The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology (2021), 45(1), 118-128

ORIGINAL RESEARCH PAPER

CONCENTRATING ROSMARINIC ACID FROM *ORTHOSIPHON ARISTATUS* EXTRACT FOR HIGH ANTIOXIDATIVE CANDIES

JIN RU LIM[1], LEE SUAN CHUA[1,2]*

[1]*Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor Bahru, Johor, Malaysia.*

[2]*Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor Bahru, Johor, Malaysia.*

*corresponding author: *chualeesuan@utm.my*

Received on 23 March 2021
Revised on 5 May 2021

Abstract

Extraction and fractionation were carried out to concentrate the rosmarinic acid from *Orthosiphon aristatus* in order to increase the value of herbal candy formulated using polyphenolic rich extract. The herb was extracted and fractionated in column chromatography, and then analyzed by LC-MS/MS. The collected plant fractions with similar chromatographic profiles were combined and determined for antioxidant capacities expressed in radical scavenging activity. The results showed that the antioxidant capacity was in good agreement with the concentration of rosmarinic acid in the combined fractions. The combined fraction II showed the highest rosmarinic acid content, 3.8%w/w and the highest antioxidant capacity (IC50=14.922 ppm). The incorporation of rosmarinic acid rich extract into candy formulation did not statistically affect the antioxidant capacity. Hence, the rosmarinic acid rich extract could be another choice of ingredient to enhance the beneficial property of candies. Candy is another form of carrier to deliver herbal ingredient for health promotion.

Keywords: *Orthosiphon aristatus*, rosmarinic acid, candy, column chromatography, radical scavenging

Introduction

Malaysia is one of the well-known countries in South East Asia actively using traditional herbal medicine to this day. Recently, the application of herbs and herbal products for overall well-being are increasingly accepted by public. *Orthosiphon aristatus* which is locally known as 'Misai Kucing' (Cat's whisker) belongs to the Lamiaceae family and commonly consumed as herbal tea. It is a medicinal plant that is native in temperate and tropical areas, such as: India, Malaysia, Indonesia, Thailand, the Pacific and some parts of tropical Australia (Movahedi *et al*., 2014).

https://doi.org/10.35219/foodtechnology.2021.1.08

O. aristatus is traditionally used to treat diuresis, rheumatism, diabetes, urinary lithiasis, oedema, hypertension, renal calculus, gall-stone and hepatitis (Amzad Hossain and Mizanur Rahman, 2011).

Previous scientific studies demonstrated that *O. aristatus* extract contained phenolic and polyphenols, such as *rosmarinic* acid, eupatorine, sinensetin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and salvigenin (Amzad Hossain and Mizanur Rahman, 2011; Saidan *et al*., 2015). Rosmarinic acid was the major phytochemical which is likely to be the marker compound of the plant *(*Akowuah *et al.,* 2004*;* Pariyani *et al.,* 2015; Shafaei *et al*., 2016). The rosmarinic acid is a phenylpropanoid, which demonstrates high medical value due to its pharmacological significance, particularly on antioxidant (Tepe *et al*., 2007; Erkan *et al*., 2008), antimicrobial (Matejczyk *et al.,* 2018; Benedec *et al.,* 2015), anti-inflammatory (Rocha *et al*., 2015) activities and hepatic protection properties (El-Lakkany *et al.,* 2017), as well as in inhibiting angiotensin I-converting enzyme (Shafaei *et al.,* 2016). Dixon *et al.* (2005) revealed that therapeutic benefits of medicinal plants are mainly attributed to their antioxidant properties.

A plant extract is a highly complex mixture of phytochemicals with different characteristics. Column chromatography could concentrate bioactive phytochemical(s), instead of crude extract for the formulation and development of value added products (Sasidharan *et al*., 2011). Column chromatography was proven to be a reliable separation technique to fractionate the crude extract of *O. stamineus* (Chua and Lau, 2017). The presence of bioactive rich plant extract would increase the biological activity of formulated products.

Since natural product samples have a very complicated phytochemical composition, hyphenated techniques, like the liquid chromatography tandem mass spectrometry (LC-MS/MS), represent a reliable technique for high throughput phytochemical screening. The system integrates a powerful separation liquid chromatography with an excellent identification instrument. This also explains the wide application of LC-MS/MS in polyherbal formulations (Lau *et al*., 2003; Hossain *et al.,* 2010). Some researchers used the chemometric approach to analyze large multivariate data for data mining (Shi *et al.,* 2017). The principal component analysis and hierarchical cluster analysis are commonly used by researchers to visualize and identify key compounds in a group of samples (Sim *et al.,* 2003).

