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Original Article

Effect of different drying methods on antioxidant properties, stevioside and rebaudioside A contents of stevia (*Stevia rebaudiana* bertoni) leaves

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Abstract

The effect of different drying treatments of stevia leaves on antioxidant activity, ferric reducing power (FRAP), total phenolic content as well as stevioside and rebaudioside A content were evaluated. Drying treatments that were applied were oven drying (80°C and 60°C), sun drying, microwave drying and freeze drying. Results of all samples were compared with fresh leaves. Antioxidant activity of dried leaves was evaluated using DPPH radical scavenging activity and ferric reducing power (FRAP) by spectrophotometer. Stevioside and rebaudioside A content were evaluated using HPLC. Among all drying treatments, microwave was found to be the highest in scavenging the DPPH radical activity with no significant different with freeze dried and fresh leaves ($P > 0.05$). Inhibitory concentration at 50% of microwaved leaves was the lowest compared to other dried leaves. In addition to that, microwave dried leaves exhibit highest total phenolic content at 53.95 ± 2.83 mg/g gallic acid equivalent. As for stevioside and Rebaudioside A, no degradation happened in comparison with fresh leaves after drying treatment. Stevioside appeared to be higher in content than rebaudioside A. This indicate that microwave can be good drying method, without altering the stevioside and rebaudioside A content inside the leaves, thus maintaining the sweetening properties of the leaves.

Keywords: Stevia, Drying methods, Antioxidant properties, Stevioside, Rebaudioside

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Introduction

The increase prevalence of obesity related disease spark health concern among people and until now there is no cure yet to been found. Besides, the new tax on sweetened beverage have been announced in Malaysian Budget 2019 indicate the concern on the high usage of caloric sweetener among population (The Star, 2018). Thus, consumers as well as industry are searching for an alternative food that offers good health properties that derived from natural sources. Since stevia was a non-caloric natural sweetener and 300 times sweeter than sucrose (Carbonell-Capella et al., 2015), thus it attract other countries like Japan, China and South Korea to grow stevia and commercially produce the sweetener from it. Japan

might be the biggest country which vastly used sweetener extracted from stevia in more than 100 food product in their market besides South Korea which 40% of sweetener in their market was occupied with stevioside (Kinghorn et al., 2001). Stevia is a sweet plant which named as *Stevia rebaudiana* Bertoni and originally from Paraguay. This plant belongs to Asteraceae family, and was reported to be the sweetest species (Barroso et al., 2016).

Stevia leaves come with diterpene glycosides or steviol glycosides which is a group of compound that is responsible for its sweet taste. There were five minor compounds which are, rebaudioside B, rebaudioside D rebaudioside F, steviolbioside, rebaudioside E, rubusoside and steviolmonoside (Ashwell, 2015) with each of them content less than 1% , and four major diterpene which is stevioside (5-10%), rebaudioside A (2.0-4.0%), rebaudioside C (1-2.0%) and dulcoside A (0.4-0.7%) which grouped into steviol glycosides cluster. Acceptable daily intake of steviol glycosides was 0-4mg/kg per body weight which projected from toxicity and carcinogenicity study made on rat (Xili et al., 1992) and in 2008 FDA approved refined form of stevia extract and acceptable as general regarded as safe food additive (GRAS)(U.S. Food and Drug Administration, 2008). Stevia leaves are often consumed as infusions because of their properties in antioxidant and their stem possess high flavonoid and phenol content (Periche et al., 2015). Stevia also rich in ascorbic acid, β carotene other mineral content like chromium, cobalt, magnesium, iron, potassium, phosphorous, riboflavin, thiamin, tin, zinc, and so forth (Goyal et al., 2010). In addition to these compounds, proximate analysis proved that stevia also contain vitamin C, all of the indispensable amino acids except for tryptophan and phenolic acid (Lemus- Mondaca et al., 2012) such as caffeic acid derivatives (Barroso et al., 2016) and gallic acid (Periche et al., 2015).

