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Effects of pre-processing on the active compounds before drying *Eucommia ulmoides* leaves

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Abstract

Aiming to provide a new way that preserves more active components *in Eucommia ulmoides* (EU) leaves, this paper utilizes 4 methods, i.e., microwave, steam, air heating, and frying, to process EU leaves before drying, and their respective effects on the leaves were evaluated. The antioxidant activity of EU leaves *in vitro* was measured by the DPPH free radical method; the contents of total flavonoids (TFC) and total phenols (TPC) were analyzed by spectrophotometry; and the contents of main active components were determined by high-performance liquid chromatography (HPLC). The results showed the pre-process improved the quality of the EU leaves. As a simple and fast process, the microwave process is the most effective technique in the retention of flavonoids and four active ingredients except aucubin. High power (800 W) and short time (2.5 min) are more conducive to the retention of active ingredients, with high antioxidant activity. Appropriate steam and frying processes also exhibited benefits for the active components. In particular, the samples treated with steam for 10 minutes showed great potential for industrial application with the highest antioxidant activity and polyphenol content. Therefore, proper pre-processing before drying should be conducted to effectively protect the active components of EU leaves.

Keywords: *Eucommia*; polyphenol; flavonoids; microwave; steam.

Practical Application: The research confirmed the traditional simple method of steaming can improve the active ingredient content of EU leaves if used properly. Because the steam treatment does not require special equipments, and its low processing cost, which is favorable method of many small EU processing plants.

1 Introduction

The cortex of *Eucommia ulmoides* Oliver (EU) is a type of traditional Chinese medicine and its application in China dates back to about 2,000 years ago. EU leaves were discovered to have similar chemical compositions and medicinal effects to the bark in recent years. In the past 30 years, EU leaves have become a popular functional healthy food in China and Japan as they were found to be abounding with bioactive compounds (polyphenolic acids, flavonoids, and iridoids) and nutrients (amino acids, vitamins, and minerals) (Wang et al., 2019). Besides, they showed strong antioxidant activity both in vivo and in vitro. The leaves extract can inhibit lipid peroxidation in experimental models (Yen & Hsieh, 1998); while, the leaves can scavenge chemical free radicals and reactive oxygen species, reduce cholesterol and "fatty liver", and restrict oxidative damage in deoxyribose and DNA (Hussain et al., 2016). EU leaves also displayed stronger antioxidant activities in vitro (Yen & Hsieh, 1998; Zhang et al., 2007). It was reported that the leaves can reduce blood pressure, suppress mutagenicity and chromosome aberration (Hussain et al., 2016). The benefits to health mentioned above explain the increasing attention on EU leaves as an industrial material for preparing medicine and functional foods.

In harvest seasons, tons of EU leaves are sent to the factory to be dried and stored. During drying, some active ingredients are oxidized and decomposed, and the content decreases, reducing the quality of plant tissues (Rocha et al., 2011; Khan et al., 2022; Thamkaew et al., 2021). As for tea, the most studied leaf processing products, short-term heating techniques named Sha-qing in Chinese is common for tea processing, which maintains its color, aroma, and taste. Other process before drying like steam is adopted to make dried green vegetable products. Therefore, the application of similar techniques to EU leaves could retain active ingredients. Instead, the postharvest process of EU leaves, especially the pre-process before drying, is rarely studied. Steaming and frying, are the traditional ways in China to process green vegetable or tea products. Air heating with electric ovens and microwaves has also been used to process plants in recent years (Rocha et al., 2011; Thamkaew et al., 2021; Qin et al., 2022). The microwave process is considered a better method as it can avoid browning, reduce drying duration, and control undesirable biotransformation in foods (Rodríguez-Lora et al., 2022; Vadivambal & Jayas, 2007; Aydar, 2021).

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Therefore, the methods of microwave, steam, air heating, and frying were adopted for the EU leaves process respectively in this paper to clarify their effects on EU leaves antioxidant activity and main active compounds. The active compounds contents, antioxidant activity, and contents of TPC and TFC of the samples were also elaborated.