Therefore, the present study was conducted to fractionate the crude extract of *O. aristatus* in order to concentrate rosmarinc acid using column chromatography. The antioxidant capacity of the fractionated samples was further analyzed for their antioxidant capacity, in terms of radical scavenging activity. The stability of the rosmarinic acid was also proven even after incorporating into candy formulation.

Materials and methods

Reagents and chemicals

Analytical grade of ethanol (96%) was purchased from QReC (Asia) Sdn. Bhd. (Rawang, Malaysia). Both analytical and HPLC grades of methanol were purchased

from Fisher Scientific (Loughborough, UK). Acid washed sea sand and glass wool were purchased from R&M Chemicals (Semenyih, Malaysia). Silica 60 (0.063–0.2 mm) was purchased from Macherey-Nagel GmbH (Duren, Germany). DPPH (2.2-Diphenyl-1-picrylhydrazyl and rosmarinic acid (≥98%) were purchased from Sigma-Aldrich Corp. (St. Louis, USA).

Reflux extraction of Orthosiphon aristatus

The dried and ground *O. aristatus* (5.0 g) was extracted by water (250 mL) using a reflux system in a stirring heating mantle (MTOPS® MS-ES303, South Korea) for one hour. The dried and ground sample was immersed in water for one hour before extraction. The extract solution was filtered and concentrated by a rotary evaporator, and further dried completely in an oven at 50°C. The yield of extract was recorded and kept in a refrigerator for further analysis.

Fractionation of crude extract

A silica 60 packed column (50 g, 3 x 16 cm) was prepared and used to fractionate the crude extract of *O. aristatus* using the solvent systems of 96% ethanol, 50% ethanol + 50% water, 100% water and acidified water with acetic acid (pH 2.0) in sequence. The crude extract (0.5 g) was reconstituted in water and ethanol (5 mL), and loaded on top of the packed column for fractionation. The first solvent system which was 96% ethanol was slowly added and eluted through the packed column concurrently at a constant flow rate (5 mL/min). The solvent system was used as a carrier to elute compounds with similar polarity in the crude extract and collected in test tubes. Thirteen tubes with 5 mL of sample solution were collected as plant fractions for each solvent system in increasing polarity. The fractions were dried in an oven at 50°C, and the weight of each fraction was recorded.

High throughput phytochemical screening

A high throughput phytochemical screening was carried out by a hyphenated system of liquid chromatography tandem mass spectrometer (LC-MS/MS). The liquid chromatography was from Dionex Corporation (Ultimate 3000; Sunnyvale, CA) integrated with a diode array detector and a C18 reversed phase XSelect HSS T3 column (2.1 x 100 mm, 2.5 µm). The mass spectrometer was a quadrupole and time-of-flight (QTOF) system from AB SCIEX (QSTAR Elite; Foster City, CA). All samples were screened by LC-MS/MS. Samples were filtered by nylon membrane filter (0.2 µm) prior to injection (5 µL). Solvent A (water with 0.1% formic acid) and solvent B (acetonitrile) were used as the binary mobile phase at the flow rate of 150 µL/min. The gradient was programmed as: 0-10 min, 90% A; 10-20 min, 90-15% A; 20-25 min, 15% A; 25-26 min, 15-90% A; 26-30 min, 90% A. The mass range of screening was set from m/z 100-1000. The voltages of ion spray were set at 4500 V and 5500 V for negative and positive ion modes, respectively. The potentials of declustering and focusing were set at 40 V and 250 V, respectively. Nitrogen was used as the nebulizing (40 psi) and curtain gas (20 psi).

Determination of rosmarinic acid

The chromatograms of samples in each tube were analysed at 254 nm. Samples with similar chromatographic profiles were combined and named as Fraction I to VII. The

concentrations of rosmarinic acid in the combined fractions were determined from a calibration curve. The calibration curve was constructed using a serial standard solution of rosmarinic acid ranged from 20-100 ppm.

Preparation of herbal candies

The ingredients for candy preparation included table sugar (100 g), light corn syrup (40 g) and water (40 g). The ingredients were mixed and heated slowly until the solution was boiled. The syrup solution was continuously stirred at 120 °C. Then, the combined fractions were added and well mixed in the syrup solution before transferred into a mould. The concentration of the combined fractions was ranged from 40-200 ppm in the syrup solution. The herbal syrup was cooled at room temperature for overnight. The volume of each candy was 3 mL.

