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Full Paper

Effect of Extraction Techniques on Phytochemicals and Antioxidants Activity of *Garcinia quaesita* Leaves

Shanthirasekaram Kokilananthan, Vajira P. Bulugahapitiya*, Harshi Manawadu, and Chinthaka S. Gangabadage

Department of Chemistry, Faculty of Science, University of Ruhuna, Matara 81000, Sri Lanka

Email Correspondence: vajira@chem.ruh.ac.lk (V. P. Bulugahapitiya)

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Abstract

Garcinia quaesita is an endemic plant in Sri Lanka with a wide array of domestic and medicinal values, yet very limited studies have been reported. The goal of this study was to investigate the phytochemicals and antioxidants capacity of leaves of *G. quaesita* under different extraction techniques in order to monitor the effect of extraction techniques on the above parameters. Water was used as the solvent and four extraction methods, namely sonication (EN01, one-hour, RT, 40 kHz), Soxhlet (EN02, six-hours, 105 ^{o}C), maceration with agitation (EN03, six-hours, RT, 1000 rpm), and maceration with agitation upon heating (EN04, six-hours, 60 ^{o}C, 1000 rpm), were applied. Phytochemical screenings and quantification of polyphenolics (TPC), flavonoids (TFC), tannins (TTC), terpenoids (TC), saponins (SC), and alkaloids (AC) were performed using standard methods, whereas antioxidants capacity was assessed using FRAP and DPPH assays. The results revealed that *G. quaesita* contains a wide range of phytochemicals and the TPC, TTC and SC profiles appeared to be higher in the extraction process of EN04 (60.73 ± 0.06 mg GAE/g, 60.12 ± 0.06 mg TAE/g, and 257.64 ± 0.72 mg SE/g, respectively) whereas those were lower in the technique EN01. In addition, TFC and TC levels were high in EN02 (3.02 ± 0.00 mg QE/g and 17.75 ± 0.04 mM LE/g respectively) and besides, TFC was low in method EN03, whereas TC was low in EN01. Interestingly, AC was found to be higher in EN03 (1.16 ± 0.03 mg AE/g) and lesser in EN02. Total antioxidant capacity (FRAP value) was calculated to be higher in extraction method EN04 (72.08 ± 0.00 mg Trolox Eq/g) whereas it was lower in extraction method EN01. The method, EN03 gave the lower IC_{50} value in DPPH radical scavenging assay (10.49 ± 0.12 mg/ml), indicating high scavenging activity. In conclusion, the extraction technique EN04 is well suitable to extract polyphenolics and antioxidants from *G. quaesita leaves.* Though *G. quaesita* leaves contain a diverse range of bioactive compounds and antioxidants properties, it is important to choose the correct extraction technique based on the objectives of the desired research work, as the number of phytochemicals extracted, and antioxidant capacity varies depending on the extraction technique used.

Keywords: *Antioxidants, extraction methods, Garcinia quaesita, phytochemicals*

Introduction

In Sri Lanka, the genus Garcinia is represented by 10 species, including five endemic species (*Garcinia quaesita*, *Garcinia zeylanica*, *Garcinia hermonii*, *Garcinia terpnophylla* and *Garcinia thwaitesii*), three Indo-Sri Lankan species (*Garcinia morella*, *Garcinia echinocarpa,* and *Garcinia spicata*), one species cultivated for more than seventy-five years (*Garcinia mangostana)* and an introduced species (*Garcinia xanthochymus*) which is now considered as a semi-naturalized species. Except for *G. spicata*, the other nine species have distributed in the wet zone of Sri Lanka [1]. Garcinia species are rich in bioactive compounds with important pharmacological properties, such as anti-inflammatory property, antipyretic activity, analgesic properties [2, 3], antioxidant activity [4], antinociceptive effects [3], and leishmanicidal activity [5]. A great variety of

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compounds, mainly flavonoids, polyisoprenylated benzophenones, guttiferone A, guttiferone analogue, guttiferone G, and xanthones have been isolated from Garcinia (Clusiaceae) species [6-8]. It has been reported that Garcinia species contain prenylated and oxygenated xanthones [8] with biological activities in Human Immunodeficiency Virus (HIV)-inhibitory [6], antifungal [9], anti-inflammatory [10], antioxidant [11, 12] and neuroprotective activities [13].

