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Selection and optimisation of extraction technique for the preparation of phenolic- and flavonoid-rich extract of leafy vegetable, *Coccinia grandis* (Linn.) Voigt

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Abstract

Coccinia grandis (L.) Voigt (family: Cucurbitaceae) is a popular leafy vegetable in Sri Lankan diet. *C. grandis* is high in phenolics and flavonoids. The present work attempted to determine a suitable extraction technique, and further optimise it to obtain phenolic- and flavonoid-rich extract from *C. grandis* leaves, with an aim at developing a nutraceutical targeting the dietary management of diabetes mellitus. Acetone extraction (AE), methanol extraction (ME), pre-warmed water extraction (PWE), electric shake extraction (ESE), reflux extraction (RE), ultrasonication with water (UE_w), ultrasonication with ethanol (UE_e), ultrasonic assisted-reflux extraction (URE), and reflux assisted-ultrasonic extraction (RUE) were chosen as the extraction techniques. URE was selected as a satisfactory extraction technique for further optimisation for the preparation of phenolic- and flavonoid-rich extract based on the contents of phenolics (32.97 ± 0.41 mg of equivalent gallic acid/g of extract) and flavonoids (4.50 ± 0.04 mg equivalent quercetin/g of extract). The highest yield of 32.8% was obtained by the URE technique. The optimal extraction conditions for URE were determined with an ultrasonic time of 19 min, refluxing time of 168 min, and liquid:solid ratio of 16.4 mL/g. This is the first attempt to investigate the selection and optimisation of an extraction technique for obtaining phenolic- and flavonoid-rich extract from *C. grandis* leaves. The present findings would be useful in the development of a commercially viable nutraceutical using a phenolic- and flavonoid-rich extract of *C. grandis.*

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Introduction

Coccinia grandis (L.) Voigt (family: Cucurbitaceae; English name: ivy gourd) is a leafy vegetable native to Asia, and can be found in India, Malaysia, and Sri Lanka (Jayaweera, 1982; Satyavati *et al.*, 1987). It is a perennial climber with slender, cylindrical, and glabrous stems with simple tendrils. The leaves are plain, alternate, and have five lobes. The length and width of a leaf are approximately 5 - 10 and 4.5 - 9 cm, respectively. Flowers are regular and unisexual. Fruits are small and fusiform-ovoid or cylindrical in shape. Traditional Sri Lankan and Ayurvedic medicine use the leaves to treat a variety of diseases including diabetes mellitus, urinary tract infections, bronchitis, itchy skin rashes, and ulcers (Jayaweera, 1982). Apart from its therapeutic applications, people in Asian countries have also prepared salads from the tender leaves of *C. grandis* mixed with grated coconut and consumed with or without cooking. *C. grandis* fruits have been shown in scientific studies to have antioxidant properties (Meenatchi *et al.*, 2017; Hegazy *et al.*, 2019). Furthermore, the leaves of *C. grandis* have been shown to have a wide range of bioactivities such as antihyperlipidemic, antibacterial, anti-inflammatory, analgesic, antipyretic, hepatoprotective, antidiabetic activities, and as a potential immunomodulator against visceral leishmaniasis (Tamilselvan *et al*., 2011). In fact, *C. grandis* could be used as a dietary supplement in the management of patients with impaired glucose tolerance and/or mild hyperglycaemic state, and majority of the bioactivities have been studied using leaves of the plant (Munasinghe *et al*., 2011; Wasana *et al.*, 2021). In Wistar rats, the *in vivo* safety of *C. grandis* leaves has been scientifically proven (Attanayake *et al.*, 2013). Previously, the results of chemical

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standardisation of *C. grandis* grown in Sri Lanka and India were published (Attanayake *et al.*, 2016; Kaviya and Shukla, 2019). The ash content, moisture content, heavy metal analysis, thin layer chromatography (TLC), and high-performance liquid chromatography (HPLC) fingerprints could serve as reference standards in the quality control assessment of *C. grandis* grown in Sri Lanka, and could be differentiated from *C. grandis* grown in other countries (Attanayake *et al.*, 2016). *C. grandis* leaves have also been suggested as a potential source of natural phenolics and flavonoids for the development of commercially viable nutraceuticals (Attanayake *et al.*, 2016). According to Indian researchers, the water extraction of *C. grandis* yielded the highest extractive value (Kaviya and Shukla, 2019).