Stevia need to be conserved before use and the common method used was drying and often recommended to conserve, add value and extend food shelf life (Pinela et al., 2011). Samsuddin and Aziz (2013) reported that stevia that is dried within 8 hour is essential in order to maintain its sweetness. Dehydration of plant can be done by many methods with oven drying might be the simplest drying methods and faster than sun drying (Veerakumar et al., 2014). Microwave drying give shorter drying time and low water activity (Chong and Lim, 2012), while freeze drying is proven to be an efficient method to preserve antioxidant and other biochemical compounds (Pinela et al., 2011). However, all of this drying might alter the chemical composition of the plant in particular. Thus the best drying method with minimal loss of nutritional composition inside stevia leaves should be identified. But there is limited study on the effect of drying method on this composition inside stevia leaves. Thus, these justifications lead to the aim of this study.

Material and Methods

Plant material and preparation of sample

The fresh and matured leaves of *Stevia rebaudiana* were obtained from Kg Tempinis, Mukim Jabi Besut Terengganu. Stevia plants that reached maximum growth stage prior to flowering (approximately after 45-60 days) and leaves that are about two inches long and three quarters of an inch in width are considered mature. The samples treated with five drying treatments which were direct sunlight for 12hour, oven drying (60°C) for 5 hour , oven drying (80°C) for 3 hour, microwave at 1200W for three minutes, and freeze drying for 48hour,. All of these drying methods were done until moisture content reach ≤20% of weight sample. For fresh sample, the leaves were dried at 34°C for 48h, in order to express the results in dry weight basis to be comparable with the other dried samples, thus the fresh sample were designated as fresh dried sample.

Extraction of sample

10 g of dried stevia leaves from each treatment were weighed and put into the conical flask that previously wrapped with aluminium foil to minimize the light exposure. Then 100ml methanol was added into the conical flask and shaken at 150 rpm for 24 hours at room temperature on orbital shaker,. Macerated methanol solution then was filtered with filter paper (Smith, United Kingdom), solution was collected and the extraction procedure was repeated 2 times to maximize the extraction process,. The extract was then concentrated using rotary evaporator (BUCHI Rotavapor R-200). The concentrated extract was collected into airtight amber bottle.

DPPH radical scavenging activity

100µL of sample were load into 96-well plates (Kartell, Italy) 100µL of DPPH solution that previously dissolved in methanol at 100µM concentration was added into the sample. The mixture

of DPPH solution with sample in the 96-well plates was left in the dark for 30 minutes for the reaction to occur. After 30 min, samples were read at 520nm using microplate reader (Multiskan, United States)(Wang et al., 2008). Antioxidant activity of sample was calculated as a percentage inhibition (%). Antioxidant activity for each sample at different concentration also was evaluated to obtain inhibitory concentration 50 (IC_{50}) value.

Ferric reducing power assay (FRAP)

For FRAP assay, the method applied was from Thaipong et al. (2006) with some modification.100µL sample extract were added to solution contained 900µL distilled water and 2ml FRAP in different test tubes for 30 min in the dark. After 30 minutes, the colour mixture of sample and FRAP reagent was read at 593nm. $FeSO_4$ was used as a standard and the concentration of FRAP content in the sample extract was reported as $FeSO_4$ equivalent (µM).

Total phenolic content

The total phenolic content in the sample extracts were analyzed using Folin- Ciocalteu method (Akay et al., 2011). 100µL of sample from different concentrations was made up to 10ml with distilled water. 500µL of 0.2N Folin-Ciocalteu's reagent was added into the sample solution and vortex before left to stand for 5 minutes. After 5 minutes, 1.5ml of 7.5% Na_2CO_3 was added into the solution and left at room temperature for one hour. The absorbance was recorded at 760nm using spectrophotometer (Merck Milipore, United States) and gallic acid was used as a standard, result was expressed as a gallic acid equivalent (GAE/g dry weight basis).

