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Comparative Analysis of Drying Methods and Solvent Polarity on Phytochemicals Contents and Antioxidant Activities of *Colubrina asiatica* Leaves

(Analisis Perbandingan Kaedah Pengeringan dan Kekutuban Pelarut ke atas Aktiviti Fitokimia dan Antioksidan pada Daun *Colubrina asiatica*)

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ABSTRACT

Colubrina asiatica is a traditional Malaysian vegetable known for its high phenolic content and antioxidant activity. However, studies on the effect of drying methods and solvent polarity on the extraction efficiency of *C. asiatica* leaves remain limited. Similarly, the phytochemical profile of *C. asiatica* has been significantly underreported. Therefore, this study aimed to evaluate the effects of low-temperature drying method (freeze-dried (FD) at -50 °C) and high-temperature drying method (oven-dried (OD) at 40 °C) and solvents of varying polarity (ethanol:water ratio of 0:100, 20:80, 40:60, 60:40, 80:20, 100:0) on the phytochemical content and antioxidant properties of *C. asiatica* leaf extracts. The results showed that the 100% ethanol OD extract had the highest ($p<0.05$) phenolic, flavonoid, vitamin C content and ferric reducing power (FRAP) compared to the other extracts. In contrast, the highest DPPH radical scavenging activity ($p<0.05$) was observed in both OD and FD extracts (80% and 100% ethanol). Phytochemical profiling using UHPLC-QTOF-MS identified the presence of phenolic acids, flavonoids, alkaloid, terpenoids and saponins in both OD and FD (100% ethanol) extracts. Quantification by HPLC-PDA further showed that the 100% ethanol FD extract (FD100) had higher concentrations of kaempferol-3-O-rutinoside (K3R) and rutin compared to 100% ethanol OD extract (OD100). In summary, oven drying combined with 100% ethanol was the most effective method for obtaining *C. asiatica* leaf extract with high phenolic, flavonoid, vitamin C content and FRAP activity. However, freeze-dried *C. asiatica* leaf extracted with 100% ethanol contains the highest concentration of K3R and rutin.

Keywords: Antioxidant; chromatography; flavonoid; mass spectrometry (MS); phenolic

ABSTRAK

Colubrina asiatica merupakan sayuran tradisional Malaysia dengan kandungan fenol dan aktiviti antioksidan yang tinggi. Namun begitu, kajian terhadap kaedah pengeringan dan kekutuban pelarut yang berkesan untuk pengekstrakan daun *C. asiatica* dan profil fitokimianya masih terhad. Oleh itu, kajian ini bertujuan menentukan kesan kaedah pengeringan suhu rendah (pengeringan sejuk beku (FD) pada -50 °C) dan pengeringan suhu tinggi (pengeringan ketuhar (OD) pada 40 °C) serta kesan pelarut dengan kekutuban yang berbeza (nisbah etanol kepada air: 0:100, 20:80, 40:60, 60:40, 80:20, 100:0) kepada ciri fitokimia dan aktiviti antioksidan ekstrak daun *C. asiatica*. Hasil kajian menunjukkan ekstrak OD, 100% etanol mengandungi fenol, flavonoid, vitamin C dan kuasa penurunan ferik (FRAP) yang tinggi ($p<0.05$) berbanding ekstrak lain. Manakala aktiviti pemerangkapan DPPH yang paling tinggi ($p<0.05$) telah direkodkan oleh ekstrak OD dan FD (80 dan 100% etanol). Pencirian daun *C. asiatica* menggunakan teknik kromatografi (UHPLC-QTOF-MS) telah mengenal pasti kehadiran asid fenol, flavonoid, alkaloid, terpenoid dan saponin di dalam ekstrak OD dan FD (100% etanol). Pengkuantitian sebatian flavonoid oleh HPLC-PDA menunjukkan ekstrak FD 100% etanol (FD100) mengandungi kaempferol-3-O-rutinosida (K3R) dan rutin yang lebih tinggi berbanding ekstrak OD 100% etanol (OD100). Secara keseluruhan, kajian ini menunjukkan pengeringan ketuhar (OD) dan pelarut 100% etanol merupakan kaedah pengeringan dan kekutuban pelarut yang terbaik untuk menghasilkan ekstrak daun *C. asiatica* dengan kandungan fenol, flavonoid, vitamin C serta aktiviti FRAP yang tinggi. Manakala, daun *C. asiatica* yang dikeringkan secara sejuk beku menggunakan pelarut etanol 100% menghasilkan ekstrak yang mempunyai kandungan K3R dan rutin.

Kata kunci: Antioksidan; fenol; flavonoid; kromatografi; spektrometri jisim (MS)

INTRODUCTION

Colubrina asiatica (L.) Brongn, a traditional Malaysian vegetable from the Rhamnaceaea family, has been reported to contain high levels of phenolic, vitamin C and exhibit strong antioxidant activities (Mohd Shukri, Alan & Site Noorzuraini 2011; Mohd Shukri et al. 2013). A previous study by Nik Hairiah et al. (2016) demonstrated that deionized water extracts of *C. asiatica* leaves contain flavonoids, terpenoids, saponins, and tannins. Quantitative HPLC analysis has confirmed the presence of flavonoids such as quercetin-3-O-rhamnoside, kaempferol-3-O-glucoside, and kaempferol-3-O-rutinoside in methanolic extracts of *C. asiatica* (Mohd Shukri, Alan & Site Noorzuraini 2011). According to Desai et al. (2010), flavonoids and polyphenols are the bioactive compounds contributing to the antioxidant activity of *C. asiatica* leaf extract. Notably, the phenolic content of methanolic *C. asiatica* extract was found to be significant correlated ($p<0.0001$) with total antioxidant activity as measured by ferric reducing antioxidant power (FRAP) assay (Mohd Shukri, Alan & Site Noorzuraini 2011). Additionally, water extracts of *C. asiatica* leaves have been reported to scavenge various reactive species, including DPPH, hydrogen peroxide, nitric oxide and superoxide anion radicals (Desai & Gaikwad 2014).