A plant's bioactivities are caused by the presence of bioactive phytoconstituents (Jain *et al.*, 2019; Ram Bindurani and Anoop, 2020). The isolated compounds of *C. grandis* have been purified and characterised using various chromatographic and spectral techniques, respectively. The ethanol, chloroform, and hexane fractions were thoroughly investigated using NMR spectral data which found that taxaterone and tinsoporin compounds A and B were present (Ram Bindurani and Anoop, 2020). Phenolics, among plant metabolites, show promise in regulating a variety of bioactivities such as antidiabetic, anti-inflammatory, antioxidant, and hepatoprotective (Mojzer *et al.*, 2016; Abbas *et al.*, 2017; Chen *et al.*, 2019). Phenolics are classified into four subgroups based on the presence of phenolic groups and structural elements; flavonoids, stilbenes, lignans, and phenolic acids (Manach *et al*., 2004; Abbas *et al*., 2017).

To date, several phenolic compounds have been isolated from the leaves of *C. grandis* including gallic acid, ferulic acid, methyl caffeate ligstroside, and trans-*p*-coumaric acid, and their antidiabetic activity has been scientifically proven through *in vitro* and *in vivo* studies (Gandhi *et al*., 2011; Narasimhan *et al*., 2015; Oboh *et al*., 2016; 2022; Al-Madhagy *et al*., 2019a; Siddiqua *et al*., 2021). Flavonoids with potent antidiabetic activity have been isolated from *C. grandis* including quercetin 3-*O*-neohesperidoside, kaempferol 3-*O*-rutinoside, kaempferol 3-O-β-D-glucoside, and rutin (Zanatta *et al*., 2008; Niture *et al*., 2014; Al-Madhagy *et al*., 2019b).

In general, the extraction technique could affect the yield and therapeutic efficacy of a plant extract. However, there is no standard extraction procedure for preparing phenolic- and flavonoid-rich extracts; thus, it is dependent on applications of interest, selectivity, and matrix characteristics of the plant (Stalikas, 2007). As a result, it is critical to investigate the optimal conditions in extraction techniques for effective utilisation of natural products, particularly for the plant of interest. In the present work, an attempt was made to determine the influence of various extraction techniques on the extraction efficiency and efficacy of phenolic compounds and flavonoids from *C. grandis* leaves. In addition, the selected extraction technique was further optimised.

Materials and methods

Chemicals and reagents

Gallic acid (CID: 370) and quercetin (CID: 5280343) were purchased from Sigma Chemical Co, USA. Chemicals and reagents were of analytical grade, and used without further processing.

Plant material

C. grandis leaves were collected during the flowering stage from the southern province of Sri Lanka (6°04'05"N, 80°13'35"E), and authenticated by comparing with the preserved specimens at the Herbarium, Botanical Gardens, Kandy, Sri Lanka. *C. grandis* leaves were washed and dried in an oven at 40°C for three days until a constant weight was reached. The water content of the plant material was calculated using weight loss after the oven drying process. The dry plant material was then pulverised.

A vibratory sieve shaker was used to determine the particle size of the plant material (Analysette 3 Spartan Apparatus, Fritsch, Germany). The powdered plant sample was filtered through several sieves with mesh sizes of 315, 212, and 180 µm. The powder retained on a 180 µm sieve plate was collected, packed, and stored at 4°C until the extracts were prepared.

Extraction methods for phenolics and flavonoids

Several extraction techniques were used to determine the best extraction method for phenolics and flavonoids. The methods were briefly described below, and all dried extracts obtained from each extraction technique were properly labelled and stored in an airtight container at 4°C. The yield was calculated as a percentage of the dry weight of the sample taken.