2 Materials and methods

2.1 Materials and reagents

The EU leaves employed in the study were collected from branches in the middle part of EU trees (30a) in a garden of Northwest A&F University, Yangling, China in July 2021, and stored in a refrigerator until processed within 24 hours.

Rutin trihydrate, quercetin, chlorogenic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and Folin-Ciocalteu's polyphenol reagent were purchased from Sigma-Aldrich Co. (Shanghai, China), while Geniposidic acid and aucubin from National Institutes for Food and Drug Quality Control (Beijing, China). All other chemicals were of analytical grade.

2.2 Methods

Process

Every sample weighs about 40 g, and the pre-process is as follows.

Natural way (control). Samples were dried in the shade at room temperature (15-25 °C) until the water content was lower than 15 wt%.

Microwave. Leaves with a thickness of *ca* 1 ± 0.2 cm were tiled on the glass tray in a microwave oven (G8023YSL-V1, 2450 MHz, Foshan Galanz Electric Company), and treated with different power (160W-800W) for different periods.

Steam. After water boiling in a steamer for 5 min, samples with a thickness of *ca* 1 ± 0.2 cm were tiled on a grate in the steamer for different time periods.

Air heating. Leaves with a thickness of *ca* 1 ± 0.2 cm were tiled on a tray in an oven with ventilation (DHG-9240A, 2050W, Shanghai Jingmi instrument company) at different temperatures for different time periods.

Frying. Leaves with a thickness of *ca* 1 ± 0.2 cm were tiled in a round frying pan on a cooker (SK2103, Midea electric company) and stir-fried at different temperatures for different time periods.

Then, all samples were air-dried in the shade at room temperature (15-25 °C) until the water content was lower than 15 wt%, and powdered and stored at -18 °C before extraction. Each process was conducted three times.

Determination of contents of active compounds

Extract preparation

Each sample of the air-dried and grounded EU leaves (10 g) was extracted twice with 60% (v/v) ethanol solution (150 mL) at 60 °C for 60 min. The two extracts were combined, filtered, and evaporated to dryness under vacuum at 50 °C to obtain brown residue. The sealed extracts were stored at -18 °C before analysis.

Total Flavonoids (TFC)

TFC was determined using a modified colorimetric method (Zhishen et al., 1999). TFC was calculated with a rutin standard curve, and expressed as rutin equivalents in milligrams per gram of dry plant sample.

Total Polyphenolics (TPC)

TPC was estimated according to a protocol with a minor modification (Singleton et al., 1999). The mixture of sample solution (one mL), distilled water (5 mL), and 1 mol/L Folin-Ciocalteu's polyphenol reagent (0.5 mL), was allowed to react for 5 min, and then added with 5 g/100 mL Na_2CO_3 (1 mL). Thereafter, it was thoroughly mixed and placed in the dark for 1 hour, and the absorbance was measured at 725 nm with a spectrophotometer. A gallic acid standard curve was obtained to calculate TPC, which was expressed as gallic acid equivalents (GAE) in milligrams per gram of dry plant material.

Chlorogenic acid and geniposidic acid

HPLC was performed to determine the contents of chlorogenic acid and geniposidic acid in the samples (Dong et al., 2011). A Shimadzu HPLC system (LC-10AT) equipped with a UV detector (SPD-10AVP), and a Shim-pack VP-ODS column (150 mm × 4.6 mm, 5 μm) were used. The mobile phase was ethanol/water/acetic acid (24/75/1, v/v), with a flow rate of 1 mL/min, injection volume of 10 μL, and detection wavelength of 240 nm. The contents were calculated based on the peak areas in the chromatogram.

Rutin and quercetin

HPLC was employed again to measure the contents of rutin and quercetin in the leaves. The mobile phase was methanol/water/phosphoric acid (50/49.5/0.5, v/v), with a flow rate of 1.0 mL/min, injection volume of 20 μL, and detection wavelength of 270 nm.

Aucubin

An improved method was adopted by using dimethylaminobenzaldehyde as a colorimetric reagent to determine the content of aucubin (Dong et al., 2011).