Most plant samples require drying prior to extraction to reduce microbial growth, enzymatic activity and biochemical reactions. However, the drying process can also influence the phytochemical content, bioactivity and physicochemical properties of the sample (Ho et al. 2018; Pham et al. 2015; Roshanak, Rahimmalek & Goli 2016). Oven drying is a commonly used high-temperature method due to its cost-effectiveness, however, prolong thermal exposure may negatively impact on the antioxidant capacity of the sample (Mbondo et al. 2017; Mphahlele et al. 2016). Therefore, low-temperature drying method (such as freeze drying) may serve as effective alternatives for better retention of antioxidant compounds (Roshanak, Rahimmalek & Goli 2016).

Solid-liquid extraction is one of the most employed techniques for extracting bioactive compounds from plants. These bioactives comprise a wide range of compounds with diverse chemical characteristics and polarities, which interact differently with solvents (Norra, Aminah & Suri 2016). Compounds of interest are more efficiently extracted using solvents with similar polarity (Altemimi et al. 2017). Ethanol and water are widely used as extraction solvents due to their effectiveness in extracting plant bioactive and their safety for human consumption (Lim et al. 2019; Pabón-Baquero et al. 2018; Roy & Datta 2019). However, previous studies on *C. asiatica* extract have not thoroughly investigated the influence of different drying methods and solvent polarities on the phytochemical content and antioxidant activities. Moreover, no comprehensive phytochemical profiling of ethanolic *C. asiatica* extracts using advanced high-performance liquid chromatography (HPLC) techniques has been reported to date. Therefore, this study aimed to evaluate the effects of low- and high-temperature drying methods (oven-drying and freeze-drying) and solvent polarity (ethanol:water ratios of 0:100, 20:80, 40:60, 60:40, 80:20, 100:0) on the phytochemical composition and antioxidant activities of *C. asiatica* leaf extract. The qualitative and quantitative phytochemical analyses using UHPLC-QTOF-MS and HPLC-PDA were conducted on *C. asiatica* extracts that exhibited the highest levels of total phenolic content, total flavonoid content, vitamin C and antioxidant activity.

MATERIALS AND METHODS

DRYING AND EXTRACTION OF *C. asiatica* LEAVES

C. asiatica leaves were collected from Baling, Kedah, Malaysia. To ensure consistency in sample selection, leaf samples were chosen based on their maturation stage, as indicated by their size (3 cm wide and 5 cm long) and dark green colour (Figure 1). The leaves were dried using two different methods; oven drying (OD) at 40 °C (Memmert, Germany) and freeze drying (FD) at -50 °C (Martin Christ, Germany). The dried leaves were taken out of the oven and freeze dryer when weight of the leaves remained constant (approximately 48 h). The samples were ground using a laboratory blender (7011HS 2-Speed Food Blender, Waring Commercial, USA) and subsequently sieved using a 550 µm mesh filter to obtain dried sample powder of uniform particle size. Based on Nik Hairiah et al. (2016), 50 g of *C. asiatica* dried powder was extracted with 250 mL of ethanol (99.8%, undenatured) and water in six different polarities; 100:0, 80:20, 60:40, 40:60, 20:80, 0:100. The mixture was placed in a mechanical shaker for 24 h at 27 °C with a speed set to 200 rpm. The aqueous extract was filtered with Whatman No. 1 filter paper and freeze dried to obtain the dry extract.

TOTAL PHENOLIC CONTENT (TPC)

According to Ain Ibrahim et al. (2023), 0.5 mL of 5 mg/mL extract was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent. After 5 min, 2 mL of 7.5% sodium carbonate (Na_2CO_3) was added to the mixture. The mixture was vortexed and after 2 h, the absorbance reading of the mixture was measured with Epoch™ Microplate Spectrophotometer (BioTek, USA) at 750 nm. TPC was calculated based on gallic acid standard curve (0.00 - 0.30 mg/mL) and expressed as mg gallic acid equivalent (GAE) per g extract.

TOTAL FLAVONOID CONTENT (TFC)

According to Nik Hairiah et al. (2016), 1 mL of 5 mg/mL extract was mixed with 4 mL distilled water followed with 0.3 mL of 10% sodium nitrate ($NaNO_3$). After 5 min, 0.3 mL of 10% aluminium chloride ($AlCl_3$) and 2 mL of 1% sodium hydroxide (NaOH), were added to the mixture.

FIGURE 1. Fresh *Colubrina asiatica* leaf

Absorbance reading of the mixture was measured with Epoch™ Microplate Spectrophotometer (BioTek, USA) at 510 nm. TFC was calculated based on quercetin standard curve (0.00 – 5.00 mg/mL) and expressed as mg quercetin equivalent (QE) per g extract.

VITAMIN C CONTENT

According to Elgailani et al. (2017), 10 mL of 0.2 mg/mL extract was mixed with 1 mL of 0.01 M potassium permanganate ($KMnO_4$) (in 0.1 M sulphuric acid (H_2SO_4)). After 5 min, the absorbance reading of the mixture was measured with Epoch™ Microplate Spectrophotometer (BioTek, USA) at 530 nm. Vitamin C content was calculated based on the ascorbic acid standard curve (0.00 - 5.00 µg/mL in 0.5% oxalic acid) and expressed as mg vitamin C per g extract.