Acetone extraction (AE)

Plant powder (20 g) was added to a beaker containing 200 mL of acetone solution (70%, v/v), and incubated at 4°C for 90 min. The mixture was centrifuged at 3,500 rpm at 4°C for 20 min, and the supernatant was collected before the solvent was evaporated at 40°C, using a rotary evaporator (Marimoutou *et al.,* 2015).

Methanol extraction (ME)

In a flask, the plant powder (20 g) was weighed and soaked in 200 mL of methanol. The flask was capped and shaken for 30 min prior to incubation for 24 h. The extract was filtered and dried in a rotary evaporator at 40°C (Akolade *et al.,* 2019).

Pre-warmed water extraction (PWE)

The plant powder (20 g) was added to a flask containing 200 mL of pre-warmed (30°C) distilled water. The mixture was shaken in an incubator at 170 rpm with 30°C for 20 min, then filtered and dried in a vacuum oven at 40°C (Cuevas *et al.,* 2019).

Electric shake extraction (ESE)

The plant powder (20 g) was mixed with distilled water (200 mL), and shaken (50 - 60 Hz, 450 rpm) for 24 h using an electric shaker (Thermo Fisher, Scientific, USA). In a rotary evaporator, the extract was filtered and concentrated under reduced pressure. The resulting concentrate was then extracted for 4 h with absolute ethanol. The mixture was centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant was collected and evaporated under reduced pressure at 40°C using a rotary evaporator (Alabi *et al.,* 2018).

Reflux extraction (RE)

The plant powder (20 g) was added to a flask and refluxed with distilled water (200 mL) for 3 h. Then the resulting mixture was filtered and dried using a rotary evaporator at 40°C (Dhanani *et al.,* 2017).

Ultrasonic extraction (UE)

Ultrasonic extraction was carried out in an ultrasonic bath (Bandelin Sonorex, UK; inner dimension: 150 × 140 × 100 mm) with an ultrasound power of 240 W, and a frequency of 35 kHz.

Ultrasonic extraction was optimised in four ways: extraction with (a) distilled water and (b) ethanol (in order to select the best solvent), respectively, (c) reflux extraction followed by ultrasonic extraction, and (d) ultrasonic extraction followed by reflux extraction, using the most suitable solvent.

Ultrasonic extraction with water (UE_w)

Plant material (20 g) was mixed with distilled water (200 mL), and immersed in an ultrasonic bath at 60°C for 1 h. The resulting product was filtered and dried at 40°C in a rotary evaporator (Lameirão *et al.,* 2020).

Ultrasonic extraction with ethanol (UE_e)

Plant material (20 g) was added to a flask containing 200 mL of ethanol, and immersed in an ultrasonic bath at 60°C for 1 h. The resulting mixture was filtered and dried at 40°C in a rotary evaporator (Savic and Gajic, 2020).

Reflux-assisted ultrasonic extraction (RUE)

The plant powder (20 g) was added to a flask, refluxed with 200 mL of distilled water for 1 h, and ultra-sonicated at 60°C for another hour. The extract was filtered and dried at 40°C in a rotary evaporator.

Ultrasonic assisted-reflux extraction (URE)

The plant powder (20 g) was added to a flask containing 200 mL of distilled water, ultra-sonicated for 1 h, and refluxed for another hour at 60°C. The extract was filtered and dried at 40°C in a rotary evaporator.

Quantification of total polyphenol and total flavonoid content

The total phenolic content (TPC) and total flavonoid content (TFC) of each extract obtained using the aforementioned extraction techniques were determined. The TPC was calculated using the gallic acid calibration curve, and the results were expressed in terms of mg gallic acid equivalents/g of extract (Singleton *et al.,* 1999). The TFC was calculated using the quercetin calibration curve, and the results were expressed in mg of quercetin equivalent/g of extract (Meda *et al.,* 2005).

Optimisation of the best extraction technique

Among the extraction techniques mentioned above, URE was chosen to be optimised further in terms of (a) ultrasonication and refluxing time, and (b) liquid and solid ratios.