DPPH Radical Scavenging activity

DPPH solution (0.1 mM) was freshly prepared by dissolving 4 mg DPPH in 100 mL methanol. Three candies (~ 10 g) were melted in 9 mL hot water. A 1 mL sample solution was added into the DPPH solution (1 mL) and incubated at 25 °C for 30 min in the dark place. The absorbance of the solution was measured at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). The DPPH radical scavenging activity of the combined fractions I to VII was calculated using Equation 1.

$$\% \text{ Inhibition} = \frac{Ab - Aa}{Ab} \; 100 \qquad (1)$$

Ab is the absorption of blank sample and Aa is the absorption of fractions. The DPPH radical scavenging activity is expressed as IC_{50} which is the required concentration of sample to exhibit 50 % inhibition against free radicals.

Statistical Analysis

A multivariate data analysis technique based on principal components was used to analyse the large datasets of mass spectrometric data. Principal component analysis (PCA) with Pareto scaling was used to analyse highly complex mass spectra under unsupervised condition (MarkerView 1.2.1, Foster City, CA). The minimum spectral peak width and mass tolerance were set at 0.05 and 0.10 Da, respectively. The retention time tolerance was 0.5 min and the maximum number of peaks was 10,000.

Results and discussion

Column chromatography of crude extract

The phytochemicals in herbal plants are usually extracted and prepared in the form of decoction. In the present study, the extract of *O. aristatus* was further dried into crude extract powder for storage and product formulation. The water extraction using reflux system was found to produce higher yield (22 %) than alcoholic extract (3-14 %) in previous studies (Chua and Lau, 2017). The dried water extract was dark and sticky because of hygroscopic nature of polysaccharides. Even though higher yield, the phytochemicals with chromophore property were less than those extracted by

ethanolic solvent, especially after compounds detected at the retention time after 17 min. The crude extract was then fractionated into several fractions using column chromatography with increasing polarity of eluent. The color and dry weight of individual fractions collected by the 4 solvent systems is presented in Figure 1.

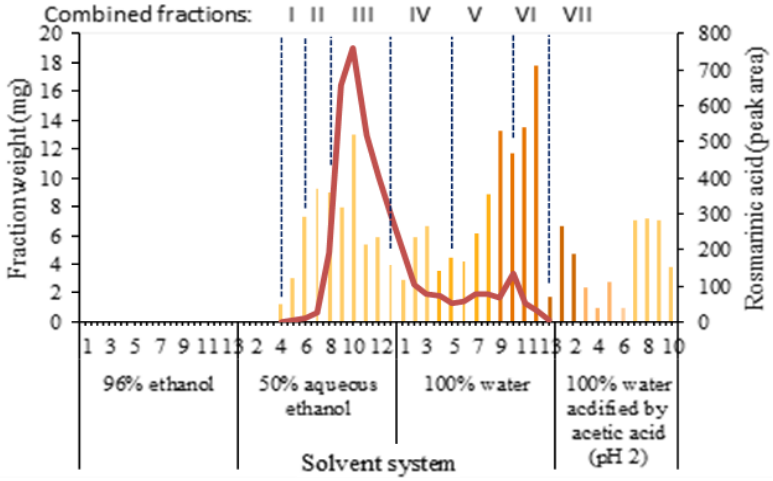

Figure 1. Weight of fractions collected from column chromatography using 4 solvents system in increasing polarity.

The color of the fractions was changed from yellowish, to brown and light brown. The dried weight of fractions collected by each solvent system; 0 mg (100% ethanol), 66.0 mg (50% ethanol 50% water), 100.6 mg (100% water) and 43.6 mg (acidified water at pH 2.0). It was found that only 42% of crude extract could be recovered from column chromatography. There were 58% of crude extract still remaining in the packed column as residue. The remaining compounds strongly bound to the surface of silica. The principle of work in column chromatography is depended upon the adsorption strength between molecules in mobile phase and the stationary phase of column. This observation shows that the residue could be highly polar compounds like polysaccharides. The results of fractionation by column chromatography also showed that only 13.2% of crude extract could be recovered by intermediate polarity of ethanolic solvent. The solvent system of 100% water could achieve the highest recovery, 20.1% since water was used as solvent for reflux extraction. The addition of acetic acid may increase the number of protons in the eluent to weaken the interaction of molecules on the surface of silica. This would accelerate the elution of compounds out from the column. The rest of compounds in the crude extract belonged to highly polar substances (residue).