G. quaesita is an endemic plant to Sri Lanka, where it is known as "Rath Goraka (Red-Goraka, Red fruited)" and is more commonly known as "Goraka" [1]. Garcinia is cultivated expressly for the purpose of harvesting its fruits, which are then utilized in cooking as a spice/condiment as well as in various ayurvedic/traditional medicine. The rind and extract are frequently used as components and condiments in Sri Lankan cuisine [14]. In addition, the fruits, seeds, bark, and leaves of *G. quaesita* have been used to cure a variety of ailments and disorders such as fevers, fractures, hyperlipidemia, wounds, hemorrhoids, and many more in Sri Lankan traditional medicine [15]. Aside from the fruits and seeds, the leaves are known to contain a variety of chemical compounds with substantial pharmacological activities [16]. Since fruits are seasonal and the leaves are available all over the year, it is most worthwhile to strengthen the scientific research on leaves for finding novel compounds and applications. Since the extraction techniques have a great impact on the selective separation of traces of important phytochemicals, this research was focused on qualitative and quantitative analysis of bioactive compounds present in the *G. quaesita* leaves along with their antioxidant capacity using four distinct extractions techniques.

Materials and Methods

Plant collection: Fresh *G. quaesita* leaves (one kilogram) were harvested from a home garden in Matara, Sri Lanka (latitude 5.9478 ºN, longitude 80.5483 ºE). The plant has been authenticated in Peradeniya botanical garden, Sri Lanka. Healthy garcinia leaves were washed with tap water and distilled water several times after plant collection and air-dried for one day in order to remove the water drops on the surface of the washed leaves., Then the plant leaves were crushed using a grinder (Philips mixer grinder HL 7756 09) and the crushed leaves were employed in the extraction procedure.

Extractions: Four different extraction approaches (EN01-EN04) were employed to extract the bioactive components from *G. quaesita* leaves and air-dried crushed leaves (100.00 g) and distilled water (500 mL) was used for each extraction.

EN01: Sonication was done for one hour at room temperature with 40 kHz frequency in an ultrasonic cleaner (ROCKER Ultrasonic cleaner, Model: SONER 202H) [17].

EN02: Soxhlet apparatus (extraction) was used for six hours at 105 ºC.

EN03: The maceration was performed at 1000 rpm agitation at room temperature for 6 hours.

EN04: The maceration was performed at 1000 rpm agitation at 60 ºC temperature for 6 hours.

Extracts were concentrated using a freeze dryer (Model: FE-10-MR, S/No: FD 2020062222), and stored at -30 ºC for further use [18]. All four extraction methods were triplicated in order to validate the results with statistical analysis.

Phytochemical screening: Phytochemical screening tests for alkaloid, flavonoid, terpenoid, saponin, polyphenol, tannin, glycoside, coumarin, anthocyanin, phytosterol, quinones, betacyanin, and chalcones

were performed in triplicates using standard procedures as described in the literature [19-26].

Alkaloids: A 3.0 mL of concentrated plant extracts were placed in a test tube, and 1.0 mL of 1% HCl was added. The mixture was then gently boiled for 20 minutes, and then cooled, and filtered. The filtrates were then treated with Mayer's reagent, Wagner's reagent, and Dragendroff's reagent to confirm the presence of alkaloids [20-22, 24].

Flavonoids (Shinoda test): The concentrated plant extracts were mixed with magnesium ribbon fragments, and concentrated HCl was added dropwise. The appearance of orange, red, pink, or purple coloration indicated the presence of flavonoids. In addition, some other tests such as the lead acetate test, the alkaline reagent test, the NH_4OH test, and the $AlCl_3$ test were also conducted for the confirmation of flavonoids [19, 22-24].

Terpenoids (Salkowski's Tests): About 2.0 mL of chloroform was added to the plant extracts (2.0 mL). Then 2.0 mL of concentrated sulfuric acid was carefully added gently shaken and allowed to stand for a while. A reddish-brown coloring of the interphase was generated indicating the presence of terpenoids. Similarly, the Liebermann-Burchardt test has also been used to determine terpene conformation [19, 21-23].

Diterpene (Copper acetate Test): The plant extracts of 0.05 g were dissolved in water and then treated with ten drops of copper acetate solution. The presence of diterpenes was indicated by the formation of the emerald-green color [24].

Saponins: The plant extracts were placed in a test tube and violently shaken with distilled water; the production of stable foam was noted to confirm the characteristics of saponins. In addition, the olive oil test was carried out to confirm the presence of saponins [20, 23, 24].

