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Original Research Article

Comparative Evaluation of Different Extraction Techniques on Phytochemicals and Antioxidant Activity of *Psidium Guajava* L. Leaves

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ABSTRACT

The extraction process can play a significant role in the separation of desired bioactive compounds from plant-based material. Therefore, this study was aimed at comparing different extraction techniques on the isolation of potent phytochemicals and their antioxidant capacity in order to find the optimal extraction process. *Psidium guajava* leaves were selected for the study as it is known to contain diverse range of phytochemicals and used in many healthcare applications. Water was employed as the extracting solvent and four extraction methods were applied: sonication (E1, one hour, RT, 40 kHz), Soxhlet (E2, six hours, 105ºC), maceration with agitation (E3, six hours, RT, 1000 rpm), and maceration with agitation upon heating (E4, six hours, 60ºC, 1000 rpm). Standard tests were carried out for phytochemical analysis, and antioxidant capacity was assessed using Ferric Reducing Antioxidant Power (FRAP) and 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH*) radical scavenging assays. The results revealed that *P. guajava* contains vast number of phytochemicals. Polyphenolics, tannins, and terpenoids appeared to be higher in the extraction process E4, flavonoids, and saponins appeared to be higher in E2, and alkaloids were higher in E3. Total antioxidant capacity was greater in extraction method E4 (432.57 ± 0.51 mg Trolox Eq/g) and the IC_{50} value of the DPPH radical scavenging assay was low in E3 (273.81 ± 0.07 ppm), indicating higher scavenging activity. In conclusion, the quantity of phytochemicals extracted, and its antioxidant capacity vary depending on the extraction technique. According to FRAP and polyphenolic content, the extraction technique E4 gives the best antioxidative properties.

Keywords: Antioxidants, Extraction techniques, Phytochemicals, *Psidium guajava* L, Spectrophotometric methods.

Introduction

Extraction is the separation of potential constituents present in plants or animal tissues using appropriate extraction methods.[1] Plants extracts themselves have been widely used in traditional and folk medicines and also many applications such as pesticides, perfumes, food flavors, food preservatives, etc. Plants contain a wide array of specific secondary metabolites with different pharmacological properties along with antioxidant capacity. Alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, polyphenolics, terpenoids, saponins, flavonoids, etc. are the main bioactive classes of such secondary metabolites. Those are produced and deposited in different parts of the plants, making them used for several purposes in plants themselves such as defense and regulatory functions etc.[2,3] Due to the structural complexity of secondary metabolites and their availability as traces, the choice of the extraction method is of great impact to get them selectively extracted. An inappropriate choice may cause the entire isolation process to be a failure or not released the desired components satisfactorily from the matrix.[4] Therefore, the precision and accuracy of qualitative and quantitative measurements of plant-based phytochemicals and the stability of chemical compositions isolated are largely dependent on the extraction method performed.[5,6]

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Psidium guajava L. is a native of Mexico that has spread over South America, Europe, Africa, and Asia. It grows throughout all of the world's tropical and subtropical regions, adapting to a variety of environmental circumstances but preferring drier climes.[7] It is generally referred to as guava (Myrtaceae family) and has long been used as herbal medicine for various diseases all over the world.[8] In traditional medicine, the leaves of this plant are used to cure a wide range of diseases, such as wounds, gastroenteritis, lung difficulties, dysentery, ulcer, rheumatism diarrhea, etc.[8,9] The leaf extracts have been reported to show various pharmacological activities.[10-22] Different phytochemicals have been identified from the leaves extracts which are linked with its diverse pharmacological properties.[7,8,23-28] Despite that, no comparative studies reported so far on how different extraction techniques affect on phytochemical profile and antioxidant capacity of guava leaves in order to find optimal extraction techniques to isolate potential components and to used in many applications. Therefore, this study aimed at filling the gap of that in order to support the researchers in the field of Natural Product Chemistry.

