Philippine Journal of Science
150 (4): 735-742, August 2021
ISSN 0031 - 7683
Date Received: 08 Feb 2021

Effects of Drying Temperature on the Amount of Secondary Metabolites and Antioxidant Activity of *Orthosiphon aristatus* (Blume) Miq. Tea Extracts

Narin Taokaenchan[1]*, **Sakchai Satienperakul**[2], **Yuto Ueda**[3], **Shuhei Tsutsumi**[3], and **Shin Yasuda**[3]

[1]Department of Medicinal Plant Science, Faculty of Agricultural Production
[2]Department of Chemistry, Faculty of Science
Maejo University, Chiang Mai 50290, Thailand
[3]Department of Bioscience, School of Agriculture
Tokai University, Kumamoto City, Kumamoto 862-8652 Japan

***Orthosiphon aristatus* (Blume) Miq. tea is a traditional herbal tea in Thailand. This herb is used to prevent and treat a wide range of diseases such as diuresis, hypertension, and diabetes. In this study, three different drying temperatures used for herbal tea processing were evaluated (40, 50, and 60 °C) for their secondary metabolites [total phenolic content (TPC), caffeic acid (CA), rosmarinic acid (RA), and total flavonoid content (TFC)] and antioxidant activity. The herbal tea processed at 40 °C showed significantly ($P < 0.05$) higher TPC [485.26 ± 49.43 mg gallic acid equivalent (GAE)/ 100 g], CA (202.17 ± 1.00 mg/kg), RA (469.99 ± 1.56 mg/kg), TFC [356.86 ± 41.81 mg catechin equivalent (CE)/ 100 g], and 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity than those at other drying temperatures. Therefore, a drying temperature of 40 °C was the optimum condition for the *Orthosiphon aristatus* (Blume) Miq. leaves because it retained the secondary metabolite content with strong antioxidant potential.**

Keywords: caffeic acid, DPPH radical scavenging activity, rosmarinic acid, total flavonoid content, total phenolic content

INTRODUCTION

In recent decades, herbal teas have become more familiar in many countries outside of Thailand, as they are an essential source of antioxidants and secondary metabolites (Moraes-de-Souza *et al.* 2008; Poswal *et al.* 2019). Various researchers have found that Thai herbal teas may cure diseases such as cancer, diabetes, and hypertension (Chusri *et al.* 2015; Tipduangta *et al.* 2019). The *Orthosiphon aristatus* (Blume) Miq. tea is the most famous tea in Thailand because of its preventative and curative properties.

*Corresponding Author: narin_t15@hotmail.com

Orthosiphon aristatus (Blume) Miq., which is called "yaa-nuat-maeo" locally, is one of the traditional folk medicines used extensively in Southeast Asia – particularly Thailand – for the treatment of a wide range of diseases. It is used as a diuretic, treatment for rheumatism, abdominal pain, kidney and bladder inflammation, edema, gout, and hypertension (Chai *et al.* 2014). This herb belongs to the Lamiaceae family and is planted throughout Thailand for medicinal purposes. It is a perennial herb that is 25–100 cm tall and with a quadrangular stem. The leaves of this plant are opposite, ovate to rhomboid, 3–7 cm long, and 2–5 cm wide. The flowers are inflorescence

terminal, with many white or pale-lilac flowers and long-protruding stamens (Figure 1). Yaa-nuat-maeo is a source of flavonoids, terpenoids, phenolic acids, antioxidants, and polyphenol compounds – particularly, CA and RA.

The processing of Thai herbal teas corresponds to the process used for *Camellia sinensis* tea – including withering, rolling, fermenting, and drying steps (Teshome 2019). Only the drying step – which uses a hot air oven – is of interest for reducing time, energy, and costs (Hao *et al.* 2018; Nguyen and Chuyen 2020). Several studies have suggested that the drying process exposes the plants, vegetables, and herbs to high temperatures, resulting in serious damage to qualities and components such as color, antioxidant properties, nutrients, and bioactive compounds (Said *et al.* 2013; Hihat *et al.* 2017). Hence, the present work aimed to study whether three different drying temperatures (40, 50, and 60 °C) used during the processing of *Orthosiphon aristatus* (Blume) Miq. into tea can influence the amount of secondary metabolites (TPC, CA, RA, and flavonoid content) and antioxidant activity. These temperatures have been applied during the herb's processing into tea, and we evaluated appropriate drying conditions.