HPLC determination of Stevioside and Rebaudioside A in stevia water infusion

Sample preparation

0.5g dried leaves of *Stevia Rebaudiana* bertoni were extracted by threefold extraction using 10ml hot water (85°C) for 30 minutes for each times. The extracts were filtered using filter paper and adjusted to 100ml in volumetric flask.

Chromatographic condition

The condition was adopted from Joint FAO/WHO expert committee on food additives (JECFA). 5µl sample injected under following conditions, column temperature was 40°C with mobile phase acetonitrile: deionized water (32:68) at flowrate 1.0ml/min and UV detector at 210nm wavelength, the chromatogram was recorded for 30 min using Syncronics C18 (length: 250mm inner diameter: 4.6mm, particle size: 5µm) column (Phenomenex, USA) in high performance liquid chromatography system module.

Statistical Analysis

All data was subjected to one way ANOVA followed by Tukey test at 5% significant level ($p<0.05\%$) using SPSS software.

Results and Discussion

Effect of different drying treatment on DPPH inhibition activity

DPPH radical scavenging activity showed that microwave, freeze dried and fresh leaves possess the highest antioxidant activity respectively with 89.16%, 88.52% and 88.97%, respectively (Table 1). Inhibition concentration at 50% inhibition was assessed in our study and fresh stevia leaves give the lowest concentration of IC_{50} which is 27.65 µg/mL thus show the highest antioxidant capacity due to its ability to scavenge 50% of free radical activity at lowest concentration while microwave showed second highest antioxidant capacity at 27.84 µg/mL. Therefore microwave drying was found to have a positive effect on the antioxidant activity of stevia leaves, showing potential to be used as a drying method.

Table 1. Percentage inhibition and inhibitory concentration 50(IC_{50}) value of different dried leaves

Treatment	Percentage Inhibition (%)	IC_{50} Value (µg/ml)
Freeze drying	88.52 ± 0.29^{a}	35.44
Microwave	89.16 ± 0.38^{a}	27.84
Sun Drying	88.20 ± 0.00^{ab}	54.15
Oven Drying (60°C)	67.18 ± 1.45^{c}	100.6
Oven Drying (80°C)	59.05 ± 1.35^{d}	174.5
Fresh	88.97 ± 0.19^{a}	27.65

Note: Values are expressed as means ± SD. Means followed by different letters in the same column are significantly different at $P<0.05$

IC_{50}: Inhibitory concentration at 50%

There were some works previously and recently in regards on the levels and antioxidant capacity of stevia leaves at different drying methods, hot air drying at

180°C (126 mg Trolox equivalent/g) (Periche et al., 2016), oven dried 30°C IC_{50} at 22.87 µg/mL (Barroso et al., 2016), air dried IC_{50} at 83.45 µg/mL (Shukla et al., 2012) and IC_{50} of 626.37 µg/weight of sample with water extract and 683.90 µg/weight of sample with methanolic extract by sun drying at temperature ranged 25-30°C for 24-48h (Abou-Arab and Abu-Salem, 2010). The lowest antioxidant capacity was for sample that oven dried at 80°C which is 174.5 µg/ml, sample that dried oven at 60°C also show lowest radical scavenging activity 100.6 µg/ml.

Effect of different drying treatment on Ferric Reducing Antioxidant Power (FRAP)

Fresh leaves showed significantly higher in antioxidant activity (41.27± 2.87 µM FeSO4 Equivalent) compared to dried leaves from other drying method (Figure 1). Meanwhile dried leaves from oven drying at temperature 80°C recorded the lowest antioxidant activity (6.69± 0.23 µM FeSO4 Equivalent), meanwhile freeze drying and microwaved dried leaves showing good reducing capability of Fe3+ into Fe2+, compared to the other dried leaves.