DPPH FREE RADICAL SCAVENGING ACTIVITY (IC_{50})

Based on Noorhadi et al. (2024), 2 mL of extract (0.2 – 5.00 mg/mL) was added to 2 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol. After 30 min, the absorbance reading of the mixture was measured with Epoch™ Microplate Spectrophotometer (BioTek, USA) at 517 nm. The extract 50% inhibition concentration (IC_{50}-) of DPPH free radical was calculated based on standard curve of extract concentration againts percent of DPPH inhibition activity using the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance blank} - \text{Absorbance sample})}{\text{Absorbance blank}} \times 100$$

FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

Based on Ling et al. (2020), 2 mL of FRAP reagent (10 acetate buffer : 1 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) : 1 iron (III) chloride hexahydrate ($FeCl_3$.6H2O)) mixed with 0.1 mL of 5 mg/mL extract. After 30 min, the absorbance reading of the mixture was measured with Epoch™ Microplate Spectrophotometer (BioTek, USA) at 593 nm. FRAP value was calculated based on Trolox standard curve (0.00 - 0.60 mg/mL in methanol) and expressed as mg Trolox equivalent (TE) per g extract.

PHYTOCHEMICALS PROFILING BY UHPLC-QTOF-MS

Ultrahigh-perfomance liquid chromatography coupled with a quadrupole-time-of-flight mass spectrometer (UHPLC-QTOF-MS) (Waters, USA) was used to identify the phytochemicals of selected OD and FD extracts, following the method described by Deng et al. (2016). The UHPLC was performed on ACQUITY UPLC 1-CLASS system with a binary pump, a vacuum degasser, an auto-sampler and a column oven. ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm × 1.8 µm) was used for the compounds separation at 40 °C. A linear binary gradient of water (0.1% formic acid) and acetonitrile (mobile phase B) was used as mobile phase A and B, respectively. The mobile phase composition was changed during the run as follows: 0 min, 1% B; 0.5 min, 1% B; 16 min, 35% B; 18 min, 100% B; 20 min, 1% B. The flow rate was set to 0.6 mL/min and the injection volume was 1 µL.

The QTOF MS equipped with Lock Spray ion source operated in negative electrospray ionization (ESI) mode with the following conditions: capillary voltage, 1.50 kV; reference capillary voltage, 3.00 kV; source temperature, 120 °C; desolvation gas temperature, 550 °C; desolvation

gas flow, 800 L/h, and cone gas flow, 50 L/h. Nitrogen (>99.5%) was employed as desolvation and cone gas. Data were acquired in high-definition MSE (HDMSE) mode in the range m/z 50 - 1500 at 0.1 s/scan. Thus, two independent scans with different collision energies (CE) were alternatively acquired during the run: a low-energy (LE) scan at a fixed CE of 4 eV, and a high-energy (HE) scan where the CE was ramped from 10 to 40 eV. Argon (99.999%) was used as collision-induced-dissociation (CID) gas.

Waters UNIFI (USA) scientific information system and Waters Traditional Medicine Library [UNIFI 1.8 EN] were used to obtain MS data, data mining and identification based on the accurate mass, molecule formula and chemical structure of the compounds. Tentatively identified compounds with good match, responses higher than 10,000 and accurate mass information (± 5 ppm) were shortlisted. The identity of the compounds was then confirmed by comparison with standard or previous literature reported on the MS fragments of the compounds.

QUANTIFICATION OF RUTIN AND KAEMPFEROL-3-O-RUTINOSIDE BY HPLC-PDA

Rutin and Kaempferol-3-O-Rutinoside (K3R) of selected OD and FD extracts were quantified using high-perfomance liquid chromatography coupled with a photodiode array detector (HPLC-PDA) (Waters, USA), following the method described by Abdul Rahman et al. (2017) with slight modification. Standards of rutin and K3R were dissolved in methanol within a concentration range of 5 - 500 µg/mL for the standard curves. The extracts were dissolved in methanol with concentrations of 10 mg/mL and 4 mg/mL, respectively. The extracts were analysed with HPLC system (Waters 600 quaternary gradient pump, Waters 717 autosampler and Waters 2996 PDA) and HPLC Phenomenex Luna C18 column (5 µm, 250 mm × 4.6 mm) with gradient solvent system consisted of two solvents; A (0.1% formic acid), B (acetonitirile) and C (methanol). The mobile phase composition was changed during the run as follows: 0 min, 20% B; 5 min, 35% B; 13 min, 35% B; 15 min, 100% B; 30 min, 100% B. Flow rate was set at 1 mL/min with sample volume of 10 µL. Retention time and maximum UV (λ_{max}) of clear peaks were used to confirm the presence of rutin and K3R. The concentration of the compounds was then determined according to the peak area of UV absorbances of the external standard at 356 nm and the standard curve plot. The average percentage of rutin and K3R were calculated as follow:

$$\text{Average percentage (\%)} = \frac{\text{Average compound concentration } (\frac{\text{mg}}{\text{ml}})}{\text{Extract concentration } (\frac{\text{mg}}{\text{ml}})} \times 100 \quad (5)$$

STATISTICAL ANALYSIS

All analyses (chemical and bioactivities) were tested for three replication (n = 3), except for the quantification of rutin and kaempferol-3-O-rutinoside which was carried out in duplicates. All data were reported as mean ± standard deviation. Data were analysed through one-way ANOVA using SPSS 22.0. Duncan test was used to analysed the difference between mean value at significant difference of $p<0.05$ and Pearson was used to determine the correlation between analysis.