MATERIALS AND METHODS

Chemicals

DMSO (dimethyl sulfoxide), DPPH, Folin-Ciocalteu phenol reagent, and gallic acid (GA) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 6-Hydroxy-2,5,7,8-

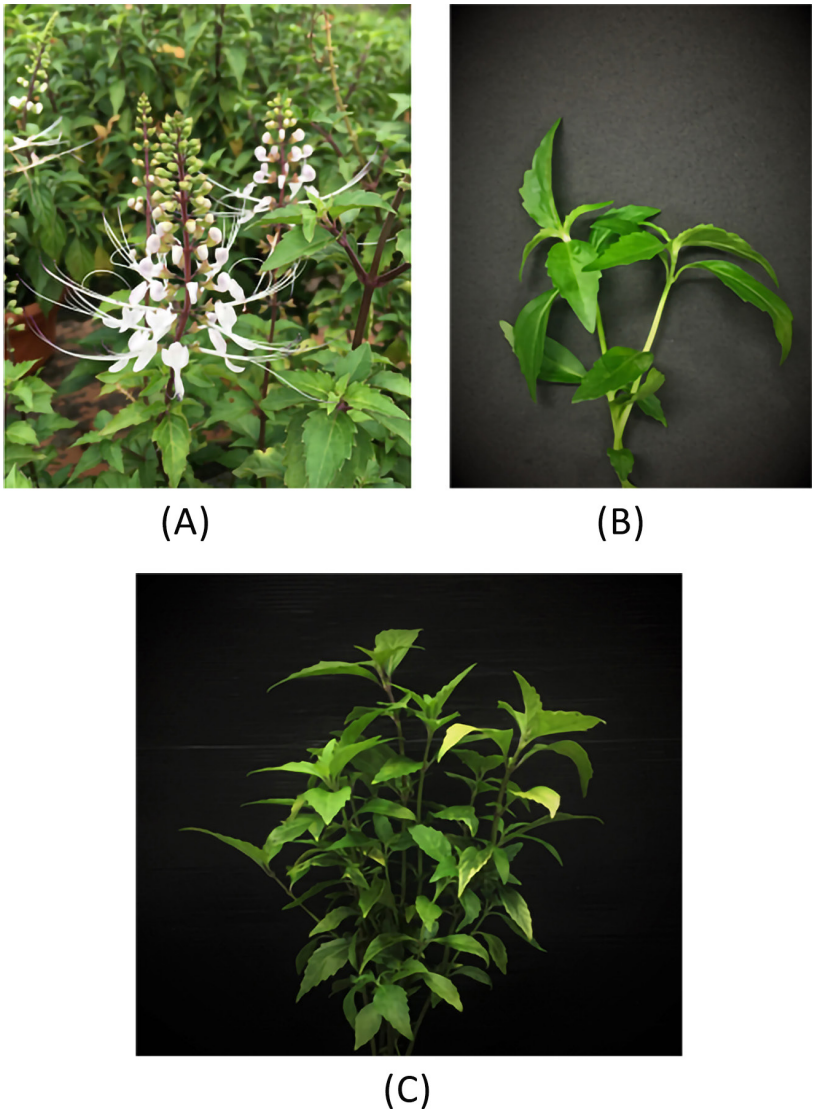

Figure 1. *Orthosiphon aristatus* (Blume) Miq: A) inflorescences, B) leaves, and C) the plant.

tetramethylchromane-2-carboxylic acid (Trolox), CA, and RA were obtained from Sigma Aldrich (Sigma–Aldrich GmbH, Germany). All chemicals and solvents were analytical grade and used without further purification.

Orthosiphon aristatus (Blume) Miq tea material

Tea leaves of *Orthosiphon aristatus* (Blume) Miq. were collected from the organic farm at Prachin Buri province, Thailand. The sample was identified by the Queen Sirikit Botanical Garden, Ministry of Natural Resources and Environment, Thailand. A voucher specimen was deposited at the Queen Sirikit Botanical Garden Herbarium (QBG no. 109754). The leaves were washed thoroughly with tap water and stored at 5 °C until further dry process.