Polyphenolics: The extract (2.0 mL) was combined with 2.0 mL of a 2 % $FeCl_3$ solution, and the presence of polyphenolics/ tannins was identified by a blue-green or black color [20, 23, 24].

Tannins (Lead Acetate Test): When 2.0 mL of extract was mixed with a few drops of 1 % lead acetate, a reddish precipitate formed indicating the presence of tannins [25].

Glycosides (Keller-kilani test): In a test tube, 2.0 mL of the plant extracts were mixed with 2.0 mL of glacial acetic acid containing 2 drops of 2% $FeCl_3$, followed by 2.0 mL of concentrated sulfuric acid was added. A brown ring at the interphase showed the presence of glycosides. Other established assays, such as the modified Borntrager's test and the Legal's test, were also employed to confirm the presence of glycosides [20, 22-24].

Coumarin: About 3.0 mL of 10% NaOH was added to the 2.0 mL of plant extracts, and the development of a yellow color showed the presence of coumarins [24, 25].

Anthocyanins: 2.0 mL of concentrated plant extracts were treated with 2.0 mL of 2 M HCl and NH_3, the development of pink-red to the blue-violet color indicated the existence of anthocyanin [24, 25].

Phytosterols (Salkowski's test): 2.0 mL of the concentrated plant extracts were treated with chloroform and filtered, and the filtrate was treated with a few drops of concentrated H_2SO_4 and shaken. The development of golden color shows that the test was positive [24].

Quinones: 2.0 mL of the concentrated plant extracts were treated with 1.0 mL of concentrated H_2SO_4. The development of a reddish hue confirmed the presence of quinones [26].

Betacyanin: The concentrated plant extracts were treated with 1.0 mL of 2 M sodium hydroxide and heated for 5 minutes at 100 ºC. The formation of yellow color indicated the presence of Betacyanin [26].

Chalcones: About 2.0 mL of NH_4OH was added to 2.0 mL of concentrated extract, and the appearance of the red color showed the presence of chalcones [24].

Quantification of phytochemicals: Aqueous extracts of *G. quaesita* leaves (0.10 g) were dissolved in 0.25 mL of DMSO and made up to 100.0 mL with methanol to achieve a 1000 ppm concentration, which was then utilized for spectrophotometric measurement of polyphenolics, tannins, flavonoids, terpenoids, alkaloids, and saponins.

Total phenolic content (TPC) and total tannin content (TTC): TPC and TTC were determined using a modified Folin-Ciocalteu (FC) technique [27, 28]. In summary, a 2.5 mL FC reagent was introduced to 0.5 mL of prepared sample extract and left to stand for 5 minutes. Then 2.0 mL of 7.5 % w/v Na_2CO_3 solution was added and incubated for 30 minutes. The absorbance was measured at 765 nm. TPC in aqueous extracts was calculated using a gallic acid standard curve and represented in milligrams of gallic acid equivalents (mg GAE/g extract). TTC of aqueous extracts was calculated using a tannic acid standard curve and represented in tannic acid equivalents (mg TAE/g extract).

Total flavonoid content (TFC): TFC was quantified using a spectrophotometric method as described in the literature [29, 30]. In brief, the above-prepared extracts (1.0 ml) were treated with 0.5 mL of 2 % $AlCl_3$ solution and 0.5 mL of distilled water and left to stand for 10 minutes before measuring absorbance at 425 nm. TFC of aqueous extracts was calculated using a quercetin standard curve and represented in Quercetin equivalents (mg QE/g extract).

Terpenoid content (TC): The TC was determined using a significantly modified spectrophotometric technique [27]. In a short, 1.0 mL of 5 %, the aqueous phosphomolybdic acid solution was progressively added to 1.0 mL of sample extract, followed by 1.0 mL of the conc. H_2SO_4. The mixture was well mixed and allowed for 30 minutes before being diluted to 5.0 mL with MeOH. The absorbance was measured at 700 nm. To compute TC, a Linalool standard curve was employed, and TC of leaf extracts was expressed in Linalool equivalents (mg LE/g extract).