RESULTS AND DISCUSSION

PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITIES OF *C. asiatica* EXTRACTS

Table 1 shows that *C. asiatica* leaves dried using the oven drying method and extracted with 100% ethanol (OD100) had significantly higher ($p<0.05$) phenolic content (25.15 ± 0.87 mg GAE/g) compared to the other extracts. According to Minatel et al. (2017), thermal treatment can deactivate the activity of the polyphenol oxidase (PPO) enzyme. The PPO enzyme is a thermolabile compound that catalyzes the oxidation of phenolic compounds to *o*-quinone, thereby affecting the stability and concentration of phenolic in the extract. The enzymatic activity of PPO was previously reported to decrease at 40 °C and to be completely deactivated at 60 °C (Boonkorn 2016).

A previous study by Zahwal et al. (2018) also reported that *Melastoma matabathricum* extracted with 100% ethanol had significantly higher ($p<0.05$) phenolic content than those extracted with 50% ethanol and 100% water. Similarly, Do et al. (2014) reported higher phenolic content in *Limnophila aromatica* extracted with 100% ethanol compared to extraction using 70%, 50%, and 0% ethanol. Ethanol is a polar solvent with a negative charge at its hydroxyl group (−OH) and positive charge at its ethyl group ($-CH_2CH_3$) (Suharni, Indarto & Cilmiaty 2021; Zhao et al. 2019). The presence of these groups allows ethanol to form hydrogen bonds with phenolic compounds and effectively extract phenolics of varying polarities compared to water. Additionally, ethanol evaporates more easily due to its low boiling point and thereby allowing the targeted compounds to be concentrated more efficiently (Sabli et al. 2019).

The current study also reported significantly higher ($p<0.05$) flavonoid content (169.48 ± 9.99 mg QE/g) in the OD100 extract compared to the other extracts (Table 1). Thermal treatment at 40 °C can reduce the enzymatic activity of PPO, preventing the degradation of flavonoid compounds in the extract (Boonkorn 2016). This factor may have contributed to the higher retention of flavonoids in the oven-dried samples compared to the freeze-dried sample in this study. Do et al. (2014) also reported higher flavonoids content in the ethanol extract of *L. aromatica* compared to the water extract. *L. aromatica* contains aglycones and glycosides of flavone, which have similar polarity to the flavonols found in *C. asiatica* (Gorai et al. 2014). Quercetin-3-*O*-rhamnoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside were previously identified as flavonol glycosides in *C. asiatica* extract (Mohd Shukri, Alan & Site Noorzuraini 2011).

TABLE 1. Total phenolic content (TPC), total flavonoid content (TFC), vitamin C, DPPH free radical scavenging activity (IC_{50}) and ferric reducing antioxidant power (FRAP) of *C. asiatica* leaves extracts

Drying method	Ethanol: water	TPC (mg GAE/g)	TFC (mg QE/g)	Vitamin C (mg/g)	DPPH IC_{50} (mg/mL)	FRAP (mg TE/g)
Oven drying (OD)	0:100	15.74 ± 1.61^{bcd}	36.56 ± 1.82^{e}	67.03 ± 8.32^{e}	3.07 ± 0.35^{a}	34.12 ± 2.41^{cde}
	20:80	10.71 ± 1.53^{e}	41.83 ± 1.73^{de}	75.71 ± 5.48^{e}	ND	16.96 ± 2.94^{f}
	40:60	11.36 ± 2.14^{de}	32.51 ± 0.59^{ef}	94.80 ± 12.09^{d}	2.34 ± 0.99^{abc}	22.99 ± 5.67^{ef}
	60:40	12.74 ± 1.87^{de}	32.61 ± 1.39^{ef}	109.33 ± 14.74^{cd}	2.42 ± 0.36^{ab}	35.32 ± 15.26^{cde}
	80:20	20.02 ± 2.29^{b}	51.25 ± 3.75^{d}	152.24 ± 18.19^{b}	0.59 ± 0.12^{e}	62.51 ± 10.25^{b}
	100:0	25.15 ± 0.87^{a}	169.48 ± 9.99^{a}	179.85 ± 7.44^{a}	0.62 ± 0.06^{e}	81.57 ± 3.36^{a}
Freeze drying (FD)	0:100	17.04 ± 1.85^{bc}	41.74 ± 0.28^{de}	64.02 ± 7.48^{e}	2.12 ± 0.70^{bc}	31.06 ± 2.02^{def}
	20:80	12.42 ± 3.77^{de}	22.07 ± 3.58^{fg}	66.04 ± 2.92^{e}	ND	21.15 ± 12.31^{ef}
	40:60	12.47 ± 3.12^{de}	31.86 ± 5.39^{ef}	71.60 ± 10.07^{e}	2.27 ± 0.28^{abc}	21.29 ± 6.88^{ef}
	60:40	14.31 ± 1.47^{cde}	14.63 ± 1.02^{g}	94.34 ± 14.02^{d}	1.52 ± 0.51^{cd}	34.98 ± 6.59^{cde}
	80:20	17.67 ± 1.31^{bc}	86.17 ± 4.10^{c}	122.18 ± 3.51^{c}	0.95 ± 0.04^{ed}	44.34 ± 7.75^{cd}
	100:0	18.37 ± 4.02^{bc}	155.64 ± 21.02^{b}	142.43 ± 4.17^{b}	1.00 ± 0.37^{ed}	48.27 ± 13.75^{bc}

Values showed mean ± standard deviation, mean (n = 3).

ND: not determined.