Drying Procedure

Orthosiphon aristatus (Blume) Miq. leaves were placed on a flat tray and dried in a hot air oven at different temperatures of 40, 50, and 60 °C. A part of the samples was taken every hour to check the moisture content until indicating below 10%. Finally, the dry solid content was determined using a moisture balance (AMB110, AE Adam, England).

Preparation of Tea Herb Extracts

The extracts from *Orthosiphon aristatus* (Blume) Miq. tea leaves were prepared by the maceration method with some modifications. Briefly, approximately 30 g of dried powder tea leaves were soaked in 250 mL of ethanol in the stoppered container flask at 40 °C for 3 h. The extract solution was filtered and evaporated to near dryness. The concentrate was further evaporated to dryness using a vacuum oven at 45 °C. The final crude extracts were stored at 5 °C until assay.

Secondary Metabolites and Antioxidant Analysis

TPC. The TPC in the tea extract samples was determined by an established procedure (Ueda *et al.* 2019). Briefly, the mixture containing test samples with 1.25–5 mg/mL (25 µL) concentrations and 10-times diluted Folin-Ciocalteu phenol reagent solution (125 µL) was kept with 10% sodium carbonate solution (125 µL) for 10 min at room temperature. The assay mixture was allowed to a colorimetric measurement at 600 nm using a microplate reader (SH1000Lab, Corona Electric, Ibaraki, Japan). GA was used as the standard for the calibration curve. In case the photometric absorption of samples/reagents may interfere with the data, a parallel experiment as background at each point was carried out. The phenolic content was expressed as mg GAE/ 100 g dry basis.

CA and RA determination. The method of Wang *et al.* (2004) was modified and used to measure the amounts of CA and RA. Briefly, approximately 100 mg of dried leaves were accurately weighed into a 50-mL tube and added 15 mL of 30% methanol (v/v). Samples were extracted using an ultrasonic bath (Sonica 2200 S3, Soltec, Italy) for 10 min. The extract solution sample was filtered through filter paper (Whatman No.1) and transferred to a 50-mL volumetric flask. The residues were extracted with 30% methanol (v/v) two times and filtered. The filtrate solutions were combined and made to 50 mL with 30% methanol (v/v). All sample solutions were passed through a 0.45-µm nylon filter (Ligand, Thailand) before injection for the high-performance liquid chromatography (HPLC) analysis.

The CA and RA reference standard compounds were used as standards. Stock standard solutions were prepared by accurately weighing 10 mg CA and RA reference standards in separate 10-mL volumetric flasks and dissolving in methanol with sonication. Mixing working standard solutions (5–100 µg/mL) were prepared by dilution from the stock standard solutions with 30% methanol (v/v).

The HPLC method, including an external calibration, was used to quantify CA and RA concentration in terms of mg/kg of dried herb. HPLC analysis was performed with the Agilent 1100 series HPLC system (Agilent, USA) equipped with an automatic injector, a column oven, and an ultraviolet detector. A Hypersil ODS-C18 column (Thermo Fisher Scientific, Sweden, 4 x 250 mm, 5-µm particle size) was used. The column temperature was maintained at 30 °C, 10 µL of a standard, and sample solution was injected with a flow rate of 1 mL/min. The detection wavelength was set to 330 nm, a mobile phase was obtained with 0.1% (m/v) orthophosphoric acid in water and 0.1% (m/v) orthophosphoric acid in methanol with the ratio of 50:50.

TFC. The amount of TFC was determined by an established procedure (Chang *et al.* 2006). Briefly, the test samples (25 µL) were mixed with water (125 µL) and 5% sodium nitrite solution (7.5 µL) for 6 min and then mixed with 10% aluminum chloride solution (15 µL) for 5 min at room temperature. After that, 1 mM NaOH (50 µL) and water (27.5 µL) were added to the mixture (total 250 µL) and incubated for 5–10 min with continuous shaking. The assay mixture was subjected to colorimetric measurement at 510 nm using a microplate reader (SH1000Lab, Corona Electric, Ibaraki, Japan). Catechin was used as a control standard to prepare a calibration curve. TFC was expressed as mg CE/ 100 g dry basis.