Saponin content (SC)**:** The SC was evaluated using the spectrophotometric approach described in [31, 32]. Simply, 8 % vanillin (1.0 mL) was treated with an equivalent amount of prepared sample extract before being placed in an ice-water bath, followed by 8.0 mL of 77 % H_2SO_4 (v/v) was added. Before placing the test tube in a 60 °C oven for 30 minutes, it was shaken. The solution was chilled in an ice-water bath for 10 minutes before being brought to RT for UV examination. At 540 nm, the absorbance was measured. Using a Saponin standard curve, the SC of aqueous extracts was expressed in Saponin equivalents (mg SE/g extract).

Alkaloid content (AC)**:** The AC was determined using a spectrophotometric technique described in [33, 34]. A fraction of the plant leaf aqueous extract was dissolved in the 2 M HCl solution prior to filtration. A milliliter of this supernatant was rinsed with 10 mL of chloroform after being passed through a separatory funnel (3 times). The pH of this prepared sample was adjusted to neutral using 0.1 M NaOH. This solution was then mixed with newly prepared Bromocresol Green (BCG) solution (5.0 mL) and phosphate buffer solution (pH 4.7, 5.0 mL). After dynamically shaking the produced solution, the complex combination was re-extracted using $CHCl_3$ (1.0, 2.0, 3.0, and 4.0 mL). The extracted complex mixture was then transferred to a volumetric flask (10.0 mL) and diluted and adjusted with $CHCl_3$. The complex's absorbance in $CHCl_3$ was measured at 470 nm on the spectra. Using an Atropine standard curve, the AC of aqueous extracts was quantified in Atropine equivalents (mg AE/g extract).

Antioxidant analysis:

Ferric reducing antioxidant power (FRAP) assay**:** The FRAP value of all aqueous extracts of *G. quaesita* leaves was measured using a standard method described in the literature [35-37]. A 100 µL sample solution was treated with 3.0 mL of newly prepared FRAP reagent [300 mM acetate buffer (pH-3.6): 10 mM TPTZ (in 40 mM HCl): 20 mM $FeCl_3$ in a 10:1:1 ratio]. After 30 minutes of incubation at 37 °C, the absorbance at 593 nm was measured. For calibration, a Trolox solution was utilized.

DPPH radical scavenging assay**:** The free radical scavenging activity of aqueous extracts of *G. quaesita* leaves was evaluated using a standard operating approach described in the literature with small changes [38, 39]. A 0.06 mM DPPH solution in MeOH (3.9 mL) was thoroughly mixed with 100 µL of aqueous extracts at various concentrations. After 30 minutes in the dark (Incubation in the dark to minimize the radical's response to light), the absorbance at 517 nm was measured. The IC_{50} value for free radical scavenging activity was calculated using a percentage of scavenging effect vs. concentration plot. As standards, ascorbic acid and Trolox were utilized.

Statistical analysis: To evaluate and compare the data, ANOVA, T-test (LSD) (LSD-Least Significant Difference), and non-parametric statistics (Cochran's Q test) were performed. The statistical analysis was carried out using SAS and R-studio. The data were reported as means and standard deviations.

Results and Discussion

Extraction: Figure 1 illustrates the extraction yield of four different extraction techniques of aqueous extracts of *G. quaesita* leaves, which clearly indicates that each extraction technique has yielded different quantities. Interestingly, as shown in Figure 2, statistical analysis data of extraction yield of four different

extraction techniques also revealed that the four different extraction techniques yielded in different percentages at a 5 % significant level. The extraction technique EN02 yielded a higher percentage than the other methods. The second highest yield was observed in EN04 followed by EN03 and lastly EN01.

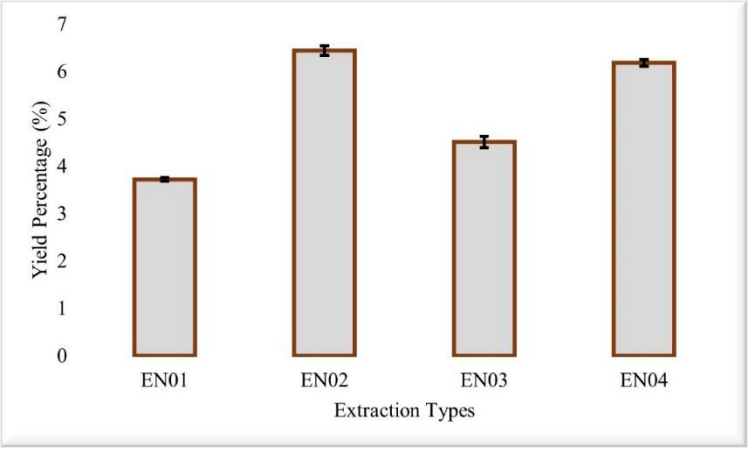

Figure 1: Comparison of extraction yield of four different extraction techniques *G. quaesita* leaves aqueous extracts