Pattern recognition of mass spectrometric data

The mass spectrometric data of samples were analyzed using principal component analysis statistically. The score and loading plots of both negative and positive ion modes are presented in Figure 2.

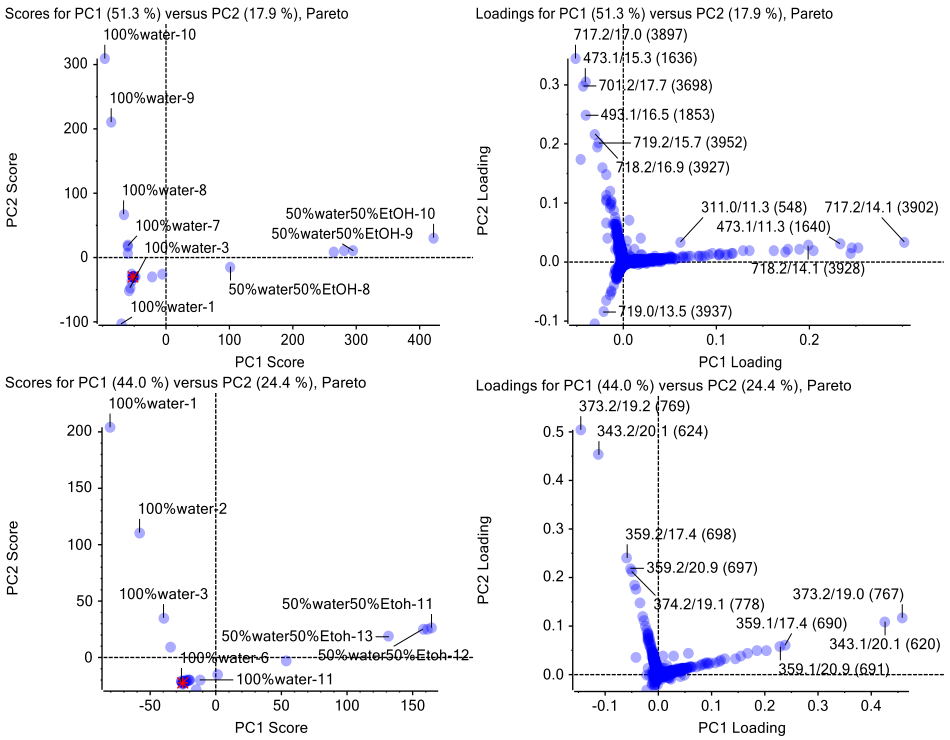

Figure 2. Score and loading plots of principle component analysis for phytochemicals detected at the negative (a and b) and positive (c and d) ion modes.

An opposite trend can be seen from the score plots of negative and positive ion modes. The first two principal components accounted for 69.2% of total variance for negative ion mode, whereas 68.4% of total variance for positive ion mode. Phytochemicals in the fractions collected from the solvent system of 50 % ethanol and 50 % water appeared to contribute to the largest variance as shown in the first principal component. The phytochemicals in the green circles are located far away from the central point of the loading plots. They were likely to be the major phytochemicals differentiating the fractions. The fragments of the compounds were matched to the data published in literature. The results found that 5 major compounds such as rosmarinic acid (m/z 359), xylosyglucosyl caffeic acid (m/z 473), deoxysalvianolic acid B (m/z 701), salvianolic acid (m/z 717) and dirosmarinic acid (m/z 719) were identified in the negative ion mode. They are caffeic acid derivatives which are preferably ionized by losing a proton. While, 4 compounds such as tetramethoxyflavone (m/z 343), eupatorin (m/z 345), 5-hydroxy-6,7,3',4'-tetramethoxyflavone (m/z 359) and sinensetin (m/z 373) were detected as the major phytochemicals in the positive ion mode. They are polymethoxyflavones which are prone to accept a proton during ionization (Cho *et al.*, 2014). Table 1 shows the fragment ions of the major compounds in both negative and positive ion modes.

Table 1. The detected major phytochemicals which are tentatively identified from their mass fragmentation at the negative and positive ion modes.