Antioxidant activity assay

Antioxidant activity of the processed leaves was investigated by IC_{50} of DPPH radical scavenging activity, and the effect of scavenging DPPH radical was measured according to a previous method (Zhang et al., 2013). The sample solution in ethanol (2 mL) was added to 500 μmol/L DPPH free radical solution (1 mL), shaken, and placed in the dark at room temperature for 20 min. The absorbance of the mixture was measured at 517 nm. Solution (1 mL) of 500 μmol/L DPPH free radical mixed with

alcohol (2 mL) was used as the control. The radical scavenging activity of samples was calculated according to Equation 1.

$$Inhibition\ effect\ (\%) = 100 - \left(\frac{absorbance\ of\ sample}{absorbance\ of\ control}\right) \times 100 \quad (1)$$

Statistical analysis

All results were obtained from three independent experiments and expressed as mean ± SD. Differences between treatments ($p < 0.05$) were determined by Duncan's multiple range test. Data analysis was performed using SAS statistical software (SAS Institute Inc., Cary NC).

3 Results and discussion

3.1 Effects of different processes on TFC

Flavonoids, which can be found in many plants, are generally antioxidants and act as free radical scavengers, as they are potential reducing agents and prevent the occurrence of oxidative reactions inside the body (Dias et al., 2021). Flavonoids, as the main active components in EU leaves, are in the range of 10.0 – 30.0 mg/g in content. Besides, the content is much higher in leaves collected in May than in those harvested in other months (Zhang et al., 2013). Figure 1 illustrates the TFC of EU leaves processed with microwave, steam, air heating, and frying, which, as mentioned, is expressed as rutin equivalents in milligrams per gram of dry leaf sample.

Figure 1a confirms the significant role of microwave's output power on TFC in samples. In other words, higher output power is accompanied by higher TFC with the process time period unchanged, and leaves processed with 800 W for 2.5 min exhibited the highest TFC, 26.2 mg/g, which shares the results of the study on *Eucommia* male flower (Dong et al., 2011). Such phenomenon can be attributed to the requirement for high output power by enzyme deactivation, such as polyphenol oxidase. Obviously, output power greater than 480 W was in a superior position to receive high TFC because of the higher TFC of leaves processed with such power than those handled with lower power.

In addition, the processed time also mattered. The samples treated for 1 min (160 W - 800 W) exhibited the lowest contents, which proved the lack of time for microwave action. The condition of lower output power (160 W and 320W) and time period (2.5 min) better protected flavonoids than 1 min and 5 min. The lower TFC of leaves treated for 5 min (480 W – 800 W) than samples processed for 2.5 min indicates that the time is too long to affect active compounds, which may come down to the strong thermal effect of microwave, and the great impact of high temperature on active ingredients (Zhang et al., 2006). Within the time duration designed in the experiment, the highest TFC was found in leaves treated with output power of 800 W for 2.5 min, which advocates TFC preservation in microwave with high output power and medium time.