[a-g] Mean values with different alphabet showed there was a significant difference ($p<0.05$) between extracts with different drying methods and solvent polarities of ethanol and water for each parameter (same column)

Flavonoids are generally non-polar compounds, but the presence of a sugar moiety on the aglycone backbone of flavonols increases the polarity of the compound (Dabeek et al. 2019; Rodríguez De Luna, Ramírez-Garza & Serna Saldívar 2020). The solubility of flavonoids depends on their ability to form hydrogen bonds with the solvent (Rodríguez De Luna, Ramírez-Garza & Serna Saldívar 2020). Therefore, ethanol with its medium polarity (0.654) and ability to donate hydrogen for hydrogen bond formation, was a more effective solvent than water for extracting flavonol glycosides from *C. asiatica* (Awouafack, Tane & Morita 2017; Reichardt & Welton 2010).

Table 1 also shows that the OD100 extract contained a higher vitamin C content (179.85 ± 7.44 mg/g) than the other extracts. Vitamin C is a labile organic compound with antioxidant properties that can inhibit lipid oxidation in food products (Tiwari, Brunton & Brennan 2013). A previous study by Mphahlele et al. (2016) also reported that the oven-dried (40 °C) *Punica granatum* extract prepared with 80% ethanol had significantly higher ($p<0.05$) vitamin C content than the freeze-dried sample. This is because thermal treatment during oven drying can inhibit the activity of enzymes such as ascorbate oxidase, cytochrome oxidase and peroxidase which are responsible for the degradation of vitamin C in the plant extracts (Giannakourou & Toukis 2021). Vitamin C is a cyclic polar molecule containing four hydroxyl groups and two hydrophilic oxygen atoms (Galvão et al. 2018; Neto et al. 2010). The dipole interactions of ethanol, which enable hydrogen bond formation, contributed to the effective extraction of vitamin C from *C. asiatica* (Rahman et al. 2013).

The antioxidant activities of *C. asiatica* extracts are also reported in Table 1. The DPPH free radical scavenging activity is expressed as the IC_{50} value. The IC_{50} value refers to the concentration of an extract required to inhibit or scavenge 50% of DPPH radical activity, determined through linear regression interpolation (Mohd Shukri et al. 2013). The current study showed that oven-dried samples extracted with 80% and 100% ethanol (OD80 and OD100) had significantly higher ($p<0.05$) DPPH scavenging activity (IC_{50}: 0.59 - 0.62 mg/mL) than the other extracts, except for the freeze-dried samples with similar solvent polarities (FD80 and FD100). The OD extracts demonstrated higher DPPH radical scavenging activity compared to the FD extracts, potentially due to the thermal treatment applied to OD, which may have inactivated polyphenol oxidase (PPO). Inactivation of this enzyme helps preserve phenolic compounds, which play a key role in antioxidant capacity and DPPH scavenging activity (Minatel et al. 2017). The presence of hydroxyl groups enables these compounds to donate hydrogen atoms to scavenge DPPH radicals. Consequently, the radical scavenging activity increases with the number of hydroxyl groups present in the compounds (Okon et al. 2020). Meanwhile, 20% ethanol extracts from both OD and FD samples failed to achieve 50% inhibition of DPPH activity, even at the maximum concentration of 5 mg/mL, and were therefore marked as not determined (ND).

The ferric reducing antioxidant power (FRAP) is determined based on the ability of antioxidant compounds to donate electrons for the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Desai & Gaikwad 2014). The OD80 and OD100 extracts (80% and 100% ethanol) recorded significantly higher FRAP values ($p<0.05$) compared to the FD80 and FD100 extracts (80% and 100% ethanol), respectively. Thermal treatment (oven drying) can deactivate the activities of enzymes (such as PPO, ascorbate oxidase, cytochrome oxidase and peroxidase) which may affect the stability and concentration of the phenolic, flavonoid and vitamin C (Giannakourou & Toukis 2021; Minatel et al. 2017). According to Siti Nur Aishah et al. (2020), phenolic and flavonoid compounds possess hydroxyl groups that contribute to the reducing activity of plant extracts. Vitamin C, a well-known reducing agent, can neutralise reactive free radicals by donating electrons and is subsequently oxidised to dehydroascorbic acid. Vitamin C capable of donating two electrons consecutively from the double bond between carbon atoms two and three (Padayatty & Levine 2016).

A previous study also reported that the 100% ethanol extract of *M. malabathricum* had significantly higher ($p<0.05$) reducing power than the 50% ethanol and 100% water extracts (Zahwal *et al.* 2018). As mentioned by Do *et al.* (2014) and Ho *et al.* (2018), the lower reducing power observed in the water extract compared to the ethanol extract may be attributed to the extraction of phenolic compounds with less reactive proton-donating ability. Ethanol is a polar solvent with hydroxyl groups that are able to form hydrogen bonds with antioxidant compounds such as phenolics and flavonoids (Rodríguez De Luna, Ramírez-Garza & Serna Saldívar 2020; Suharni, Indarto & Cilmiaty 2021). Previous studies have reported that phenolics, flavonoids, alkaloids, carotenoids and vitamin C contribute to the antioxidant activity of *C. asiatica* extract (Mohd Shukri 2008; Mohd Shukri et al. 2013).

The flavonoid kaempferol-3-*O*-rutinoside and the alkaloid magnoflorine have previously been identified in *C. asiatica* extracts (Mohd Shukri, Alan & Site Noorzuraini 2011; Wagner et al. 1983). Previous studies have reported that kaempferol-3-*O*-rutinoside and magnoflorine, isolated from plant extracts, exhibit DPPH radical scavenging activities (Juan-Badaturuge, Habtemariam & Thomas 2011; Naseer et al. 2015). According to Lv et al. (2020) and Okon et al. (2020), the presence of hydroxyl groups in kaempferol-3-*O*-rutinoside and magnoflorine enables these compounds to effectively scavenge DPPH radicals by donating hydrogen atoms to the free radicals. Similarly, vitamin C also able to act as an antioxidant or free radical scavenger by donating electrons (Padayatty & Levine 2016).

