recovering soluble polyphenols contains within the compartment of plant cell vacuoles while less polar solvents such as alcohols are more efficient in cell wall and seed degradation. The amount of TFC recovered interestingly was highest in water extract and believed to be widely distributed in plant vacuole which could increase water solubility.

Comparing among binary solvents, 60% ethanol had the lowest yield of TPC and TFC. The removal of 60% ethanol solvent during extraction process requires higher temperature and longer time exposure (65ºC for 2 hours) compared to 60% acetone (60ºC for 1 hour). The loss of polyphenolic compounds might be due to the usage of high heat and long exposure time during solvent removal. Higher temperatures and longer exposure times reduced extract polyphenol diversity (Vergara-Salinas *et al.,* 2012) and this might be the reason for higher TPC and TFC recovery in 60% acetone compared to 60% ethanol. Furthermore, aqueous acetone is believed to be a good choice for extracting higher molecular weight flavanols (Dai & Mumper, 2010) and phenolic acid with one -OH group (Gawel Beben *et al.,* 2015). Study by Park *et*

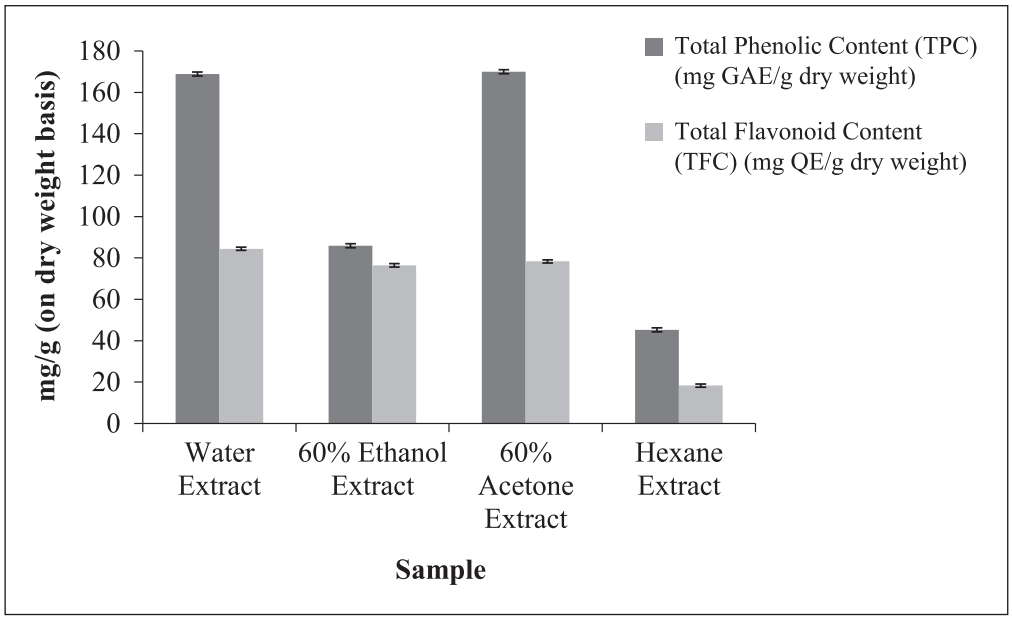

Fig. 1. The total phenolic content (TPC) and total flavonoid content (TFC) of *S. rebaudiana* extracts. TPC and TFC were expressed as mg GAE/g dry weight and mg QE/g dry weight, respectively. Error bars represent mean ± standard deviation (n=3).

al. (2014) also reported that the highest polyphenol concentrations was from the acetone extract of orange flesh, followed by the ethanol and methanol extracts, which permits them to act as hydrogen or electron donors. However, when compared to others studies, crude extracts obtained in the present study have much higher TPC and TFC as compared to water extract containing only 56.73 mg GAE/g dry weight of TPC and 15.65 µg QE/g dry weight of TFC as observed by Shukla *et al*. (2012) and Kim *et al.* (2011), respectively.

The result obtained in the present study is in agreement with previous reports that the extraction of phenolic compounds in plant material was influenced by solvent used, chemical properties, extraction method and time (Anokwuru *et al.,* 2011). The uses of organic solvents need further separation from the extracts, with some of them (i.e.: acetone, methanol) can be toxic to humans (Bhebhe *et al.,* 2016). Least toxic solvents such as ethanol, however requires higher temperature and longer exposure time during separation. Hence, water extract has performed the most potential for phenolic compounds recovery.