Materials and Methods

Plant materials

Fresh leaves of commonly available *P. guajava*, common guava (PGCG) were plucked in November 2020 from home gardens in Matara, Sri Lanka (latitude 5.9478ºN, longitude 80.5483ºE). Plant materials were authenticated in Peradeniya Botanical Garden, Sri Lanka, and deposited with the voucher No AHEAD/DOR 05/C4**.**

Chemicals

Bromocresol green (BCG), Hydrochloric acid (HCl), Glacial acetic acid (CH_3COOH), Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Sulfuric acid (H_2SO_4), Ammonia (NH_4OH), Sodium nitroprusside, Pyridine,

Sodium hydroxide (NaOH), Magnesium ribbon (Mg), Lead acetate, Aluminum chloride anhydrous ($AlCl_3$), Olive Oil, Chloroform ($CHCl_3$), Acetic anhydride, Copper acetate, Nitric acid (HNO_3), Copper sulfate ($CuSO_4$), Potassium hydroxide (KOH), Absolute Ethanol (EtOH), n-butanol, Sodium chloride (NaCl), Dimethyl sulfoxide (DMSO), Folin-Ciocalteu reagent (FC reagent), Sodium carbonate monohydrate ($Na_2CO_3 \cdot H_2O$), Anhydrous sodium sulfate, Gallic acid monohydrate, Tannic acid, Phosphomolybdic acid, 2,2′-Diphenyl-1-Picrylhydrazyl ($DPPH^*$); radical, 2,4,6-Tripyridyl-S-triazine (TPTZ), Linalool, Trolox are classified as AR grade, Hexane, Diethyl ether, Methanol (MeOH) and Benzene are classified as GC grade and Quercetin is classified as HPLC grade.

Sample preparation
Healthy leaves of PGCG were washed with tap water followed by distilled water and then air-dried for a day to remove the moisture from the surface. The air-dried leaves were ground into powder using a normal grinder and used in the extraction process.

Extraction of plant constituents
The constituents in PGCG leaves were extracted by following four extraction techniques. Air-dried ground PGCG leaves (100.00 g) and distilled water (500 mL) were used for each extraction. In the E1 method, the ground leaves were sonicated in an ultrasound-assisted extractor (ROCKER Ultrasonic cleaner, Model: SONER 202H) for one hour at 30-35ºC.[29] Likewise, other three types of extraction techniques were used namely, Soxhlet (E2), two types of maceration (agitation at room temperature (E3), and agitation at 60ºC (E4)). The extraction time was about 6 hours except for sonication which was done in one hour. After freeze-drying of aqueous extracts (Model: FE-10-MR, S/No: FD 2020062222), the resultant crude was stored at -30ºC until further use.[30]

Qualitative analysis of phytochemicals
Using standard procedures, qualitative tests for phytochemicals such as polyphenolics, flavonoids, tannins, saponins, terpenoids, alkaloids, coumarin, glycosides, anthocyanins, phytosterols, quinones, betacyanin, and chalcones were performed in triplicates for each aqueous extract.[31-33]

Quantification of phytochemicals
The aqueous extracts of PGCG leaves (0.10 g) were dissolved in DMSO and diluted with methanol (100 mL) to make a 1000 ppm solution, which was then used for spectrophotometric quantification of polyphenolics, tannins, flavonoids, terpenoids, alkaloids, and saponins.

Total Phenolic Content (TPC) and Total Tannin Content (TTC): TPC and TTC were determined using a slightly altered Folin-Ciocalteu method.[34,35] In brief, a 2.5 mL mixture of FC reagent was added to 0.5 mL of prepared sample extract and allowed to stand for 5 minutes. After adding 2 mL of Na_2CO_3 (7.5% w/v), the solution was incubated for 30 minutes. The absorbance was measured at 765 nm using a UV-visible spectrophotometer (HITACHI, UH5300). TPC was calculated using a gallic acid standard curve and represented in milligrams of gallic acid equivalents (mg GAE/g extract) whereas TTC was obtained 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 with slight modification.[36,37] In brief, prepared sample extracts (1.0 mL) were mixed with 0.5 mL of 2% $AlCl_3$ solution and 0.5 mL of distilled water and allowed to stand for 10 minutes. After vigorous shaking, the absorbance was measured at 425 nm. TFC was calculated using quercetin standard curve and represented in Quercetin equivalents (mg QE/g extract).