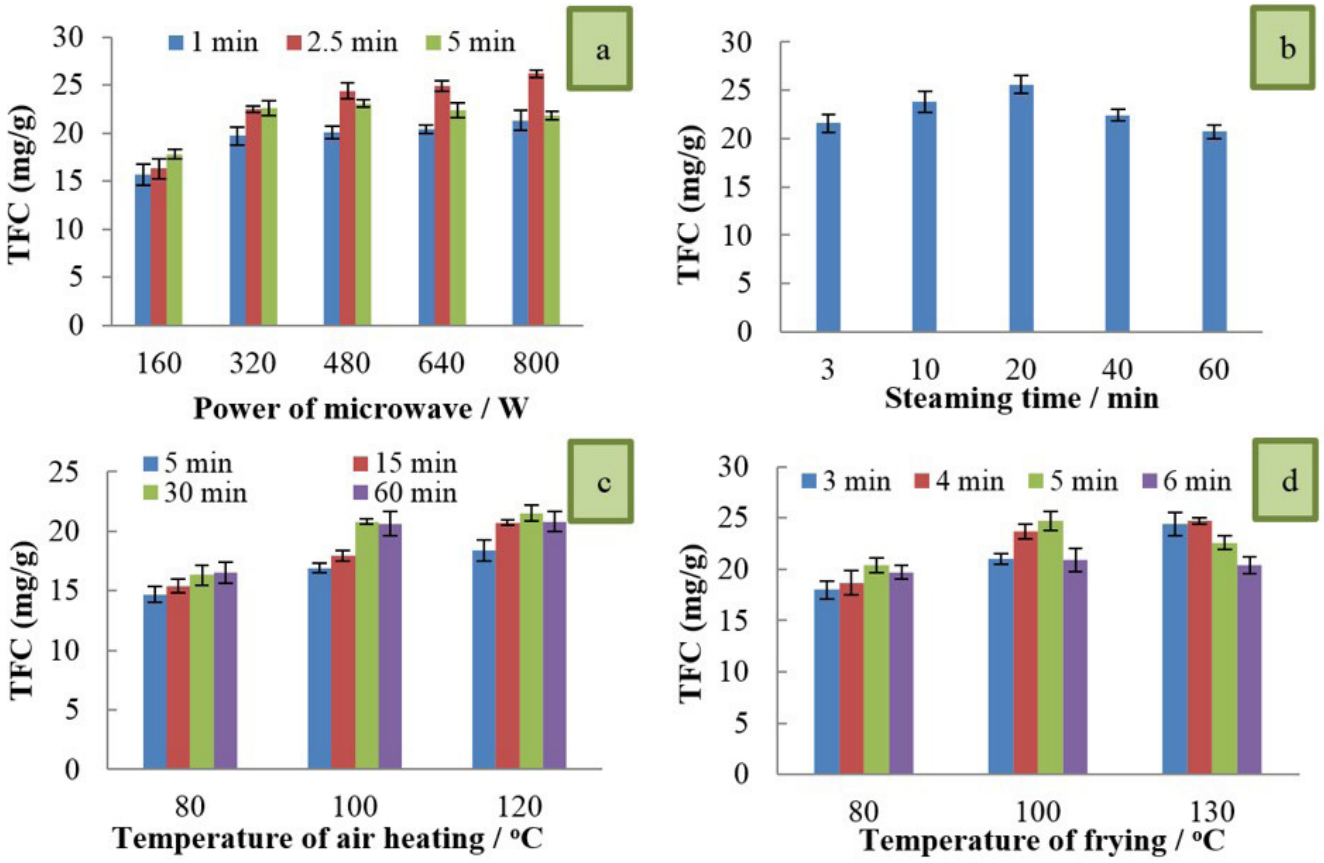

Figure 1. TFC of the processed EU leaves.

Steam is one of the traditional methods used for food production (Pongmalai & Devahastin, 2020; Sun et al., 2020). Figure 1b shows the higher TFC (25.6 mg/g) of leaves treated for 20 min than those treated for a longer or shorter time. The similar TFC in some samples processed in this way with those treated with modern techniques, such as the microwave, merits mentioning. Despite its longer time for mass and heat transfer in the leaves than microwave, the steam effectively avoids the higher temperature of samples, and protects the heat-sensitive components, which confirms the high TFC over a long period of time (10-20 min) of samples treated with steam. The steam temperature that is not high can exert certain oxygen isolation effect, which may explain the favorable performance of steam (Wu et al., 2022).

Air heating is commonly used for process of vegetables and plant medicines (Thamkaew et al., 2021). Figure 1c reveals the influence of temperature and time on flavonoid contents. To be specific, leaves heated at a lower temperature (80 °C) exhibited lower TFC regardless of the heated time, while longer process duration at higher temperatures led to higher TFC. Among all experiments, treatments at 100 °C for 30 and 60 min, and at 120 °C for 15 to 60 min resulted in favorable TFC above 20 mg/g, which still eclipsed compared with that of samples processed with microwave and steam.