Effect of ultrasonication and refluxing time on ultrasonic assisted-reflux extraction (URE)

The following combinations were made to optimise the ultrasonication time (T_U) and refluxing time (T_R). First, the powdered material (20 g) was weighed in a flask, and ultrasonicated with 200 mL of distilled water for 15 min before being refluxed for another 180 min. The flask was capped and allowed to cool to room temperature (27°C), after which it was filtered and evaporated to dryness using a vacuum rotary evaporator at 40°C (T_U and T_R, 15:180). Using the same extraction procedure, the T_U and T_R ratios were varied as follows: 30:135, 45:90, and 60:45. As previously stated, TPC and TFC were determined for each variation. Graphically, the best T_U and T_R ratio was obtained [*x*-axis: time ratio (T_U/T_R), and *y*-axis: total phenolic content].

Effect of liquid and solid ratios on ultrasonic assisted-reflux extraction (URE)

All previous experiments maintained a liquid:solid ratio of 10:1 (mL/g). As a result, this ratio was varied as follows: 5:1, 10:1, 20:1, 30:1, and 40:1 (mL/g), and subjected to URE under optimised T_U and T_R. The TPC and TFC were determined for each variation as previously described. The best liquid:solid ratio was obtained graphically [*x*-axis: liquid/solid ratio, and *y*-axis: total phenolic content].

The TPC and TFC were calculated at the best T_U:T_R and liquid:solid ratios.

Statistical analysis

Origin Pro 8 statistical software was used to statistically analyse the data. All experiments were conducted in triplicate. The data were presented as mean ± SEM. Tukey's *post-hoc* HSD test ($p < 0.05$) was used to compare all statistical comparisons using one-way analysis of variance (ANOVA). Using Origin Pro 8, spline plots were constructed to show the effect of each input variable. Five different T_U:T_R ratios and five different liquid:solid ratios were considered as input variables in the estimation of TPC and TFC.

Results

C. grandis leaves had a water content of 6.3 ± 0.2%. The highest TPC was obtained using ultrasonic assisted-reflux extraction (URE) technique followed by reflux assisted-ultrasonic extraction (RUE), reflux extraction (RE), ultra-sonication with water (UE_w), ultra-sonication with ethanol (UE_e), both methanol extraction (ME) and electric shake extraction (ESE), pre-warmed water extraction (PWE), and acetone extraction (AE) (Table 1). Similarly, URE yielded the highest TFC. However, for flavonoid extraction from *C. grandis* leaves, RE outperformed RUE, and UE_w outperformed both UE_e and ESE. As shown in Table 1, the TFC obtained from UE_e and ESE were nearly similar, and gradually decreased with ME > PWE > AE (Table 1).

Table 1. Total phenol and total flavonoid contents of *Coccinia grandis* leaves obtained from different extraction techniques.

Extraction technique	Total phenolic content (mg of GAE/g of extract)	Total flavonoid content (mg of QE/g of extract)
AE	2.99 ± 0.19^{h}	0.92 ± 0.12^{h}
ME	25.08 ± 0.11^{f}	3.04 ± 0.03^{f}
PWE	23.10 ± 0.23^{g}	1.64 ± 0.14^{g}
ESE	24.87 ± 0.56^{f}	3.14 ± 0.19^{e}
RE	30.04 ± 0.06^{c}	4.44 ± 0.05^{b}
RUE	31.56 ± 0.17^{b}	4.36 ± 0.02^{c}
URE	32.97 ± 0.41^{a}	4.50 ± 0.04^{a}
UE_w	27.11 ± 0.23^{d}	4.02 ± 0.22^{d}
UE_e	26.38 ± 0.11^{e}	3.18 ± 0.04^{e}

Data are mean ± SEM, $n = 4$. Means followed by different lowercase superscripts in a column are significantly different ($p < 0.05$). AE: acetone extraction, ME: methanol extraction, PWE: pre-warmed water extraction, ESE: electric shake extraction, RE: reflux extraction, RUE: reflux assisted-ultrasonic extraction, URE: ultrasonic assisted-reflux extraction, UE_w: ultra-sonication with water, UE_e: ultra-sonication with ethanol, GAE: gallic acid equivalent, and QE: quercetin equivalent.