Pearson correlation analysis shows that the phenolic, flavonoid and vitamin C content of *C. asiatica* extracts, obtained through both oven and freeze drying, were significantly correlated with the reducing power of the extracts (Table 2). Phenolic compounds, flavonoids, and vitamin C are among the bioactive compounds in the extract responsible for the reduction of Fe^{3+} to Fe^{2+} and are capable of acting as antioxidants (Somdee et al. 2016). Similarly, previous studies have reported a positive correlation ($p<0.001$) between the phenolic and reducing power of *C. asiatica* extracts (Mohd Shukri, Alan & Site Noorzuraini 2011). Significant correlations were also observed between phenolic, flavonoid, and vitamin C content of oven-dried *C. asiatica* extracts and their DPPH scavenging activities. In contrast, for freeze-dried *C. asiatica* extract, only phenolic and vitamin C content showed significantly correlations with DPPH scavenging activity. Oven drying, which typically involves moderate heat ranging from 40 to 60 °C, can lead to the breakdown of cell walls and the subsequent release of bound phenolic compounds, thereby increasing their extractability (Li et al. 2022). In addition, the inactivation of enzymes through thermal treatment (oven drying) helps preserve phenolic compounds that are important for antioxidant capacity and DPPH scavenging activity by deactivating enzymes (such as PPO, ascorbate oxidase) which may reduce the stability and concentration of phenolics, flavonoids and vitamin C (Giannakourou & Toukis 2021; Minatel et al. 2017). In contrast, freeze-drying (lyophilization) preserves the native structure and retains water-soluble compounds, but it may be less effective in releasing bound antioxidants (Li et al. 2022).

TABLE 2. Pearson correlation of total phenolic content (TPC), total flavonoid content (TFC) and vitamin C with DPPH free radical scavenging activity and ferric reducing antioxidant power (FRAP) of *C. asiatica* leaves extracts

Drying method	TPC		TFC		Vitamin C	
	DPPH	FRAP	DPPH	FRAP	DPPH	FRAP
Oven drying (OD)	0.745**	0.951**	0.504**	0.785**	0.810**	0.910**
Freeze drying (FD)	0.779**	0.899**	0.241	0.693*	0.474*	0.759**

Data showed r values from Pearson correlation analysis.

*Correlation was significant at $p<0.05$; ** correlation was significant at $p<0.01$

UHPLC-QTOF-MS AND HPLC-PDA ANALYSES OF OD100 AND FD100 EXTRACTS

The oven-dried (OD) and freeze-dried (FD) extracts obtained using 100% ethanol were selected for further profiling due to their notably high concentrations of phenolic compounds, flavonoids, vitamin C, and strong antioxidant activity as determined by DPPH and FRAP assays. Phytochemical profiling of *C. asiatica* leaves by UHPLC-QTOF-MS using MS/MS data acquired in high-definition MS^E ($HDMS^E$) mode identified a total of 21 and 19 compounds in the oven-dried and freeze-dried ethanol extracts, respectively. The identified compounds with accuracy errors below 4 ppm were classified into chemical families, and the parameters supporting their identification are presented in Table 3. Flavonoids were the major compounds identified in both extracts, followed

TABLE 3. Phytochemicals of 100% ethanol oven-dried (OD100) and 100% ethanol freeze-dried (FD100) extracts identified using UHPLC-QTOF-MS $[M-H]^-$

Compound	Formula	Extract	t_R (min)	m/z $[M-H]^-$	Fragment ions (m/z)	References
Phenolic acid						
Quinic acid	$C_7H_{12}O_6$	OD100	0.47	191.0565	93.0350, 111.0462, 127.0406, 173.0451	(Aabideen et al. 2020)
		FD100	0.48	191.0563	93.0342, 127.0412, 173.0460	
o-Coumaric acid	$C_9H_8O_3$	OD1000	4.91	163.0405	119.0508	(MassBank of North America, MoNA n.d.)
		FD100	4.93	163.0405	119.0508	
Elemicin	$C_{12}H_{16}O_3$	FD100	9.83	207.1032	135.0815, 163.1134, 181.1238	(Lei et al. 2018; Pandey et al. 2016)
Flavonoid						
Vaccarin	$C_{32}H_{38}O_{19}$	FD100	8.41	725.1936	605.1509	(Qi et al. 2012)
Rutin	$C_{27}H_{30}O_{16}$	OD100	8.47	609.147	151.0042, 300.0279, 301.0349, 343.0464	(MassBank Europe n.d.; MoNA n.d.; Wang et al. 2017)
		FD100	8.5	609.1473	151.0043, 300.0279, 301.0350, 343.0468	
6-Hydroxykaempferol-3-O-glucoside	$C_{21}H_{20}O_{12}$	OD100	8.66	463.0885	300.0278, 301.0349	(Tang et al. 2016)
		FD100	8.69	463.0884	300.0279, 301.0348	
Genistein-7,4'-di-O-β-D-glucoside	$C_{27}H_{30}O_{15}$	FD100	9.01	593.1516	284.0332	(Salahuddin et al. 2020)
Quercetin-3-O-α-L-arabinopyranoside	$C_{20}H_{18}O_{11}$	OD100	9.19	433.0781	243.1238, 300.0282, 301.0343	(Okonkwo et al. 2016)
		FD100	9.22	433.0783	243.1238, 300.0282, 301.0343	
Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	OD100	9.42	593.153	151.0042, 227.0354, 255.0301, 257.0453, 284.0328, 285.0407, 286.0439	(MoNA n.d.; National Institute of Standards and Technology, NIST 2014)
		FD100	9.45	593.1531	151.0043, 227.0353, 284.0330, 285.0409, 286.0441	
Quersetin-3-O-rhamnoside	$C_{21}H_{20}O_{11}$	OD100	9.62	447.0934	151.0046, 227.0352, 255.0302, 256.0348	(MoNA n.d.)
		FD100	9.65	447.0940	151.0042, 227.0354, 255.0302, 256.0349	
		FD100	9.73	623.1622	271.0255, 315.0516	