Frying treatment is popular for the process of green tea. Figure 1d demonstrates the obvious effect of temperature and treatment time on TFC. The temperature and flavonoids content was in direct proportion for samples fried for 3 min or 4 min, the temperature of 100 °C was a better choice for samples fried for 5 min, and no significant difference was observed regardless of temperature among samples fried for 6 min. Samples treated for 5 min showed a higher flavonoids content at lower temperatures of 80 and 100 °C, while leaves heated at high temperature (e.g. 130 °C), and short duration (e.g. 3 min or 4 min) presented high TFC (24.4 and 24.7 mg/g). Manual frying, which is integral to green tea production and seems like a Chinese cooking process, serves as a competitive candidate for flavonoids preservation.

3.2 Effects of different treatments on TPC

Polyphenols, another kind of main bioactive compounds in EU leaves, are about 70 -110 mg/g in content, a value much higher than that of the flavonoids. Samples harvested in summer contained more phenolic compounds than those collected in other seasons (Zhang et al., 2013). Figure 2 validates the role of the four pretreatments on the contents of phenolic compounds. Besides, TPC of samples varied in the range of 46.39- 102.93 mg/g.

Figure 2a illustrates the larger impact of output power of microwave than treatment time. Samples treated with higher

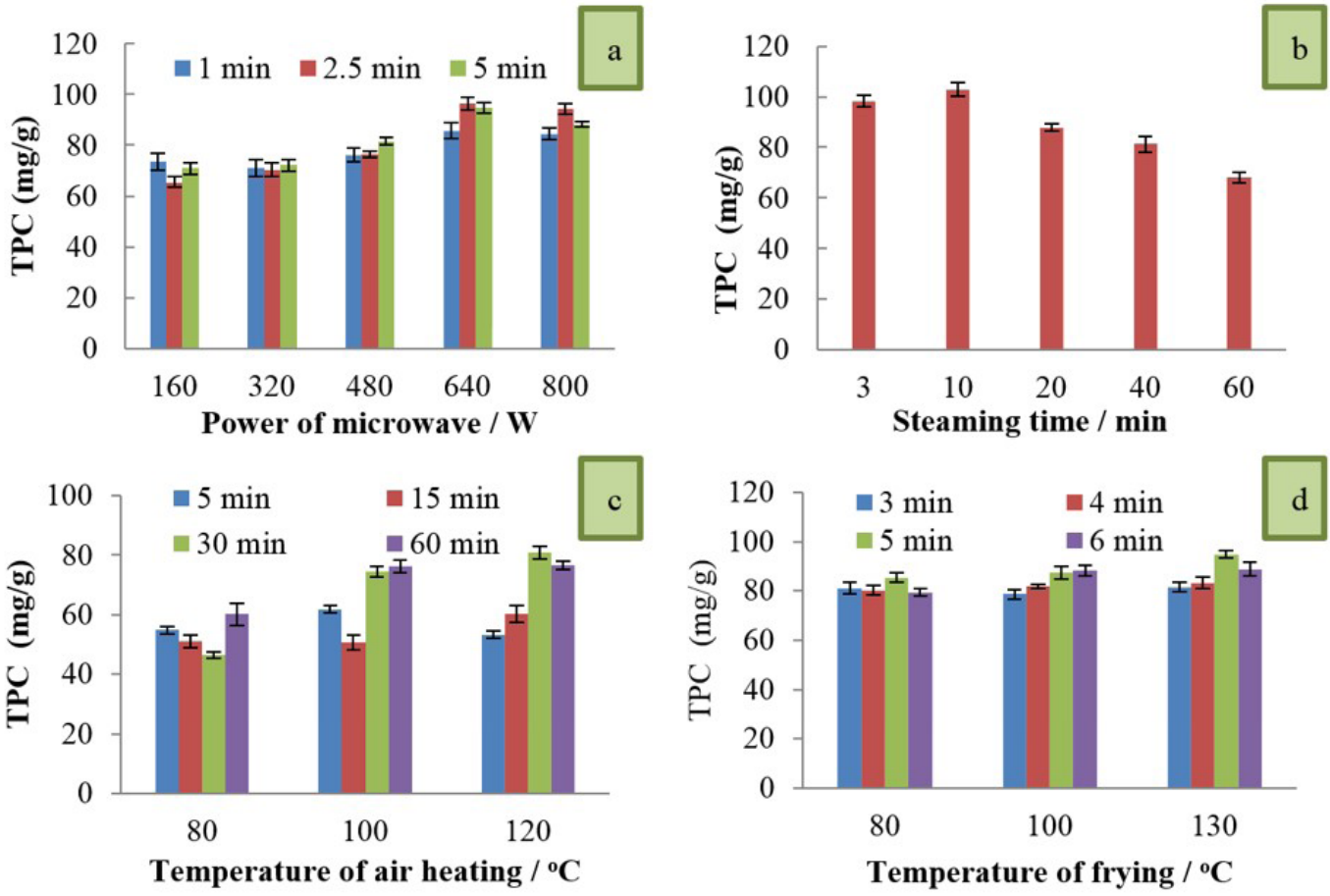

Figure 2. TPC of the processed EU leaves.

output power (640 W, 800 W) had more phenolic compounds than those treated with lower output power (160 W, 320 W, 480 W) regardless of treatment time. As for the processing time, 2.5 min was suitable to protect polyphenols under higher output power. Besides, leaves processed with 640 W for 2.5 min and 5 min, and 800 W for 2.5 min showed TPC over 94 mg/g.

Figure 2b supports the steam's ability to preserve polyphenols as a conventional method. At the experimental conditions, leaves exposed to steam for a short time of 3 min or 10 min contained much more phenolic compounds (> 98mg/g) than samples treated for a longer time (20 - 40 min), and even more than samples treated with microwave.