The yields were observed in descending order for URE (32.8%), RUE (31.3%), RE (26.0%), UE_w (23.6%), UE_e (18.7%), ME (18.0%), ESE (17.1%), PWE (10.1%), and AE (5.2%) with respect to the dry weight of the samples (Table 1). Reflux extraction was combined with ultrasonic extraction in two ways; RUE and URE. URE was further optimised by varying the ultrasonication time:refluxing time and liquid:solid ratios. The influence of ultrasonication and refluxing time on TPC and TFC was evaluated in a range of T_U and T_R of 0 - 60 and 45 - 240 min, respectively. The TPC and TFC increased over time up to a T_U:T_R of 19:168, and then dramatically decreased when the ratio between T_U and T_R was increased (Figures 1 and 2).

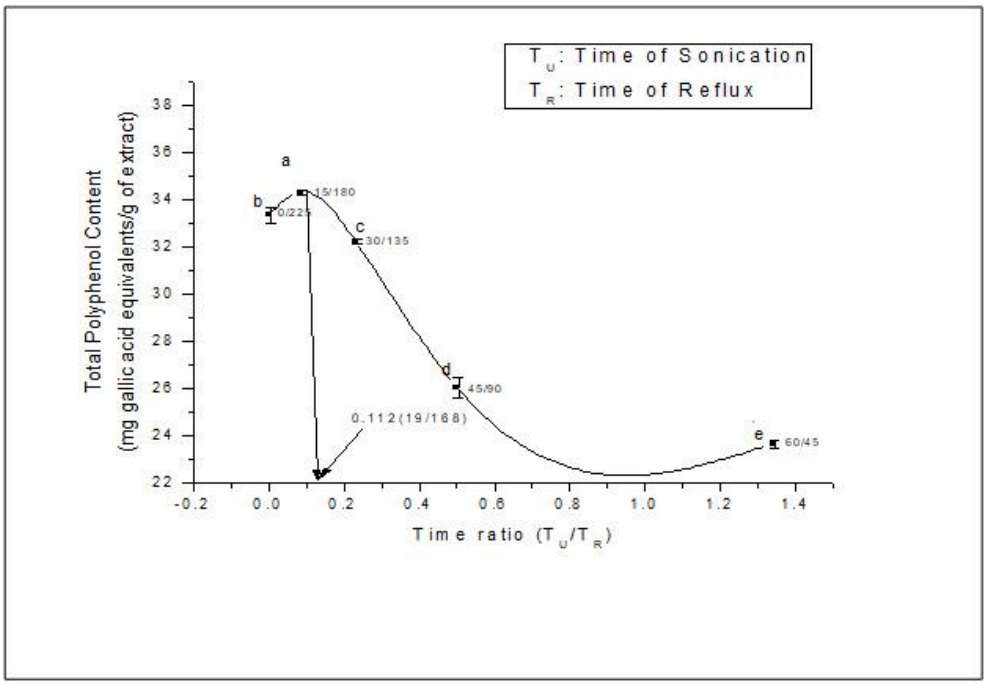

Figure 1. Variation of total phenolic contents of *Coccinia grandis* with different ultra-sonication time (TU) and refluxing time (TR) ratios in ultrasonic assisted-reflux extraction (URE).