Analysis of *S. rebaudiana* essential oil by using GC-MS

Extraction yield of EO from *S. rebaudiana* leaves was 17.0% ± 0.36 as shown in Table 1. GCMS chromatogram (Figure 2) reveals thirty one compounds representing 92.85% of the total oils from the leaves. Major constituents present are lupeol acetate (19.75%), hexadecanoic acid (4.79%), α-linoleic acid (4.43%), 2, 4-α-8, 8-tetramethyldecahydrocyclopropanaphthalene (8.28%), epiputranjivol (4.69%), lupeol (5.01%), and taraxerone (3.7%). The EO from *S. rebaudiana* leaves comprised a complex mixture containing mainly terpene hydrocarbon and oxygenated compounds, which contributing to the aromatic compounds such as aldehyde, alcohol, phenol and methoxy derivatives. In recent years, several scientists have revealed that monoterpene and sesquiterpene hydrocarbons as well as their oxygenated derivatives are the major components of EO of plant origin with massive biological potential (Siddique *et al.,* 2012; Boligon *et al.,* 2013; Adinew, 2014). Interestingly, some new compounds were identified such as γ-sitosterol (1.15%), globulol (2.56%), epiputranjivol (4.69%), betulinic aldehyde (8.30%), and ionone (2.54%) that had not been previously reported as constituents of *S. rebaudiana*. However, similar compounds identified in these extracts were also reported in other literature such as caryophyllene oxide and spathulenol (Siddique *et al.,* 2012). Many of EO compounds are known for their bioactivities and the presence of compounds detected are believed to be influenced by several factors like climate, geographical, and nutritional status, which can affect the composition of EO (Adinew, 2014; Muanda *et al.,* 2011).

Antioxidant assay of extracts and essential oil

Figure 3 shows the IC_{50} value of extracts and EO. Among crude extracts, water extract has the

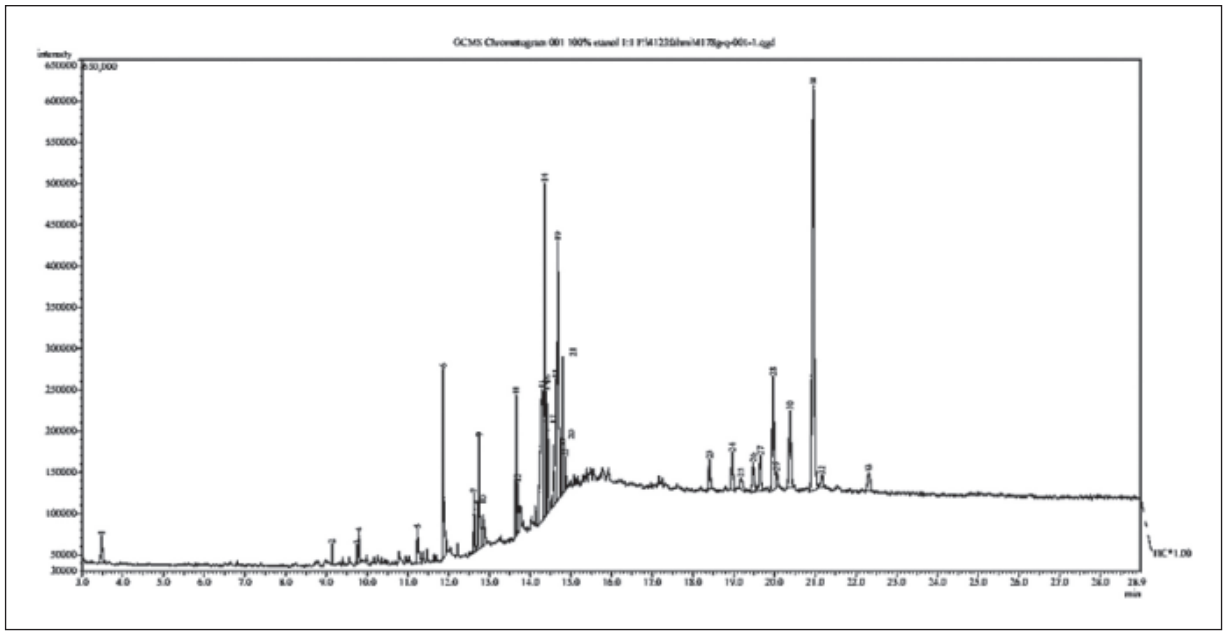

Fig. 2. GC-MS chromatograms of the constituents of *S. rebaudiana* essential oil.