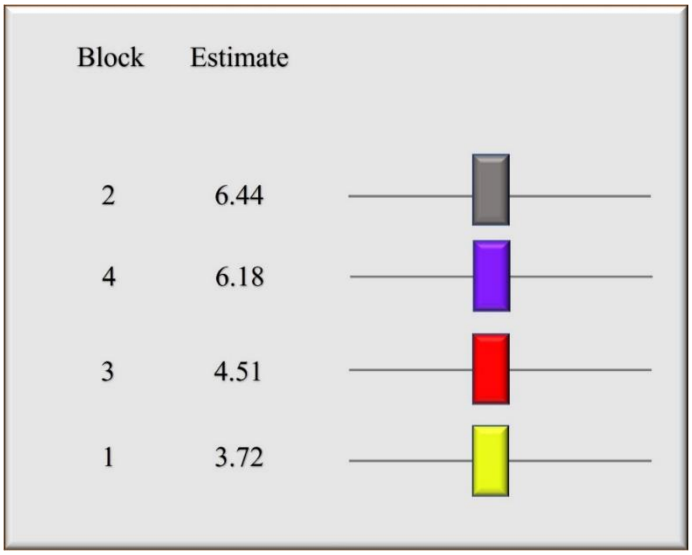

Figure 2: Statistical analysis data of comparison of extraction yield of four different extraction techniques of *G. quaesita* leaves aqueous extracts. (Alpha = 0.05, Block: 1: EN01, 2: EN02, 3: EN03, 4: EN04, Estimate: mean value, means covered by the same bar are not significantly different).

Phytochemical screening: It revealed the presence of highly important secondary metabolites, including alkaloids, glycosides, flavonoids, saponins, tannins, terpenoids, polyphenolics, coumarins, phytosterols, betacyanin, and quinones in all the aqueous extracts of garcinia leaves (Table-1). It was found that anthocyanins and chalcones were absent in all the aqueous extracts of garcinia leaves which are shown in Table 1. In the non-parametric analysis, Cochran's Q test was used to statistically determine the presence and absence of phytochemical availability in all plant extracts samples. The non-parametric analysis of Cochran's Q test demonstrated that these phytochemicals are present in all aqueous extracts of garcinia leaves.

Table 1: Phytochemicals qualitative analysis data after statistical analysis of all four different extracts from four different extraction approaches (A: Absent, P: Present).

Phytochemicals	Test method	EN01	EN02	EN03	EN04
Alkaloids	1). Mayer's Test	P	P	P	P
	2). Wagner's Test	P	P	P	P
	3). Dragendroff's Test	P	P	P	P
Glycosides	1). Keller-kilani Test	P	P	P	P
	2). Modified Borntrager's Test	A	A	A	A
	3). Legal's Test	P	P	P	P
Flavonoids	1). Alkaline reagent Test	P	P	P	P
	2). Shinoda Test/ Mg turning Test	P	P	P	P
	3). Lead acetate Test	P	P	P	P
	4). $AlCl_3$ Test	P	P	P	P
	5). NH_4OH Test	P	P	P	P
Saponins	1). Froth Test	P	P	P	P
	2). Olive Oil Test	P	P	P	P
Tannins	1). Bramer's Test	P	P	P	P
	2). Lead Acetate Test	P	P	P	P
Terpenoids	1). Salkowski's Test	P	P	P	P
	2). Liebermann- Burchardt Test	P	P	P	P
	3). Copper acetate Test	P	P	P	P
Phenols	1). Ferric Chloride Test	P	P	P	P
Coumarins	1). UV light Test	A	A	A	A
	2). NaOH Test	P	P	P	P
Anthocyanins	1). HCl & NH3 Test	A	A	A	A
Chalcones	1). NaOH Test	A	A	A	A
Phytosterol	1). Salkowski's Test	P	P	P	P
Betacyanin	1). NaOH Test	P	P	P	P
Quinones	1). H_2SO_4 Test	P	P	P	P

Phytochemical quantitative analysis:

Table 2: Phytochemical quantitative analysis of garcinia leaves' aqueous extracts. Values represent the mean ± standard deviation of triplicate sample.