Retention time (min)	Mass per charge (m/z)	Tentative compounds	Fragment ions
Negative ion			
12.0	473	Xylosyglucosyl caffeic acid	473/219(-254)/179(-294)/ 149/135
13.0	359	Rosmarinic acid	359/197/179/161
13.5	719	Rosmarinic acid dimer	719/359(-360)/297/197/ 179/161/133
14.0	717	Salvianolic acid B	717/519(-198)/339(378)/321/295/277/197/185
15.0	701	Deoxysalvianolic acid B	701/339(-362)/321/295/ 279/185
Positive ion			
19.0	373	Sinensetin	
19.4	345	Eupatorin (3',5-dihydroxy-4',6,7-trimethoxyflavone)	345/330/312/284/269/185 /147/136
20.2	343	Tetramethoxyflavone	343/327/313/299/285/254 /238/155
21.0	359	5-hydroxy-6,7,3′,4′-tetramethoxyflavone	359/343/329/315/298/283 /255/227/

Plant fractions rich in polyphenolic compounds

The fractions with similar chromatographic profiles were combined and further analysed for their antioxidant capacities in candy formulation. A total of 7 combined fractions (I-VII) was formed from the crude extract. The chromatograms of the combined fractions are presented in Figure 3. The chromatograms of combined fractions VI and VII are hardly to see any significant peak at 254 nm. The rosmarinic acid was detected at 13 min and its peak also presented at the highest concentration in the combined fractions II and III. The concentration of rosmarinic acid in each combined fraction is presented in Figure 4. The figure shows a significant increment of the rosmarinic acid content after column chromatography. The combined fraction II and III show the highest rosmarinic acid content which were increased from 0.05% in crude extract to 3.8 % and 2.3% in the combined fraction II and III, respectively. Rosmarinic acid was prone to be eluted by aqueous ethanol effectively. As seen in Figure 4, the eluent of acidified water at pH 2.0 could assist in eluting the remaining rosmarinic acid bound to the surface of silica. The competitive interaction between mobile phase and silica based stationary phase could be illustrated by the elution profile of rosmarinic acid.

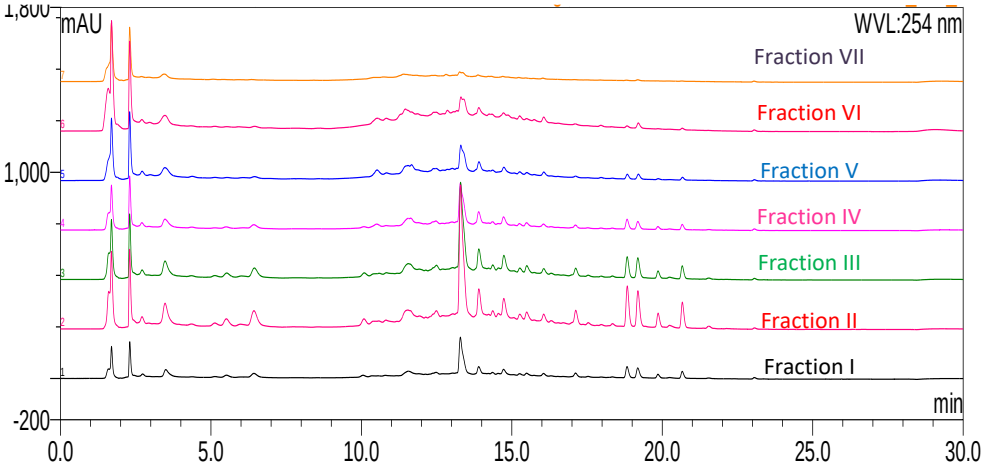

Figure 3. Chromatograms of the combined fractions from column chromatography using *Orthosiphon aristatus* extract.

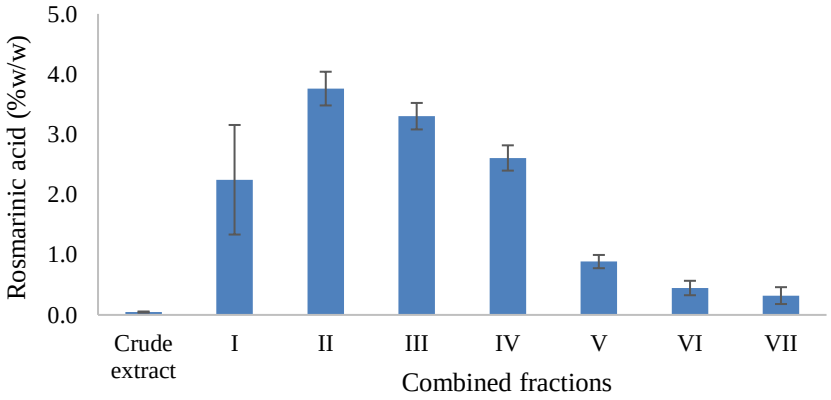

Figure 4. Rosmarinic acid content in the combined fraction I – VII.