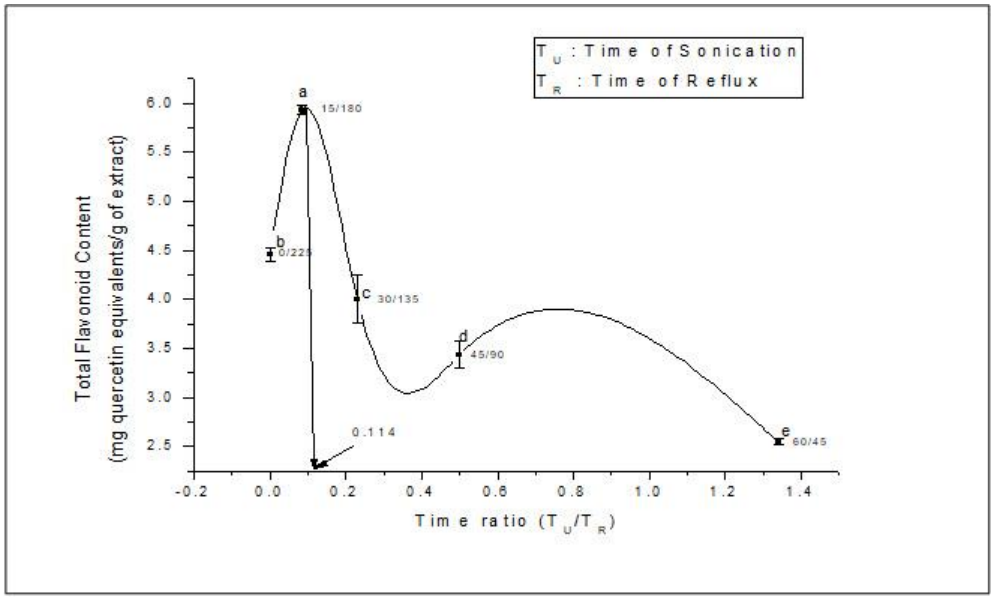

Figure 2. Variation of total flavonoid contents of *Coccinia grandis* with different ultra-sonication time (TU) and refluxing time (TR) ratios in ultrasonic assisted-reflux extraction (URE).

The maximum TPC and TFC were obtained by varying the liquid:solid ratio from 5 to 16.4 mL/g. Furthermore, the TPC and TFC in the liquid did not increase, which was 16.4 mL/g solid ratio (Figures 3 and 4). Therefore, a volume-to-solid ratio of 16.4 mL/g was chosen as the optimal ratio for extracting phenolics and flavonoids from *C. grandis* leaves. The liquid-solid ratio in the extraction process can affect the degree of interaction between the solid and the solvent, thereby affecting extraction efficacy. The best efficacy is achieved when the solution reaches saturation, and the efficacy decreases dramatically beyond the optimised liquid:solid ratio.

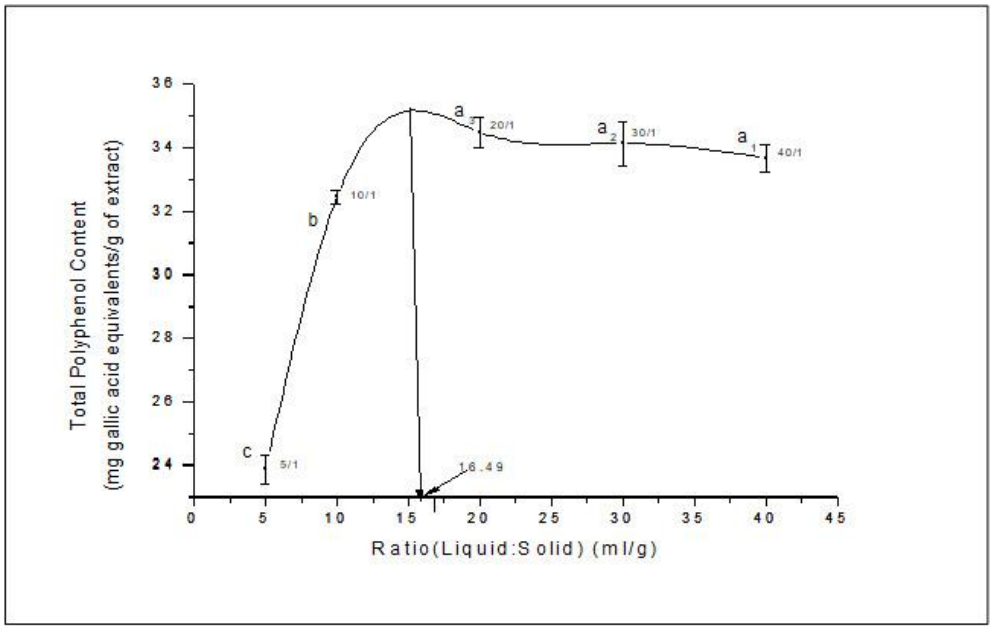

Figure 3. Effect of liquid:solid ratios on total phenolic contents in ultrasonic assisted-reflux extraction (URE).