Extraction type	TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg TAE/g)	TC (mM TE/g)	AC (mg AE/g)	SC (mg SE/g)
EN01	34.87 ± 0.06	2.22 ± 0.01	34.51 ± 0.05	02.63 ± 0.01	1.10 ± 0.07	101.21 ± 0.72
EN02	49.33 ± 0.06	3.02 ± 0.00	48.83 ± 0.06	17.75 ± 0.04	0.72 ± 0.02	161.69 ± 1.49
EN03	36.27 ± 0.06	2.16 ± 0.01	35.90 ± 0.06	14.06 ± 0.02	1.16 ± 0.03	221.21 ± 0.72
EN04	60.73 ± 0.06	2.86 ± 0.01	60.12 ± 0.06	03.51 ± 0.01	0.76 ± 0.01	257.64 ± 0.72

Quantitative analysis of polyphenolics, flavonoids, tannins, terpenoids, alkaloids, and saponins revealed that all the aqueous extracts of garcinia contain varying amounts, as shown in Table 2. According to the results of quantification, especially polyphenolics, flavonoids, tannins, terpenoids, saponins, and alkaloids, the extraction technique EN04 is the most effective method to extract polyphenolics, tannins, and saponins in garcinia leaves than the other three methods used, since these three classes of phytochemicals were high in the method EN04 (60.73 ± 0.06 mg GAE/g, 60.12 ± 0.06 mg TAE/g, and 257.64 ± 0.72 mg SE/g respectively). In contrast, those three classes of phytochemicals and terpenes content were also extracted in fewer quantities in the EN01. Interestingly, the Soxhlet extraction technique (EN02) showed as the best extraction method to extract flavonoids and terpenoids from garcinia leaves with water as the solvent due to the presence of a higher quantity of flavonoids and terpenoids (3.02 ± 0.00 mg QE/g and 17.75 ± 0.04 mM TE/g respectively) whereas the lesser quantity of alkaloids (0.72 ± 0.02 mg AE/g) was recorded in this method. Finally, the extraction method EN03 is the best technique to extract alkaloids from garcinia leaves as the quantity of alkaloid contents was high in the EN03 method (1.16 ± 0.03 mg AE/g) than in other methods.

The T-test (LSD) indicated that all of the extraction techniques used in this study extracted significantly different amounts of polyphenolics, flavonoids, tannins, terpenoids, and saponins at a 5% significance level, whereas the extracted amounts of alkaloids from pairs of extraction techniques such as EN01 and EN03 and EN02 and EN04 are not significantly different at a 5% significance level, as shown in Figure 3. More precisely methods EN01 and EN03 are the best methods to extract alkaloids from garcinia leaves with water as a solvent and the most important both methods showed significantly no differences in quantity.