Terpenoid Content (TC): A slightly modified spectrophotometric method was used to determine TC.[34] In brief, 1 mL of 5% aqueous phosphomolybdic acid solution was gradually added to 1 mL of sample extract, followed by 1 mL of the con. H_2SO_4. The mixture was completely mixed and left to stand for 30 minutes before diluting with MeOH to 5 mL and the absorbance was measured at 700 nm. Linalool standard curve was employed to calculate TC and the TC was expressed in Linalool equivalents (mM LE/g extract).

Saponin Content (SC): A slightly modified spectrophotometric method was used to determine the SC.[38,39] Simply, 8% vanillin (1.0 mL) was mixed with an equal amount of prepared sample extract before placing in an ice-water bath, followed by the addition of 8 mL of 77% H_2SO_4 (v/v). The mixture was shaken and placed in a 60°C oven for 30 minutes, after cooling in an ice-water bath for 10 minutes the absorbance was measured at 540 nm. Using a saponin standard curve, the SC was expressed in saponin equivalents (mg SE/g extract).

Alkaloid Content (AC): A slightly modified spectrophotometric method was used to determine AC.[40,41] A portion of the aqueous extract of plant leaves was dissolved in the 2 M HCl solution. About 1.0 mL of resultant supernatant was passed through a separatory funnel and washed with 10.0 mL chloroform (3 times). A solution of 0.1 M NaOH was used to adjust the pH of the prepared sample to make it neutral. This solution was then combined with freshly prepared BCG solution (5.0 mL) and phosphate buffer solution (pH 4.7, 5.0 mL). The mixture was re-extracted with $CHCl_3$ (1, 2, 3, and 4 mL respectively), then it was poured into a volumetric flask (10.0 mL) and volume up with $CHCl_3$. The absorption of the resultant solution was measured at 470 nm. Using atropine standard curve, the AC was quantified in Atropine equivalents (mg AE/g extract).

Antioxidant analysis
Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP value of the aqueous extracts of PGCG was determined using a standard method.[42-44] About 3.0 mL of freshly made 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] was mixed with 100 µL of sample solution. The absorbance at 593 nm was measured after 30 minutes of incubation at 37 °C. A Trolox solution (0 –100 ppm) was used for calibration.

DPPH Radical Scavenging Assay: The free radical (FR) scavenging activity of aqueous extracts was assessed using the standard procedure with slight alterations.[45-47] DPPH solution in MeOH (0.06 mM, 3.9 µL) was completely mixed with 100 µL of aqueous extracts of plant material at various concentrations. The absorbance at 517 nm was measured after 30 minutes of incubation in the dark. Using a percentage of scavenging effect vs. concentration plot, the IC_{50} value for free radical scavenging activity was computed. Ascorbic acid and Trolox were used as standards.

Statistical analysis
All analyses in this study were done in triplicates. The statistical software, SAS OnDemand for Academics: Studio (SAS 9.4) and R-studio were used for the statistical analysis. Phytochemical qualitative data were statistically analyzed by non-parametric statistics (Cochran's Q test) and extraction yield data, phytochemical quantitative data, and antioxidant analysis data were analyzed by one-way analysis of variance (ANOVA) and T-test (LSD - Least Significant Difference) where $p < 0.05$ was used as a significance level. The data were presented in the form of mean ± standard deviation.

Results and Discussion

Extraction
The percentage yields of all four extracts were compared and statistically evaluated, as illustrated in Figures 1 and 2. Among the four distinct extraction methods (E1, E2, E3, and E4), E2 gave the highest yield followed by E4, E3, and lastly E1. Statistical analysis indicated that the yields of E2 and E4 were statistically not different, but greater than the other two extracts. When E3 and E4 were compared, both were carried out under the same extraction conditions except heating in E4.
The higher extraction yields in E2 and E4 can be attributed to the supplying heat in the extraction processes.[48] Sonication process made the extraction in a relatively short period (one hour) at room temperature (RT). Since sonication is a non-conventional extraction technique and one of the green extraction strategies, further study is required considering other parameters.[30]