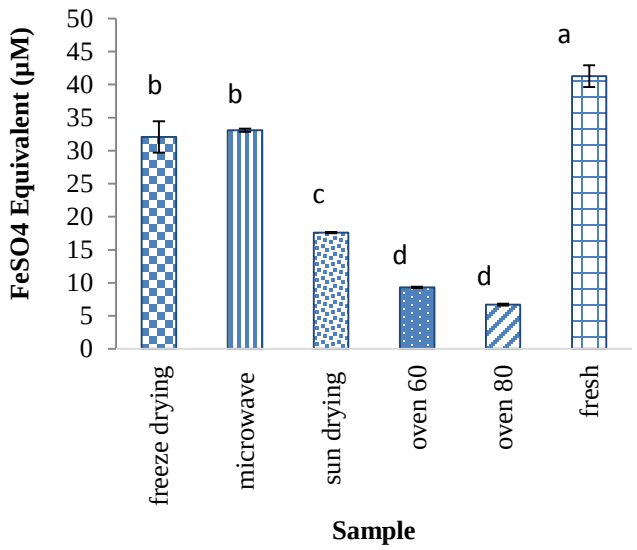

Figure 1. Ferric reducing antioxidant power assay (FRAP) value for different drying treatment. All values are expressed as micromolar ferrous sulphate (µM $FeSO_4$) equivalent. Different letters in the bar chart graph represent significantly different value *P<0.05*

Effect of different drying treatment on total phenolic content (TPC)

Phenolic compounds is some of antioxidants components that were commonly higher in fresh leaves in comparison with dried leaves due to its sensitivity upon heat during drying process. While that happen, recently there are many research that report their tolerance towards heat which mention the increase in content after drying treatment applied. Roshanak et al. (2016) reported that total phenolic content of green tea increased after drying and green tea that dried in oven 60°C showed highest radical scavenging activity and reducing power. Youssef and Mokhtar (2014) studied on the effect of drying method on the antioxidant and phytochemical content of *Portulaca oleracea* L. leaves. They found that drying by hot drying and freeze drying exhibit lowest adverse effect on antioxidant activity of the leaves. In case of our study, microwave dried leaves showed significantly ($p<0.05$) high total phenolic content recorded with 53.95 ± 2.83 mg GAE/g dry basis compared to the other dried leaves (Table 2). But microwave dried leaves were not significantly ($p>0.05$) different with freeze dried leaves. The oven dried leaves at 60°C and 80°C would not be the most competitive method in preserving total phenolic content as oven 60°C and 80°C show 23.23% and 31.47% reduction of total phenolic content in respect to fresh leaves ($p<0.05$).

Table 2. Total Phenolic Content in mg GAE/g dry weight of different dried leaves.

Drying Method	Total Phenol Content(mg GAE/g dry weight basis)
Freeze Drying	49.03 ± 1.84^{ab}
Microwave	53.95 ± 2.83^{a}
Sun Drying	47.22 ± 1.06^{ab}
Oven Drying (60°C)	$33.36. \pm 0.16^{c}$
Oven Drying (80°C)	29.78 ± 2.41^{c}
Fresh	43.46 ± 9.14^{ab}

Note: Values are expressed as mean ± SD. Means followed by different letters in the same column are significantly different at *P<0.05*

All values are expressed as milligram gallic acid equivalent per dried weight basis (mg GAE/g dry weight basis)

Correlation between total phenolic content and antioxidant activity

Figure 2 shows a good correlation between total phenolic content and antioxidant activity. This good correlation show high possibility that antioxidant activity in dried stevia leaves attributed by phenol compound inside the leaves. The similar correlation between antioxidant activity and polyphenols in ethanolic extracts stevia also was shown by Kaushik et al. (2010). Polyphenols give direct indicator to the antioxidant activity in red wine as it is higher in antioxidant activity compared to white wine (Pignatelli et al., 2006). It can be said that high antioxidant activity as seen in microwave dried leaves due to polyphenol content inside the dried leaves. Microwaves are the electromagnetic waves which can be absorbed by water containing materials and then converted into heat with more uniform heating and rapidly heated (Maskan, 2000). Plant material consists complex series of compound that can be either phytochemical, phenol and other compound with each of them has different dielectric permittivity resulting in selective heating as the microwaves energy is absorbed by the compounds with high dielectric loss factor after passing through the low transparent compounds (Hayat et al., 2010), this can explain the significant increase in gallic acid in stevia leaves after being microwaved and Hayat et al. (2010) verified this in their research which there is no significant different in gallic acid content between 15 minutes and 10 minutes microwave treatment time on citrus mandarin peel, with an exception of gallic acid, other phenolic content show significant decrease as the microwave heat treatment time increase.