Figure 2c verifies the obvious effect of temperature and time on the TPC of air heating leaves. The low temperature of 80 °C was not suitable to protect polyphenols due to the samples' low polyphenols contents (46 - 60 mg/g). For higher temperature (e.g. 100 °C and 120 °C), longer times of 30 min and 60 min were better choices. However, all samples heated in the oven contained less polyphenol than those processed by microwave and steam at proper working conditions.

Figure 2d reveals the TPC ranging from 78 to 94 mg/g in frying processed leaves, which are affected by frying temperature and time. No significant change in samples' TPC was observed at the temperature of 80 °C and 100 °C regardless of processed time, and only the treatment at 130 °C for 5 min resulted in a higher TPC, which was even close to the best samples processed by microwave and steam.

3.3 Effects of different treatments on the leaves' antioxidant activity

EU leaves showed a strong antioxidant activity in vivo and in vitro, and the association between antioxidant activity and flavonoides and polyphenols content was proposed (Wang et al., 2019, 2020). Figure 3a reveals the inverse proportion between output power and IC_{50} of DPPH, which suggests the growing antioxidant activity with higher microwave power. Besides, the strongest activity (IC_{50} = 127 μg/mL) was observed in the sample with the most flavonoides and polyphenols and treated at 800 W for 2.5 min.

Figure 3b reveals the advantage of steam in preserving antioxidant activity. The IC_{50} of samples steamed for 10 min was only 119 μg/mL, even lower than that of the best sample in microwave group. Besides, the polyphenols of samples were 102 mg/g, excelling all other samples, which strengthened the competitiveness of the traditional method.

Figure 3c displays the results of air heating. Samples heated at higher temperature (120 °C) and for longer time (30 min and 60 min) presented antioxidant activity of 166 μg/mL and