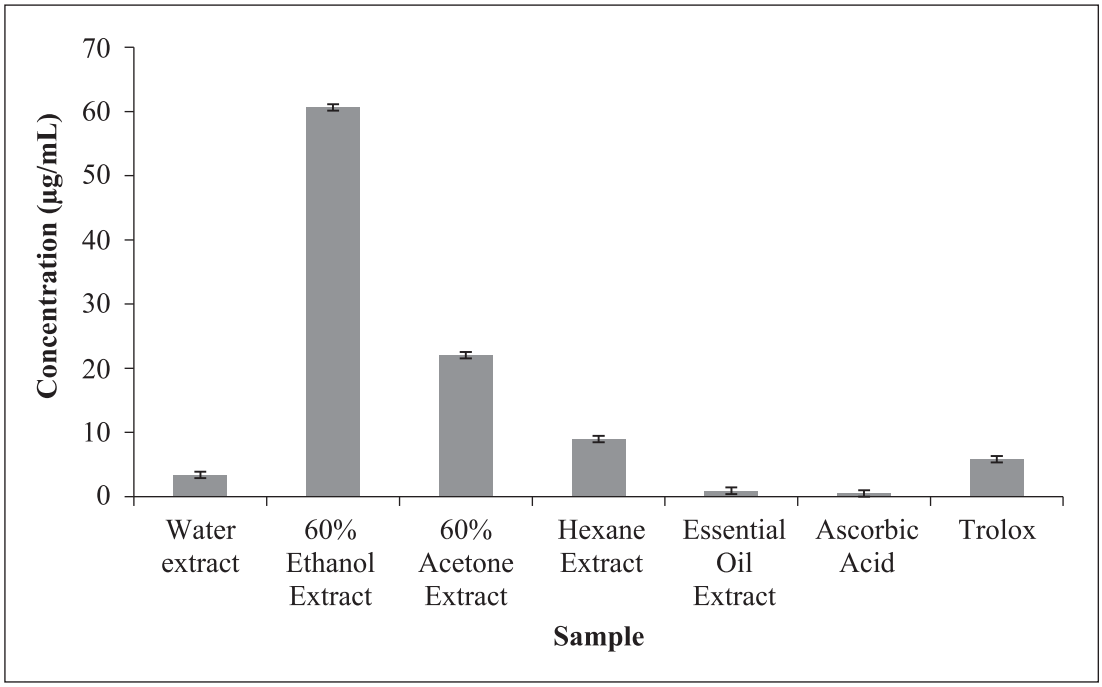

Fig. 3. The IC_{50} value of *S. rebaudiana* crude extracts and essential oil with comparison of the IC_{50} value of standard ascorbic acid and trolox. Error bars represent mean ± standard deviation (n=2). IC_{50} were obtained by plotting log (concentration) vs. % radical scavenging activity (% RSA) using GraphPad Prism software.

lowest IC_{50} which is 3.37 µg/mL, comparable to standard trolox (5.83 µg/mL), followed by 60% acetone extract (13.8 µg/mL), 60% ethanol extract (18.89 µg/mL) and the highest is hexane extract which is 27.62 µg/mL. Thus, water extract is said to contain more antioxidant contents as only small concentration is needed to reach 50% inhibition. High antioxidant activity of water extract was also in line with high TPC and TFC recovered in this study, where TFC recovered in water extract was higher than 60% acetone. A study by Wang & Xu (2014) showed that water extracts of *Agaricus subrufescens* possessed much higher antioxidant properties than organic solvent extracts (acetone

and ethanol). Muanda *et al.* (2011) and Gawel-Beben *et al.* (2015) indicated in their studies that the key compounds in water stevia extract including quercetin, caffeic acid and protocatechuic acid with the most potential therapeutic values.

It has been reported that various flavonoids are extracted with acetone, which probably describes the antioxidant activity of 60% acetone to be higher than 60% ethanol (Jimenez *et al.,* 2011). Ethanol was less efficient in the extraction of antioxidant compounds than water that may be due to the presence of the longer ethyl radical resulting in a lower solubility of the antioxidant molecules (Boeing *et al.,* 2014). For EO extract, the IC_{50} value was 0.91 µg/mL which was much lower compared to standard trolox and slightly higher than ascorbic acid standard. Antioxidant activity of EO has been ascribed to the presence of compounds such as lupeol, ionone, globulol, epiputranjivol and linoleic acid. This result shows that the *S. rebaudiana* essential oil obtained in this study is an excellent radical scavenger comparable with ascorbic acid. Based on literatures, compound such as pinene, carvacrol, and caryophyllene are known for their antioxidants properties (Kapoor *et al.,* 2009). According to Paixao *et al.* (2007), DPPH was known to react specifically with low molecular weight phenolic compounds; where the low molecular weight phenolic compounds extracted in this study might have contributed to the high scavenging activity on DPPH radicals. The results presented here may reveal to the knowledge of the antioxidant potential of the essential oil and provide some information for its uses.

CONCLUSION

Different solvents and extraction method affect the TPC, TFC and antioxidant activity of all crude samples significantly. Besides, the yield of extract recovered would not attribute to phenolic recovery or high antioxidant activity as it can be influenced by the synergistic effects of the extracted phenolic compounds itself. Among crude extracts, water extract has performed the best extraction medium for various phytochemicals, high TPC and TFC recovery with the best antioxidant activity demonstrated in this study. The *S. rebaudiana* essential oil obtained in this research shows a few new compounds detected with potent antioxidant activity. Regarding these results, both water extract and essential oil of *S. rebaudiana* can be considered as a potential source of natural antioxidant and its valuable constituents can be applied for numerous commodities of pharmaceutical, cosmetics, and medicinal attributes and to improve the efficacy of food products.

ACKNOWLEDGEMENTS

The authors would like to thank the Universiti Putra Malaysia (PUTRA Grant-IPS9471300) for funding this research activity. The authors are grateful to the Laboratory of Food and Microbiome Technology (FAMTech), Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia for providing laboratory facilities for the duration of the research project period.

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