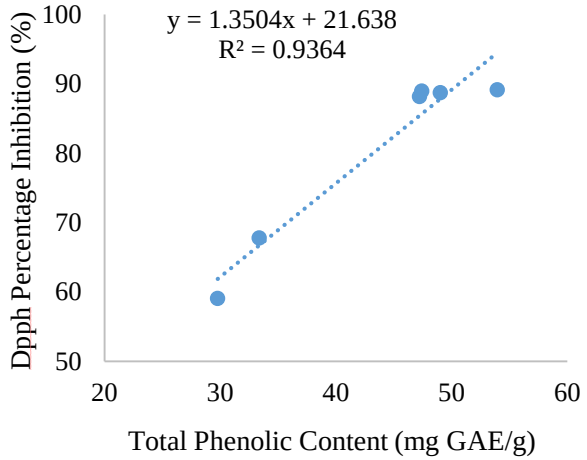

Figure 2. Correlation graph between total phenolic content and antioxidant activity

This main idea show that different phenolic content inside the plant material possessed individual resistance to microwave heating. This trend also can be seen in recent studies done by Valadez-Carmona et al. (2016) which report that their coconut husk that dried by microwaves showed an increase in four type of phenol content which is gallic, 4-hydroxybenzoic, ferulic and syringic acids when compared with fresh coconut husks. Study by (Barroso et al., 2016) indicates that oven drying method give the highest total phenolic content meanwhile (Periche et al., 2016) found that air drying exhibit higher increment in flavonoids and phenolic compounds. The reported phenolic content by Periche et al. (2015) was 76.8 mg equivalent/g (hot air drying at 180°C), 39.1 mg equivalent/g (shade drying), 31.5 mg equivalent/g (hot air drying at 100°C) and 26.2 mg equivalent/g (freeze drying).

This incident explained in paper by Hossain et al. (2010) and Roshanak et al. (2016), active enzyme present in fresh leaves may contribute to the degradation of phenolic compounds in the leaf. Low water activity present in the dried leaves will make destructive enzymes such as polyphenol oxidase. This enzyme degrades phenolic compounds via oxidation (Chan et al., 2009), inactivate and indirectly preserving the phenolic compounds inside it. All these previous studies explain that drying can still either preserve, increase or decrease the retention of antioxidant and total phenolic content but nevertheless it may vary with the drying condition applied and type of phenolic compounds inside the plant material. Miletić et al. (2013) reported that drying method induces dramatic increase in gallic acid while decreasing neochlorogenic acid in fruits of plum.

Freeze drying has the same concept with other drying methods which is to remove moisture from the samples to prevent plant material and food samples from deteriorate. The degradation of nutrient inside the plant material and food will be also preserved. Taking into consideration that total phenolic content for freeze drying dried stevia leaves recorded t at 49.03 ± 1.84 (mg GAE/g dry weight basis) with no significant different ($p>0.05$) from total phenolic content from microwave dried leaves present a bit higher phenol content maybe due to the cell disruption as a result of ice crystal formation during freezing process release certain enzyme that degrade certain phenolic compounds. This drying method can be regarded as suitable method in maintaining the stability of phenolic content inside the plant material

by releasing the phenolic compound from the cellular constituents break apart after undergoing freeze drying process (Chan et al., 2009) thereby lead to the increase of phenolic content.