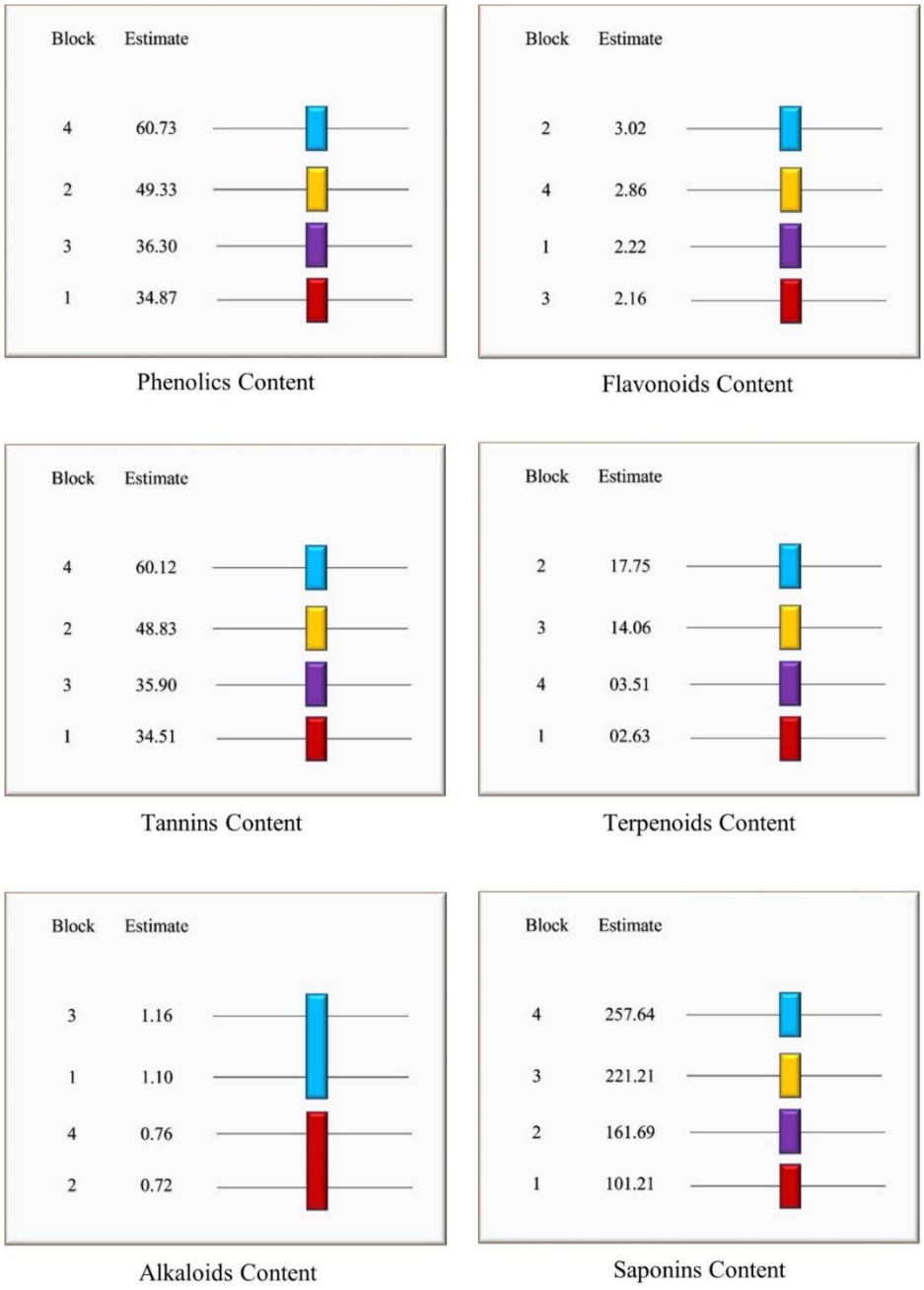

Figure 3: Statistical analysis data of comparison of phytochemicals quantification data of four different extraction techniques of *G. quaesita* leaves' aqueous extracts. (Alpha = 0.05, Block: 1: EN01, 2: EN02, 3: EN03, 4: EN04, Estimate: mean value, means covered by the same bar are not significantly different).

Antioxidant analysis:

Antioxidant analysis of four categories of garcinia leaves aqueous extracts using FRAP and DPPH assays revealed that the garcinia leaves exhibit antioxidant activity, which is shown in Figure 4 and Figure 5. Despite the fact that all four types of aqueous extracts exhibit antioxidant activity, the antioxidant capacity varies depending on the extraction technique. In precision, total antioxidant capacity by FRAP assay confirmed that extraction method EN04 (72.08 ± 0.00 mg Trolox Eq/g) has a higher antioxidant capacity

than all the others. Most importantly, all four techniques have significant differences at the 5% level, which is shown in Figure 6. The DPPH radical scavenging effect investigation showed that extraction technique EN03 (10.49 0.12 mg/ml) has relatively high scavenging activity than the other three approaches, but statistical analysis demonstrated that extraction techniques EN01, EN02, and EN04 had the same scavenging capacity at the 5 % significant level as shown in Figure 6, whereas the IC_{50} value of standards such as ascorbic acid and Trolox have revealed that 0.11 ± 0.00 and 0.10 ± 0.00 mg/ml respectively.

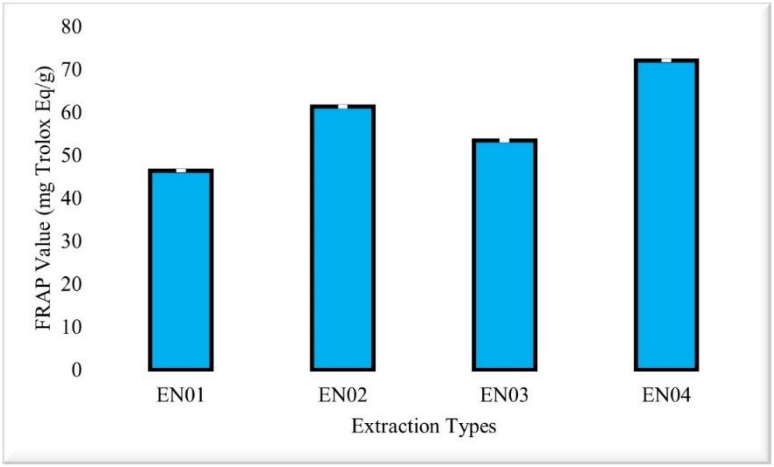

Figure 4: Comparison of total antioxidant capacity (FRAP value) of four different extraction techniques *G. quaesita* leaves aqueous extracts.