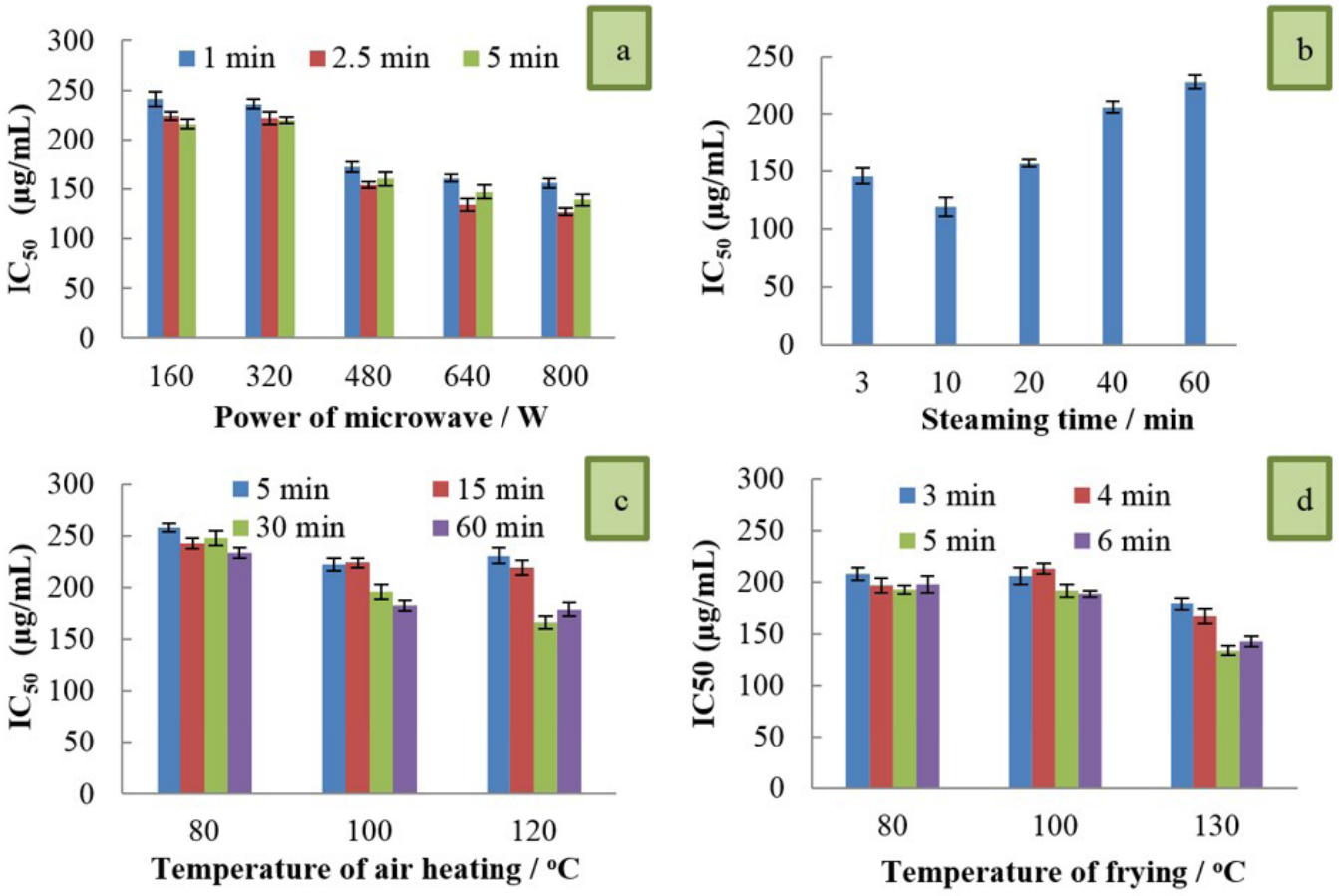

Figure 3. IC_{50} of DPPH free radicles of the process EU leaves.

Table 1. Contents of active compounds and free radical scavenging activity of dried EU leaves (mg/g).