Free radical scavenging activity of combined fractions

In the assay of radical scavenging, the purple free radicals, DPPH• would be reduced to a yellowish solution which is a stable and complex end product after reacted with antioxidant compounds (Kedare and Singh, 2011). The capability of combined fractions in reducing free radicals was expressed in IC50, which explains the required concentration of sample to scavenge 50% free radicals. Hence, the lower the IC_{50} value describes the higher antioxidant activity of sample. Figure 5 shows the free radical scavenging activity of combined fractions before and after formulated into candy solution. Obviously, the combined fraction VII shows the lowest antioxidant capacity. The antioxidant capacity of the combined fractions was found to be in rosmarinic acid dependent manner. In other words, fractions with higher rosmarinic acid would exhibit higher antioxidant capacity (Benedec *et al*., 2015).

The combined fractions of II and III shows to have lower IC50 with higher antioxidant capacity.

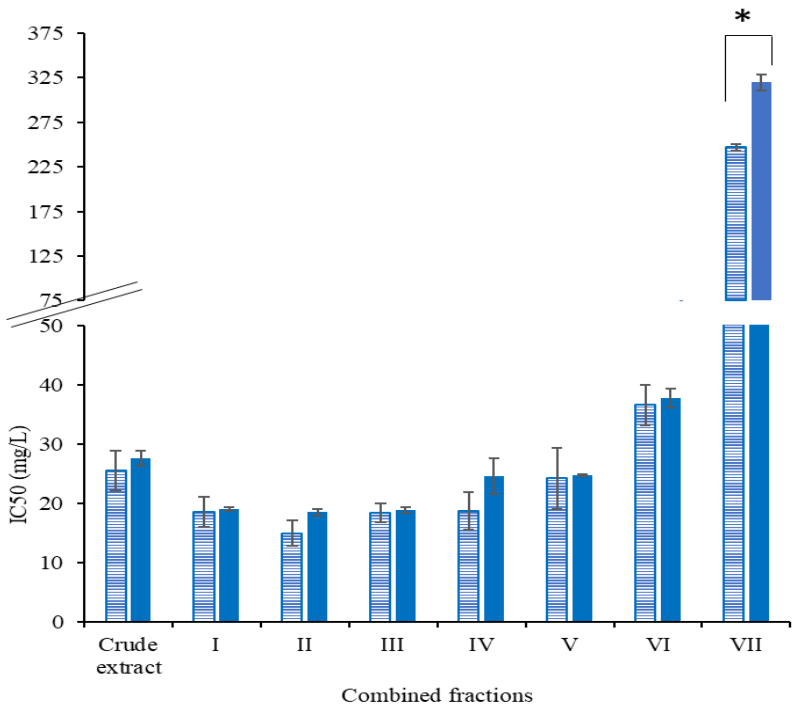

Figure 5. Effective concentration at 50% inhibition (IC50) against free radicals of DPPH exhibited by samples before (line bar) and after formulated into candies (solid bar).

*indicates the significant difference at 95% confident level using one-way ANOVA.

Figure 5 also shows to have insignificant difference ($p>0.05$) between the antioxidant capacity of combined fractions after incorporated into candy formulation. Therefore, the process of candy preparation did not degrade any compounds in the fractions, especially rosmarinic acid. It is interesting because the rosmarinic acid rich fraction could be used as bioactive ingredient in candy formulation. The condensation between the hydroxyl groups of rosmarinic acid and hydroxyl groups of sugar which lead to the formation of glycoside was not significant to reduce the antioxidant capacity of fractions (Shalaby *et al.*, 2016; Nakilcioglu-Tas, 2018).

Conclusions

This study demonstrated that column chromatography could effectively concentrate the rosmarinic acid from the crude extract of *O. aristatus*. The rosmarinic acid rich fraction could be used to prepare candy with antioxidant property. This value added

herbal candy is suggested to food manufacturing companies. The rosmarinic acid rich fraction could be another ingredient choice to enhance the value of product.

Acknowledgments

The works were supported by the research grant of Universiti Teknologi Malaysia (TDR-07G21-06G75) and (HR-08G84).

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