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Kaempferol-3-O-α-L-arabinoside	$C_{20}H_{18}O_{10}$	OD100	9.95	417.0828	285.0396	(Qing et al. 2011)
		FD100	9.97	417.0830	285.0399	
Epimedin C	$C_{39}H_{50}O_{19}$	FD100	10.44	821.2870	659.235	(Wang et al. 2010; Zhu et al. 2017)
Rhamnazin	$C_{17}H_{14}O_7$	OD100	10.73	329.0675	299.0596	(Handoussa, AbdAllah & AbdelMohsen 2019)
Nevadensin-7-O-[α-L-rhamnosyl(1→6)]-β-D-glucoside	$C_{30}H_{36}O_{16}$	OD100	11.1	651.1929	607.1609	(Kumar et al. 2021)
		FD100	11.13	651.1939	607.161	
Kaempferol-3-O-rhamnoside	$C_{21}H_{20}O_{10}$	OD100	11.15	431.0983	284.0327, 285.0393	(MoNA n.d.)
Quercetin	$C_{15}H_{10}O_7$	OD100	11.96	301.0356	151.0037, 178.9967, 273.0386, 299.0195	(MoNA n.d.)
		FD100	12	301.0362	151.0041, 178.9971, 257.0446, 273.0408	
Kaempferol	$C_{15}H_{10}O_6$	OD100	13.88	285.0407	93.0352, 151.0043, 159.0459, 229.0508, 255.0299	(MassBank Europe n.d.; MoNA n.d.; Wang et al. 2017)
		FD100	13.92	285.0406	93.0353, 151.0034, 227.0353, 239.0353, 255.0302	
Alkaloid						
Magnoflorine	$C_{20}H_{23}NO_4$	OD100	6.47	340.1561	282.1142, 297.1304	(MoNA n.d.)
		FD100	6.5	340.1560	282.1139	
Terpenoid						
Eucommiol	$C_9H_{16}O_4$	OD100	9.34	187.0979	125.0974	(He et al. 2018; Pi et al. 2016)
		FD100	9.36	187.0981	95.0505, 123.0815, 125.0975, 169.0872	
Albiflorin	$C_{23}H_{28}O_{11}$	OD100	14.66	479.158	283.1074	(Yang et al. 2021)
Albiflorin R1	$C_{23}H_{28}O_{11}$	OD100	14.9	479.1575	283.0244	(Li et al. 2009)
Saponin						
Ginsenoside Rg1	$C_{42}H_{72}O_{14}$	OD100	13.39	799.4845	637.4317	(Choe et al. 2020; Zhang et al. 2020)
Ecliptasaponin C	$C_{42}H_{68}O_{14}$	OD100	14.85	795.4531	633.3998	(Fu et al. 2019; Han et al. 2015)
Yesanchinoside B	$C_{48}H_{82}O_{20}$	FD100	11.32	977.5321	815.4795	(Zou et al. 2002)
Notoginsenoside G	$C_{48}H_{80}O_{19}$	OD100	13.85	959.5202	931.4877	(Zhang et al. 2020)
Asiaticoside	$C_{48}H_{78}O_{19}$	OD100	15.3	957.5059	323.0993, 485.1533	(MoNA, n.d.; MassBank Europe, n.d.; Liu et al. 2010)

by saponin, phenolic acid, terpenoid, and alkaloid. Quinic acid and o-coumaric acid were the phenolic acids found in both *C. asiatica* extracts and, have not been previously reported in the literature. According to Cinkilic et al. (2013) and Karaman et al. (2019), quinic acid exhibits strong antioxidant capacity due to its hydroxyl groups, which enable it to scavenge reactive oxygen species. Meanwhile, o-coumaric acid was previously reported to have a significant ($p<0.01$) correlation with antioxidant activities of *Eriobotyra japonica* (Xu, Li & Chen 2014).

Table 3 shows the presence of rutin, quercetin-3-O-rhamnoside (Q3R) and kaempferol-3-O-rutinoside (K3R) in both *C. asiatica* extracts, consistent with previous chromatographic studies on *C. asiatica* (Lee, Chen & Chen 2000; Mohd Shukri, Alan & Site Noorzuraini 2011). According to Liana et al. (2019), K3R is a bioactive with antioxidant, antimicrobial, anti-inflammatory and anticancer properties. Previous study reported that K3R, isolated from *Cassia auriculata,* was able to scavenge free radicals and inhibit lipid oxidation (Juan-Badaturuge, Habtemariam & Thomas 2011). Similarly, Q3R and rutin were also previously reported with significant ($p<0.05$) ROS and lipid oxidation inhibition activities (Cui et al. 2022; Jiménez-Aliaga et al. 2011). According to Siti Sarwani, Abdulwali and Jamaludin (2018), the presence of hydroxyl group at C-3' and C-4' positions of the B-ring in rutin, kaempferol and quercetin contributes to hydrogen donation and superoxide radical scavenging activities.