DPPH radical scavenging assay. The DPPH radical scavenging activity was measured based on the following method (Singh *et al.* 2002). The assay of DPPH radical scavenging was performed in a 96-well microplate with

50 µL of DPPH solution (0.5 mM in ethanol) and 10 µL of a sample. It was then added with 70% ethanol (90 µL) and 0.1 M sodium acetate buffer (pH 5.5, 100 µL) and allowed to stand for 30 min at room temperature. The reaction mixtures were incubated for 30 min at 25 °C in the dark and absorbance was measured at 517 nm using a microplate reader (SH1000Lab, Corona Electric, Ibaraki, Japan). The DPPH radical scavenging activity (%) was calculated using the following equation:

DPPH radical scavenging activity (%) = $\{1 - [A_{Sample} - A_{Blank}) / (A_{Control} - A_{Blank})]\}$ x 100

where A_{Sample} is the absorbance of the test sample and A_{Blank} is the absorbance measured in the presence of neither the tested sample nor the synthetic radical solution. $A_{Control}$ is absorbance measured in the absence of the sample. The EC_{50} value is defined as the concentration of the sample leading to a 50% reduction of the initial DPPH concentration. It was obtained from the linear regression of plots of the percentage of the radical scavenging activity against the concentration of the test extracts (µg/mL) obtained from three replicate assays. The antioxidant activity was also expressed as g Trolox equivalent (TEAC)/ 100 g dry basis using Trolox as a standard.

Statistical Analysis

All experiments were conducted in three replicates. The results were expressed in mean ± standard deviation. Analysis of variance using one-way ANOVA at 0.05 significance level and Duncan's new multiple range test was used. Pearson's correlation coefficient (r) was used to determine the relationship between variables.

RESULTS AND DISCUSSION

To determine the most appropriate temperature for herbal tea processing using drying in a hot air oven. The current research investigated the effects of different drying temperatures on the secondary metabolites and antioxidant activity of *Orthosiphon aristatus* (Blume) Miq. tea leaves.

Impact of Drying Temperature on Moisture Content and the Extract Yields from Herbal Teas

The moisture content of herbal teas promotes microbial growth and leads to shorter shelf life. It also affects the degradation of phenolic compounds by enzymatic oxidation (Hihat *et al.* 2017). Hence, the Thai Food and Drug Administration (FDA) recommends that herbal tea's moisture content not exceed 10% (FDA 2004). As shown in Table 1, the moisture contents of *Orthosiphon aristatus* (Blume) Miq. at all drying temperatures are less than 10%. Extract yields from the herbal teas were 4–8%, as seen in Table 1. Herbal tea dried at 40 °C had a yield of extract two times higher than at different drying temperatures.

Table 1. The moisture content and the yield of extract from herbal tea at different drying temperatures and times.

Drying temperatures (°C)	Time (h)	Moisture content (%)	Yield of extract (%)
40	14	9.99	8.86
50	6	9.67	4.83
60	4	9.38	3.53

Effect of Different Drying Temperatures on the TPC, CA, RA, and TFC of *Orthosiphon aristatus* (Blume) Miq. Tea Leaves

TPC. Phenolic compounds are found in a wide variety of vegetables and herbs. One of the beneficial effects of phenolic compounds is antioxidant activity (Huang *et al.* 2010; Yakoh *et al.* 2018). The quantity and quality of bioactive compounds, including phenolics, in vegetables and herbs have been shown to change after some kinds of food processing such as blanching or drying (Minatel *et al.* 2017).

As shown in Table 2, the amount of TPC in 100 g of tea leaves ranged from 127–486 mg. The different drying temperatures significantly affected ($P < 0.05$) the TPC: the highest TPC content obtained was 485.26 ± 49.43 mg GAE/ 100 g at 40 °C. Meanwhile, a drying temperature of 60 °C resulted in the lowest TPC (127.39 ± 15.98 mg GAE/ 100 g). This difference indicated that increasing the drying temperature led to decreasing amounts of TPC. This result agrees with the work of Afifah and Niwat (2020), who studied the effect of different drying temperatures on the TPC of moringa leaves. Raising the drying temperature reduced the amount of TPC because the excessive heat may have led to the degradation of TPC (Karaaslan *et al.* 2014).