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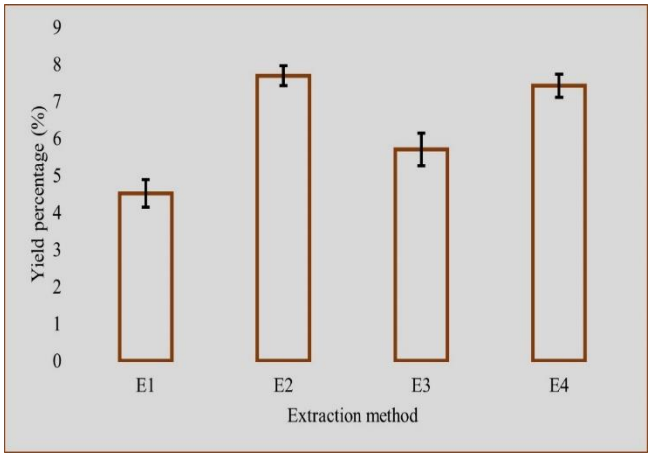

Figure 1: Comparison of crude aqueous extraction yields by four different extraction methods for PGCG.

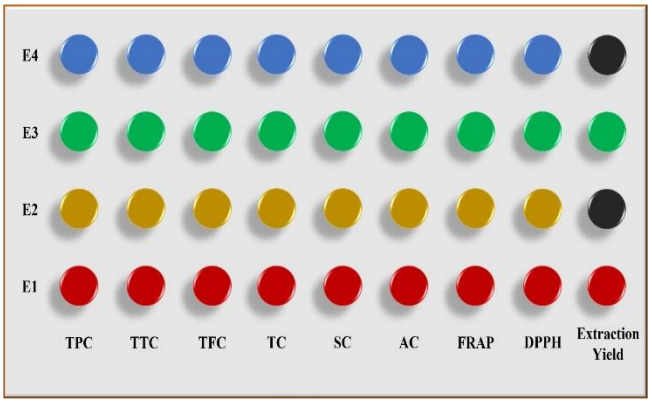

Figure 2: T-test (LSD) for phytochemical quantification, FRAP and DPPH assays, and crude aqueous extraction yields data of PGCG leaves extracts (Alpha = 0.05, circles filled with the same color in vertical alignment are not significantly different)

Table 1: Comparison of phytochemical screening data of four different aqueous extracts of PGCG leaves

Phytochemicals	Test method	E1	E2	E3	E4
Alkaloids	Mayer's Test	P	P	P	P
	Wagner's Test	P	P	P	P
	Dragendroff's Test	P	P	P	P
Glycosides	Keller-kilani Test	P	P	P	P
	Modified Borntrager's Test	A	A	A	A
	Legal's Test	P	P	P	P
Flavonoids	Alkaline reagent Test	P	P	P	P
	Shinoda Test/ Mg turning Test	P	P	P	P
	Lead acetate Test	P	P	P	P
	$AlCl_3$ Test	P	P	P	P
	NH_4OH Test	P	P	P	P
Saponins	Froth Test	P	P	P	P
	Olive Oil Test	P	P	P	P
Tannins	Bramer's Test	P	P	P	P
	Lead Acetate Test	P	P	P	P
Terpenoids	Salkowski's Test	P	P	P	P
	Liebermann- Burchardt Test	P	P	P	P
	Copper acetate Test	P	P	P	P
Polyphenols	Ferric Chloride Test	P	P	P	P
Coumarins	UV light Test	A	A	A	A
	NaOH Test	P	P	P	P
Anthocyanins	HCl & NH3 Test	A	A	A	A
Chalcones	NaOH Test	A	A	A	A
Phytosterol	Salkowski's Test	P	P	P	P
Betacyanin	NaOH Test	P	P	P	P
Quinones	H_2SO_4 Test	P	P	P	P

(P: Present and A: Absent).