Magnoflorine was the alkaloid found in both *C. asiatica* extracts (Table 3). Magnoflorine was also previously identified in *C. asiatica* extract by Wagner et al. (1983). Okon et al. (2020) reported that magnoflorine, isolated from the plant sources, exhibited significant antioxidant activity and is capable of inhibiting lipid oxidation. Its antioxidant potential is attributed to the presence of two hydroxyl groups, which contribute to the phenolic hydrogen donation, thereby disrupting the lipid oxidation chain reaction (Okon et al. 2020). Table 3 also shows the presence of terpenoid albiflorin in the OD100 extract. According to Sun et al. (2022), albiflorin demonstrated stronger antioxidant activity than quercetin and exerted protective effects against oxidative stress-induced toxicity. Similar to magnoflorine, albiflorin contains two hydroxyl groups that enable it to donate hydrogen atoms to neutralise free radicals (Zhao et al. 2018). Asiaticoside, one of the saponins detected in the OD100 extract, has been reported to possess strong free radical scavenging capacity and reducing power (Bandopadhyay et al. 2023).

Phytochemical profiling of *C. asiatica* extracts showed the presence of compounds with significant antioxidant capacity, as supported by previous cited studies. Therefore, further comprehensive investigations focusing on the isolation, purification, characterization and quantification of these compounds would be valuable for elucidating their roles in the antioxidant activity of *C. asiatica* extracts. In this study, the content of rutin and K3R in the OD100 and FD100 extracts were quantified using HPLC-PDA. As shown in Figure 2, the retention times (t_R) of rutin and K3R were 8.281 and 10.871, respectively, in the OD100 extract and 8.491 and 10.839 min, respectively, in the FD100 extract. Figure 1 also illustrates the maximum UV (λ_{max}) obtained for rutin (OD100: 255.9 nm and 355.7 nm; FD100: 264.2 nm and 347.3 nm) and kaempferol-3-*O*-rutinoside (OD100: 254.7 nm and 352.1 nm; FD100: 264.2 nm and 346.1 nm) in both extracts.

The results showed that both rutin and K3R concentrations were higher in the FD100 extract (rutin: 51.06 µg/mL; K3R: 144.73 µg/mL) than in the OD100 extract (rutin: 17.22 µg/mL; K3R: 118.43 µg/mL) (Table 4). A previous study by Mphahlele et al. (2016) similarly reported higher rutin content in the freeze-dried sample of *Punica granatum* compared to oven-dried

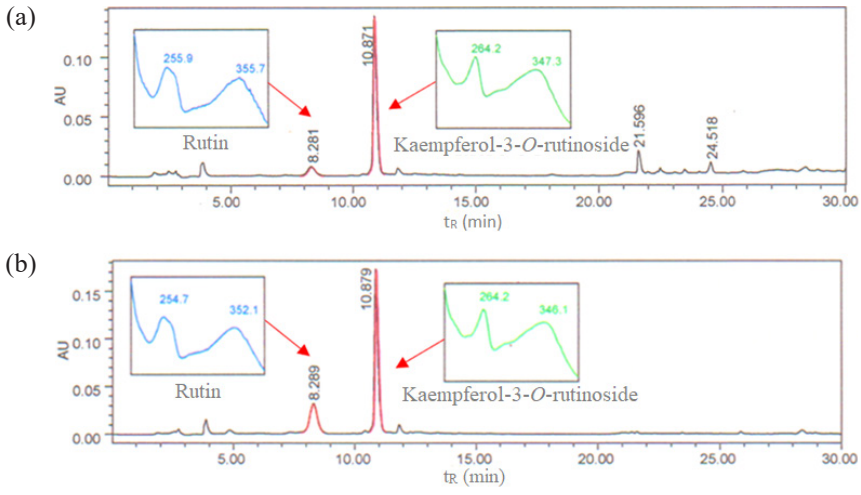

FIGURE 2. HPLC chromatogram of (a) oven-dried, 100% ethanol extract (OD100) and (b) freeze-dried, 100% ethanol extract (FD100)

TABLE 4. Total rutin and kaempferol-3-O-rutinoside (K3R) of 100% ethanol oven dried (OD100) and freeze-dried (FD100) extracts using HPLC-PDA

Extract	Rutin		Kaempferol-3-O-rutinoside	
	Average concentration (µg/mL)	Average percentage (% w/w)	Average concentration (µg/mL)	Average percentage (% w/w)
OD100	17.22 ± 3.08	0.17 ± 3.08	118.43 ± 0.33	1.18 ± 0.33
FD100	51.06 ± 2.12	1.28 ± 2.12	144.73 ± 0.74	3.62 ± 0.74

Values showed mean ± standard deviation, mean (n = 2)

(40 °C) sample. According to Chaaban et al. (2016), the stability and biological activity of flavonoids, such as rutin can be affected by temperature, with greater degradation occuring at higher thermal intensities and longer exposure. The thermal degradation of flavonoids is influenced by their molecular structure. Therefore, K3R which has a structure similar to that of rutin but with an additional hydroxyl group, is expected to exhibit a similar degradation trend (Chaaban et al. 2016; Lv et al. 2020). These findings indicate that freeze drying results in higher retention of rutin and K3R than oven drying in 100% ethanol *C. asiatica* extract.

CONCLUSIONS

This study demonstrated that oven-dried *C. asiatica* leaves extracted with 100% ethanol (OD100) had significantly higher phenolics, flavonoids, vitamin C and reducing power compared to the other extracts. Phytochemicals profiling of *C. asiatica* extracts using UHPLC-QTOF-MS successfully identified various antioxidant compounds, while HPLC-PDA analysis showed that the freeze drying method resulted in higher concentration of rutin and kaempferol-3-O-rutinoside in 100% ethanol *C. asiatica* extract compared to the oven drying method.

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