CA and RA. Chromatograms of a standard mixture of CA and RA and the *Orthosiphon aristatus* (Blume) Miq. tea leaves samples recorded at 330 nm are presented in Figure 2. The retention times of CA and RA in the standard mixture and the tea samples were 2.9 and 5.8 min, respectively. The amounts of polyphenol compounds detected in these samples are presented in Table 2 in mg/kg of dried sample. The most abundant polyphenol compound detected was RA, and CA was in minor amounts. These results are similar to those in previous literature (Chua *et al.* 2018). It is known that RA can scavenge excess radicals and may play a role in preventing diseases (Kim *et al.* 2015; Alagawany *et al.* 2017).

Table 2. TPC, CA, RA, and TFC of *Orthosiphon aristatus* (Blume) Miq. tea.

Drying temperatures (°C)	TPC (mg GAE/100g)	CA (mg/kg)	RA (mg/kg)	TFC (mg CE/100g)
40	485.26 ± 49.43[a]	202.17 ± 1.00[a]	469.99 ± 1.56[a]	356.86 ± 41.81[a]
50	149.88 ± 14.48[b]	112.99 ± 1.62[b]	294.17 ± 0.97[b]	80.53 ± 11.10[b]
60	127.39 ± 15.98[c]	52.83 ± 1.54[c]	154.96 ± 13.89[c]	51.64 ± 5.34[b]

Different letters within a column indicate significant differences at $P < 0.05$.

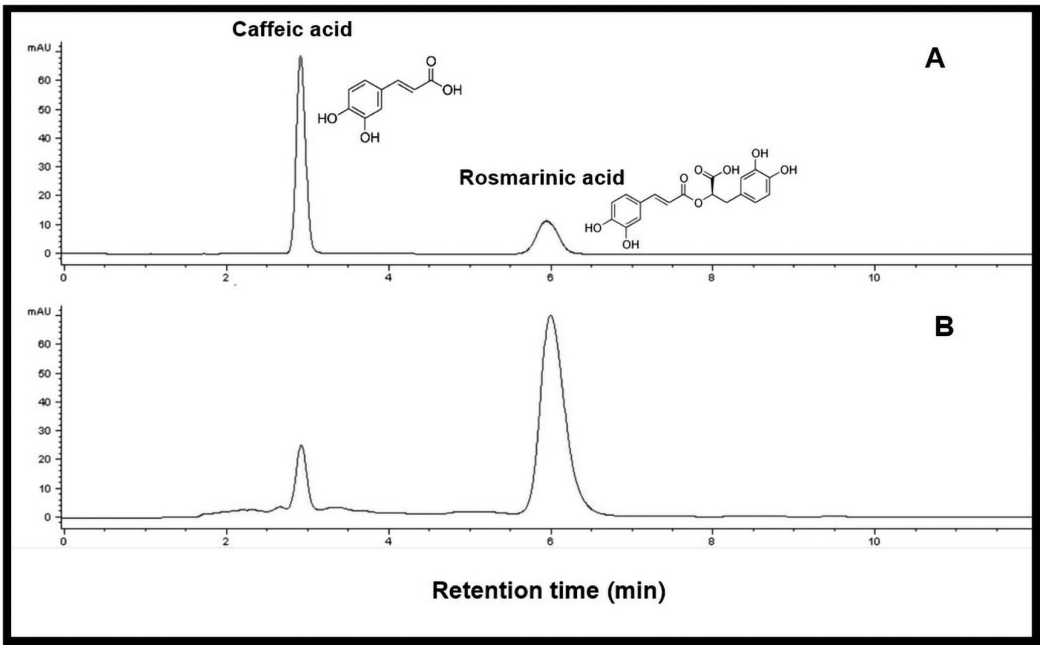

Figure 2. Chromatograms of CA and RA obtained by HPLC: A) mixed standard at 50 ppm and B) sample tea leaves extract.

Different drying temperatures had a significant impact ($P < 0.05$) on CA and RA in *Orthosiphon aristatus* (Blume) Miq. tea leaves. The highest levels of CA (202.17 ± 1.00 mg/kg dried sample) and RA (469.99 ± 1.56 mg/kg dried sample) were obtained in the tea leaves dried at 40 °C, where the tea leaves dried at 60 °C had the lowest levels of CA (52.83 ± 1.54 mg/kg dried sample) and RA (154.96 ± 13.89 mg/kg dried sample), respectively. Amounts of CA and RA in *Orthosiphon aristatus* (Blume) Miq. tea leaves were similar to the TPC results. Increasing the drying temperature reduced CA and RA amount in the tea leaves. Our findings were in agreement with previous studies on *Orthosiphon aristatus* (Blume) Miq. tea leaves, which reported that lower drying temperature resulted in higher RA yield (Abdullah *et al.* 2018). This difference can be explained by the thermal stability of CA and RA in *Orthosiphon aristatus* (Blume) Miq. tea leaves at low temperatures.