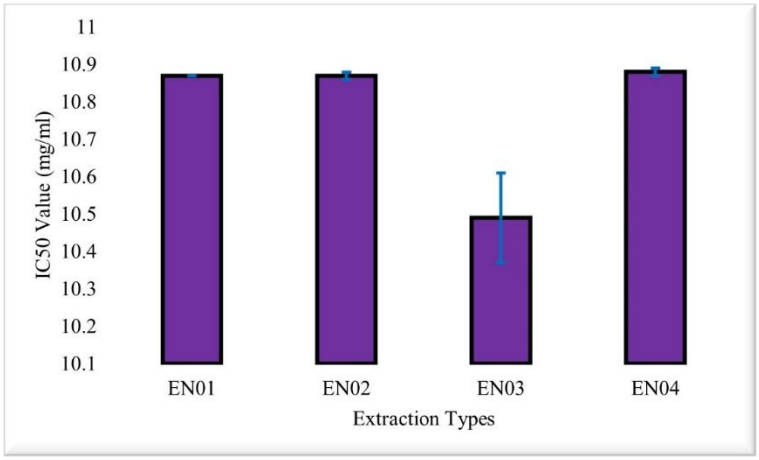

Figure 5: Comparison of radical scavenging capacity (DPPH IC_{50} value) of four different extraction techniques *G. quaesita* leaves aqueous extracts.

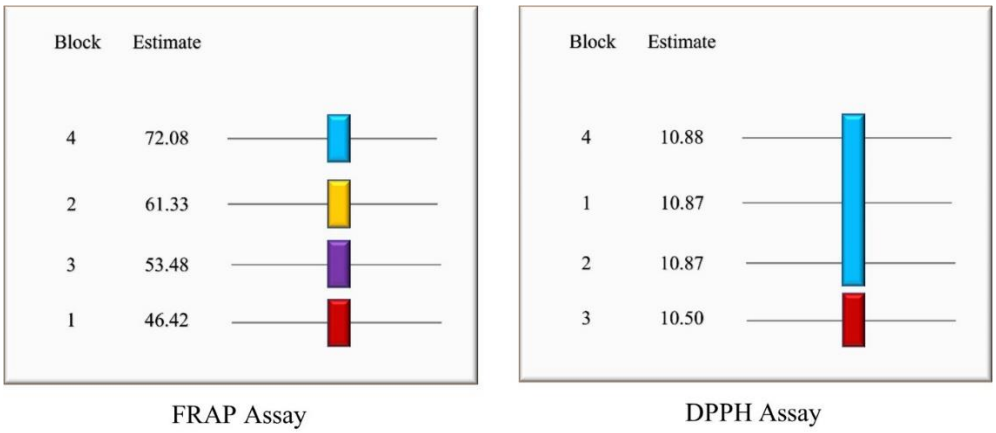

Figure 6: Statistical analysis data of comparison of antioxidant analysis data of four different extraction techniques of *G. quaesita* leaves' aqueous extracts. (Alpha = 0.05, Block: 1: EN01, 2: EN02, 3: EN03, 4: EN04, Estimate: mean value, means covered by the same bar are not significantly different).

Conclusion

Based on this study, it can be concluded that the extraction technique EN04 is a well suitable method to extract polyphenolics, tannins, and saponins, and also it is a good method to extract antioxidants from the leaves of *G. quaesita*. The extraction technique EN02 is well matched for extracting flavonoids and terpenoids whereas the extraction technique; EN03 is a recommended extraction technique for the extraction of alkaloids. This is the first study to compare extraction methods employing phytochemical analysis and the antioxidant potential of *G. quaesita* leaves. Finally, as the selection of extraction techniques is the most crucial step in the isolation of natural compounds, the finding of this study will be immensely useful for further research in the field.

Conflicts of Interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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