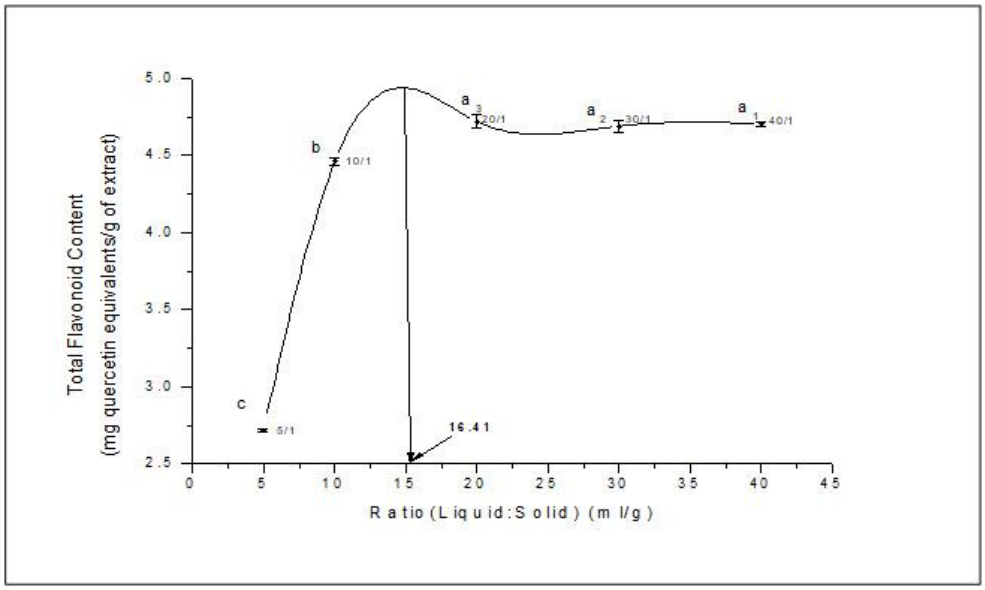

Figure 4. Effect of liquid:solid ratios on total flavonoid contents in ultrasonic assisted-reflux extraction (URE).

At the best T_U:T_R ratio, the TPC and TFC were experimentally estimated as 34.92 ± 0.56 mg of GAE/g of extract and 5.70 ± 0.04 mg of QE/g of extract, respectively. At the best liquid:solid ratio, the TPC and TFC were estimated as 35.71 ± 0.42 mg of GAE/g of extract and 4.92 ± 0.02 mg of QE/g of extract, respectively.

Graphically, the TPC and TFC were 34.50 mg of GAE/g of extract and 5.58 mg of QE/g of extract, respectively, at the best T_U and T_R ratio (Figures 1 and 2). The TPC and TFC were 35.10 mg of GAE/g of extract and 4.58 mg of QE/g of extract, respectively, at the best liquid:solid ratio (Figures 3 and 4). Accordingly, the methodologically obtained TPC and TFC in the best T_U:T_R and liquid:solid ratios were comparable to the graphical output.

Discussion

Phenolic and flavonoid compounds are phytoconstituents/secondary metabolites that contain at least one hydroxyl group on an aromatic ring (Dopico-García *et al.*, 2008; Kumar and Pandey, 2013). Phenolics and flavonoids from medicinal plants have recently gained popularity due to their numerous health benefits (Scalbert *et al.*, 2005; Rasouli *et al.*, 2017; Taamalli *et al.*, 2019). The present work attempted to select an extraction technique that yielded high phenolic and flavonoid contents from *C. grandis*.

Methanol, ethanol, acetone, ethyl acetate, and other solvents are commonly used for phenolic and flavonoid extraction. In fact, ethanol-water, methanol-water, and acetone-water mixtures are recommended due to very polar compounds which cannot be extracted using pure organic solvents (Stalikas, 2007; Biesaga, 2011). Aside from the solvents used in extraction, temperature, duration of extraction, and sample-to-solvent volume ratio are all important factors to consider (Stalikas, 2007). In the present work, URE was considered as an effective extraction technique for both phenolics and flavonoids, whereas some of the other extraction techniques tested did not show the ability to extract both phenolics and flavonoids in the same degree. Similar results were reported with *Trifolium pratense* L., a wild vegetable from Nepal; and *Hyoscyamus gallagheri* A.G.Mill. and Biagi (Esmaeili *et al.*, 2015; Aryal *et al.*, 2019; Hossain *et al.*, 2019). When choosing or optimising an extraction technique, it is critical to consider its efficiency and efficacy. The term efficiency refers to the yield of the extract, whereas efficacy refers to the potency of the extract (Jadhav *et al.*, 2009).