TFC. Flavonoids such as euapatorin; sinensetin; salvigenin; ladanein; vomifoliol; 5-hydroxy-6, 7, 3′, 4′-tetramethoxyflavone; 6-hydroxy-5, 7, 4′-trimethoxyflavone; 7, 3′, 4′-tri-O-methylluteolin; tetramethylscutellarein; and scutellarein tetramethyl ether are phytochemicals present in *Orthosiphon aristatus* (Blume) Miq. (Ameer *et al.* 2012). Flavonoids inhibit oxidation reactions and chronic inflammation-mediated pathogenesis of human illnesses such as cardiovascular disease, certain cancers, and neurological disorders (Rupasinghe 2020).

Results of measuring the TFC in herbal tea leaves showed that the drying temperature was significantly different ($P < 0.05$) from the TFC in tea leaves. From Table 2, it can be seen that the TFC in herbal tea leaves ranged from 127–458 mg CE/100 g of dried sample. High drying temperatures (50 and 60 °C) resulted in decreased TFC compared with the TFC of leaves dried at 40 °C. This is due to the degradation of the TFC in tea leaves at high drying temperatures (Tiho *et al.* 2017).

Influence of Different Drying Temperatures on Radical Scavenging in *Orthosiphon aristatus* (Blume) Miq. Tea Leaves

Free radicals are products of chemical reactions that cause aging and various diseases due to damage to tissues and cells. Thus, it is very important to find antioxidants to scavenge these free radicals from the body.

The DPPH method is one of the universal tools for estimating the antioxidant activity of different products and was used to determine antioxidant activity. First, a pilot study revealed that a sample of herbal tea extract test at 5–250 µg/mL showed 4–97% of DPPH radical scavenging activity (Figure 3). Sample herbal teas dried at 40, 50, and 60 °C resulted in 97.34, 89.52, and 91.01% of the maximum antioxidant activities. To quantitatively evaluate the antioxidative effect, the sample herbal tea extracts from different drying temperatures were further tested at 10–175 µg/mL in a DPPH radical assay. As presented in Table 3, these extracts show increased DPPH radical scavenging activity when the sample concentration increased. The EC_{50} values and antioxidant activities of herbal tea dried at different temperatures were significantly different ($P < 0.05$): the highest EC_{50} values and antioxidant activity of 51.58 ± 3.95 µg/mL and 1.25 ± 0.14 g TEAC/ 100 g, respectively, were obtained at 40 °C. In contrast, a drying temperature of 60 °C lowered the EC_{50} values and antioxidant activity (130.32 ± 6.50 µg/mL and 0.20 ± 0.02 g TEAC/ 100 g, respectively). Based on these results, the EC_{50} values and antioxidant activity decreased when the temperature was increased.

Correlation between Secondary Metabolites and Antioxidant Properties at Different Drying Temperatures

The correlation between secondary metabolites and antioxidant properties was analyzed, and the outcomes are shown in Table 4. The correlation coefficient values showed a significant ($P < 0.05$) and strong positive correlation between the TPC and CA, RA, TFC, and antioxidant activity. The Pearson correlation coefficients or r-values were 0.94, 0.93, 0.97, and 0.93, respectively, which revealed that the secondary metabolites (TPC, CA, RA, and TFC) in *Orthosiphon aristatus* (Blume) Miq.