Effect of different drying treatment on rebaudioside A and stevioside

Stevioside and rebaudioside was reported to be stable in wide condition. No photodegradation of stevioside and rebaudioside A reported by Clos et al. (2008).There was no sign of steviol glycoside decomposition shown in several food matrices (Jooken et al., 2012), and at pH 2-6.5 over 72h and at 50°C indicate the high stability of stevioside. Figure 3 summarize the effect of drying treatments on stevioside and rebaudioside A. Stevioside recorded high in concentration compared to rebaudioside A. Rebaudioside A in freeze dried stevia leaves recorded the highest at 0.0335±0.0032 mg/g with the lowest was in microwave leaves at 0.00815±0.0021 mg/g. Figure 3 show that there was no degradation of rebaudioside A in all drying treatments compared to fresh leaves. Even though microwave dried show lower in concentration of rebaudioside A in respect to fresh leaves, but ANOVA do not show different homogenous group. In contrast to our study, dehydrated stevia leaves from study by Periche et al. (2015) showed very low concentration of rebaudioside A in all drying conditions, which ranging from 0.5±0.14 mg/g (in shade drying) to 6.1 ± 1.6 mg/g (in hot air to 180°C drying).However, they conclude as no difference between the leaves thus show the stability of rebaudioside A. Meanwhile for stevioside freeze dried, sun dried and oven dried (at 60°C), leaves showed increasing in concentration by 176%, 79% and 17.6%, respectively. Stevioside showed good thermal stability at microwave and oven drying (at temperature 80°C) with no significant different in concentration compared to fresh leaves, thus all thermal and non-thermal treatment in this study does not degrade the stevioside content but might as well increase and maintain the stevioside concentration. Reported data from the other study vary greatly with no specific detail on the drying method applied, besides lack of research done on the thermal stability of stevioside in dried leaves. There was important decrease in stevioside in all dehydrated samples of stevia leaves reported by (Periche et al.,2015) compared to fresh leaves. Incubation of solid stevioside sweetener at elevated temperatures up to 120°C for 1 hour give good stability, but when exceeding 140°C, sign of decomposition was shown meanwhile no significant changes shown in stevioside content in hot coffee and tea at 80°C for 4 hour (Kroyer, 2010).

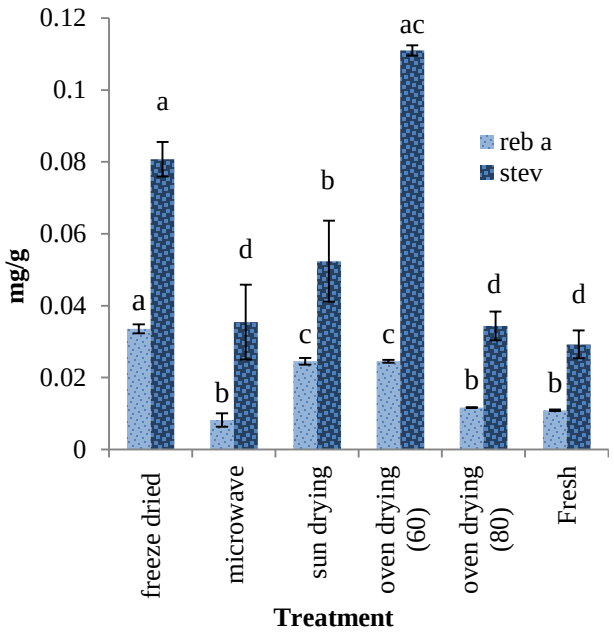

Figure 3. Bar chart of stevioside and Rebaudioside A content in different drying treatment dried leaves expressed as milligram per gram (mg/g). Different letters in the bar chart graph represent significantly different value *P<0.05*.

Conclusion

In conclusion, stevia dried with microwave (1200W) for three minutes can retain most of the beneficial properties of stevia without altering the sweetening properties of the leaves.

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Contribution of Authors

Halim AA: Carried out the experiment. And wrote the manuscript with support from other authors.
Zain ZM: Helped supervise the project in this manuscript and also helped in designing the experiment and in writing the manuscript.

Mubarak A: Contributed to the design and implementation of the research and writing the manuscript.
Ahmad FT: Conceived the present idea, developed the theory and planned the whole experiments. Supervised the project and lead the writing of the manuscript.

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