In the initial stage, classical extraction techniques such as AE, ME, PWE, ESE, and RE were used to identify the useful technique for extracting the phenolics and flavonoids of *C. grandis* leaves. Among the experiments conducted, the RE technique and water were found to be the most appropriate technique and solvent, respectively. Furthermore, based on recent literature, many researchers have used the ultrasonic extraction technique to obtain a high yield of targeted compounds (Jadhav *et al.*, 2009; Dong *et al.*, 2010; Roselló-Soto *et al.*, 2015). Ultrasonic extraction uses ultrasonic wave energy in the extraction. Ultrasounds are capable of producing cavitation, which accelerates the breakdown of the solute's biological cell walls, thus allowing the release of biologically active compounds into the solvent without heat (Zhu *et al.*, 2015). Furthermore, the advantages of ultrasound extraction include the use of less solvent, lower energy consumption, and a decrease in extraction temperature and time (Soria Cristina and Villamiel, 2010; Barba *et al.*, 2016). As a result, in the present work, we determined that ultrasonication was one of the extraction techniques for extracting the phenolic- and flavonoid-rich extract for future applications. The results of the present work corroborated the findings of Biesaga (2011), where ultrasonic extraction has been considered as a potential alternative to traditional reflux extractions of maize samples. In addition, the ultrasonic extraction technique was optimised using water and ethanol separately. Water was discovered to be the best solvent for extracting phenolics and flavonoids from *C. grandis* leaves. We discovered that URE was one of the satisfactory extraction techniques that could be used to extract phenolic-rich extracts; similarly, Li *et al.* (2017) and Chemat *et al.* (2017) extracted camptothecin and betulinic acid from *Camptotheca acuminata* Decne fruits using ultrasonic extraction followed by reflux extraction. The solvent used in an extraction technique must be carefully chosen. Organic solvents such as acetone, methanol, and ethanol were used in the present work. However, since water was discovered to be the best solute, further testing for the presence of solvent residues in *C. grandis* extract was not required.

Ultrasonic waves cause chaotic vibrations at the solvent-solid interface, disrupting cells, and hastening phenolic and flavonoid release into the solvent. As a result, it is possible to obtain a phenolic- and flavonoid-rich extract. Following that, more compounds were released into the solvent as a result of the refluxing. The target components would no longer be released after a long period of ultrasonic time, and the compounds would deteriorate with long-term reflux heating. As a result, the optimised

ultrasonic time was 19 min, and the optimised refluxing time was 168 min. T_U range, T_R range, and liquid/solid ratio were selected based on the findings of previous investigations (Dong *et al*., 2010; Li *et al*., 2017).

The present work, nevertheless, has limitations. Despite the fact that the extraction methods of URE, RUE, and RE showed some similarities in the results in terms of TPC and TFC, only the URE method was used to optimise further conditions. The availability of chemicals, as well as laboratory conditions, hampered the consideration of several extraction methods for further optimisations. This also resulted in considering a minimum of five data points in Figures 1 - 4.

Conclusion

As compared to the other extraction methods tested, the ultrasonic assisted reflux extraction technique (URE) was found to be a good choice for extracting both polyphenols and flavonoids from *C. grandis* leaves. Considering practical operation, the final optimal conditions were ultrasonication for 19 min, followed by refluxing for 168 min. The liquid:solid ratio was determined to be 16.4 mL/g. This is the first attempt to select and optimise an extraction technique to target polyphenols and flavonoids from *C. grandis* leaves. Through URE, novel nutraceutical agents will be developed using polyphenol-rich extracts.

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