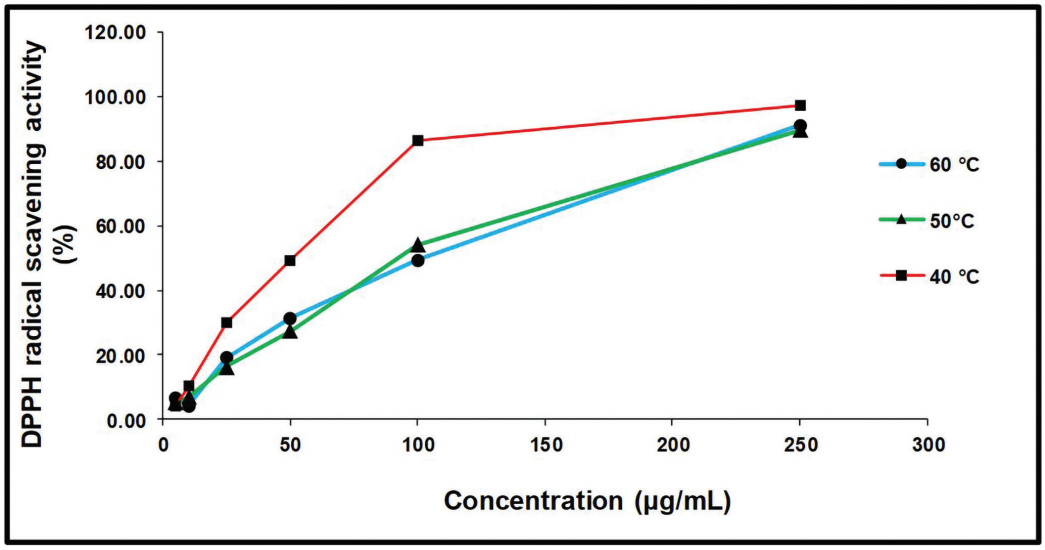

Figure 3. Antioxidant activity of tea leaves extracts in DPPH radical scavenging assay.

Table 3. Antioxidant activity using DPPH radical scavenging assay of *Orthosiphon aristatus* (Blume) Miq. tea at different drying temperatures.

Drying temperatures (°C)	DPPH radical scavenging activity (%) at various concentration					EC_{50} (µg/mL)	Antioxidant activity (g TEAC/ 100 g DW tea)
	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	175 µg/mL		
40	15.70 ± 3.74	28.95 ± 3.50	51.31 ± 6.48	80.82 ± 6.74	–	51.58 ± 3.95[a]	1.25 ± 0.14[a]
50	13.55 ± 0.45	26.28 ± 1.22	41.51 ± 2.25	72.47 ± 2.40	–	65.56 ± 1.22[b]	0.57 ± 0.01[b]
60	8.29 ± 1.25	13.87 ± 0.56	22.49 ± 2.37	37.00 ± 3.93	60.91 ± 4.53	130.32 ± 6.50[c]	0.20 ± 0.02[c]

Different letters within a column indicate significant differences at $P < 0.05$.

Table 4. Correlation matrix of the studied parameters (Pearson correlation coefficients).

	TPC	CA	RA	TFC	Antioxidant activity	Drying temperature
TPC	1					
CA	0.94	1				
RA	0.93	0.99	1			
TFC	0.97	0.96	0.94	1		
Antioxidant activity	0.93	0.98	0.98	0.95	1	
Drying temperature	–0.88	–0.99	–0.99	–0.90	–0.98	1

tea leaves mainly contribute to the antioxidant capacity. Similar results were observed by Aryal *et al*. (2019); they found a strong correlation between total phenolic and flavonoid content and antioxidant properties (DPPH assay) in wild vegetables from Western Nepal. Manaois *et al*. (2020) observed a similar trend in vegetables commonly consumed in the Philippines. Overall, the TPC, CA, RA, and TFC play an important role in the antioxidant capacity of the *Orthosiphon aristatus* (Blume) Miq. tea leaves. The correlation between drying temperature with the secondary metabolites (TPC, CA, RA, and TFC) and antioxidant capacity is shown in Table 4. There was a negative correlation for all parameters studied. These correlations supported the interpretation that increasing the processing temperature for drying *Orthosiphon aristatus* (Blume) Miq. tea leaves decreased the secondary metabolites and antioxidant activity.

CONCLUSION

The drying temperature of *Orthosiphon aristatus* (Blume) Miq. has significantly affected the retention of TPC, CA, RA, TFC and their antioxidant activity measured by DPPH. Among the three drying temperatures tested (40, 50, and 60 °C), drying at 40 °C demonstrated the best processing condition. Based on the overall results, a hot air oven drying at 40 °C is recommended as the best condition for *Orthosiphon aristatus* (Blume) Miq. tea processing.

ACKNOWLEDGMENTS

The authors would like to thank the Department of Medicinal Plant Science, Faculty of Agricultural Production, Maejo University, Thailand for the financial support.

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