Samples	Rutin	Quercetin	Chlorogenic acid	Geniposidic acid	Aucubin	TFC	TPC	DPPH μg/mL
Control	6.2 ± 0.05b	0.28 ± 0.05c	20.0 ± 0.29d	1.34 ± 0.05ab	6.8 ± 0.12a	20.4 ± 0.25d	87.53 ± 2.33c	154 ± 4c
Microwave	6.7 ± 0.19a	0.47 ± 0.04a	25.6 ± 0.61b	1.38 ± 0.02a	4.5 ± 0.09b	26.2 ± 0.38a	94.21 ± 2.19b	127 ± 4ab
Steam	6.3 ± 0.12b	0.34 ± 0.06b	28.4 ± 0.34a	1.27 ± 0.02b	2.4 ± 0.11d	23.8 ± 0.49b	102.93 ± 2.65a	119 ± 8a
Air heating	6.1 ± 0.16b	0.36 ± 0.02b	19.6 ± 0.23d	1.29 ± 0.06b	3.9 ± 0.07c	21.5 ± 0.64c	80.82 ± 2.12d	166 ± 6d
Frying	6.2 ± 0.18b	0.18 ± 0.02d	23.4 ± 0.12c	1.12 ± 0.05c	3.8 ± 0.09c	22.6 ± 0.37c	94.78 ± 1.54b	134 ± 5b

Note: letters within the column indicate a significant difference ($p < 0.05$).

179 μg/mL, respectively, and TPC about 80 mg/g, both of which outperformed those treated at 80 ºC.

According to a study on antioxidant activity, high temperature can better reserve bioactivity (Figure 3d) of frying leaves, and samples treated with at high temperature showed higher activity than other samples regardless of treatment time. In particular, samples fried for 5 and 6 minutes showed high activity, contained about 90 mg/g of TPC, and exhibited respective 134 μg/mL and 143 μg/mL IC_{50}.

3.4 Comparison of methods

Chlorogenic acid, aucubin, geniposidic acid, rutin, and quercetin are the main medicinal compounds in EU leaves (Wang et al., 2019; Zhu & Sun, 2018), and HPLC was employed to measure their contents in samples processed by the four methods and showed highest antioxidant activity. Chlorogenic acid serves as the most active compound in EU leaves, the representative of EU polyphenols, and an indicator of leaves quality. Table 1 confirmed the highest chlorogenic acid content of samples treated by steam (28.4 mg/g), and the higher chlorogenic acid contents of EU leaves treated with microwave and frying than the control. No significant difference between the air heated samples and the control was found.

Rutin is the main compound of flavonoids in EU leaves. The rutin content of samples treated by microwave (6.7 mg/g) is higher than that of the control, and other samples showed similar content to the control. As for quercetin content, samples treated with microwave contained the highest, those processed with steam and air heating outnumbered the control, and only frying leaves exhibited lower content.

Geniposide acid belonging to iridoids is also the main active component in EU leaves. Table 1 demonstrates the highest geniposide acid content of microwave treated sample, and the lower content of leaves treated by the other three methods compared with the control. Aucubin is also a kind of iridoid compound, which is very unstable and sensitive to temperature. Therefore, longer heating process destructs the structure and decreases the content of aucubin (Li et al., 2009; Zheng et al., 2012). Table 1 reveals the lower aucubin content of all the processed samples than that of the control. Therefore, the extraction of iridoid compounds should avoid thermal processing.

As shown in Table 1, leaves processed with microwave, steam, and frying presented much stronger antioxidant activity than the control, and showed higher TFC, TPC, and chlorogenic acid content. Therefore, the development of EU leaves for health food or medicines requires proper pre-process before drying, which enhances leaves quality.

4 Conclusions

Fresh leaves are commonly dried for storage before extraction. The comparison between four pre-processing methods of microwave, steam, frying, and air heating with leaves directly dried without processing reveals the failure of air heating in the ventilation oven to better preserve active ingredients, and the ability of the other three to better preserve active components and antioxidant activities. The content of active components in leaves is affected by microwave power, time, and temperature. Samples heated at 800W for 2.5 min by microwave performed the highest TFC (26.2 mg/g), and exhibited higher antioxidant activity, TPC, chlorogenic acid content, rutin content, and geniposide acid content than those of untreated samples. Samples steamed for 10 minutes exhibited a chlorogenic acid content of 28.4 mg/g, TPC of 102.93 mg/g, and the IC_{50} for DPPH radical of 119 μg/mL. Besides, leaves fried experienced an increase in chlorogenic acid content, TFC, TPC, and DPPH scavenging ability. Given their favorable performance in preserving active ingredients and no need for advanced equipment, the methods of frying and steam merit further study.

Acknowledgements

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