Phytochemical qualitative analysis

All the aqueous extracts indicated the presence of specific phytochemicals such as alkaloids, glycosides, flavonoids, saponins, tannins, terpenoids, polyphenolics, coumarins, phytosterols, betacyanin, and quinones. As shown in Table 1, anthocyanins and chalcones were found to be absent in all the aqueous extracts of PGCG leaves. Cochran's Q test, a non-parametric technique, was employed to confirm statistically, the presence or absence of phytochemicals in all aqueous extracts considered. Non-parametric analysis of Cochran's Q test showed no statistically significant variations at 5% significant level in the availability of phytochemicals evaluated qualitatively with the four aqueous extracts prepared using four distinct extraction methods. This study explains the fact that any of these four extraction approaches can be used to extract phytochemicals as no difference was observed in phytochemicals qualitative analysis at 5% significant level.

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Phytochemical quantification

The quantitative determination of polyphenolics, flavonoids, tannins, terpenoids, alkaloids, and saponins levels in the leaves indicated that all aqueous extracts of PGCG contain different quantities, as displayed in Table 2. At a 5% significance level, the T-test (LSD) indicated that all of the extraction techniques employed in this study extracted significantly different amounts of polyphenolics, flavonoids, tannins, terpenoids, saponins, and alkaloids, which is illustrated in Figure 2. Phytochemical quantification data specifically emphasizes the effect of extraction techniques.

Based on the current quantification results of phytoconstituents, specifically, polyphenolics, flavonoids, tannins, terpenoids, saponins, and alkaloids, the extraction technique E4 is more effective than the other three methods used to extract polyphenolics, tannins, and terpenoids from PGCG leaves, as these three classes of phytochemicals were high in the method E4. In contrast, those three classes of phytochemicals and saponins content were extracted in fewer quantities in the E1 method. Interestingly, E2 extraction technique was found to be the best extraction method for extracting flavonoids and saponins from PGCG leaves using water as the solvent due to the presence of more flavonoids and saponins and less alkaloids compared to the other extraction methods. Previous studies has also revealed that the Soxhlet extraction is suitable for extracting flavonoids.[7] Finally, E3 extraction technique is the best for extracting alkaloids from PGCG leaves since the number of alkaloids content was higher in method E3 than in other methods. Flavonoids were extracted in smaller quantities in E3 than in other methods.

Antioxidant analysis

All the aquoues extracts that have been resulted in four extraction techniqes exhibited antioxidant activity, however it varies significantly with the extraction process. Total antioxidant capacity by FRAP test revealed that the extraction method, E4 has better antioxidant capacity than the other three methods, as shown in Figure 3, and more interestingly all four methods have significant differences at the 5% significant level, as shown in Figure 2. The DPPH radical scavenging investigation revealed that the extraction technique E3 has relatively higher scavenging activity than the other three techniques, as shown in Figure 4. All four procedures have significant differences at the 5% level, as shown in Figure 2. Since this is the first study based on the evaluation of extraction techniques based on the phytochemicals and antioxidant capacity of guava grown in Sri Lanka, a compariation of the data in earlier findings is not possible.

Table 2: Comparison of phytochemical quantification data of four different aqueous extracts of PGCG leaves.

Phytochemicals	Extraction Types			
	E1	E2	E3	E4
Phenolic content (mg GAE/g)	187.07 ± 0.23	237.47 ± 0.23	269.47 ± 0.23	279.07 ± 0.23
Flavonoid content (mg QE/g)	26.81 ± 0.04	38.17 ± 0.03	23.80 ± 0.03	25.12 ± 0.03
Tannin content (mg TAE/g)	185.18 ± 0.23	235.08 ± 0.23	266.76 ± 0.23	276.27 ± 0.23
Terpene content (mM LE/g)	6.54 ± 0.01	7.04 ± 0.01	9.18 ± 0.01	28.61 ± 0.06
Alkaloid content (mg AE /g)	2.39 ± 0.07	1.59 ± 0.07	2.92 ± 0.20	1.80 ± 0.05
Saponin content (mg SE /g)	352.91 ± 2.18	575.29 ± 2.86	460.52 ± 4.37	536.71 ± 2.86

Values represent the mean ± standard deviation of a triplicate sample.

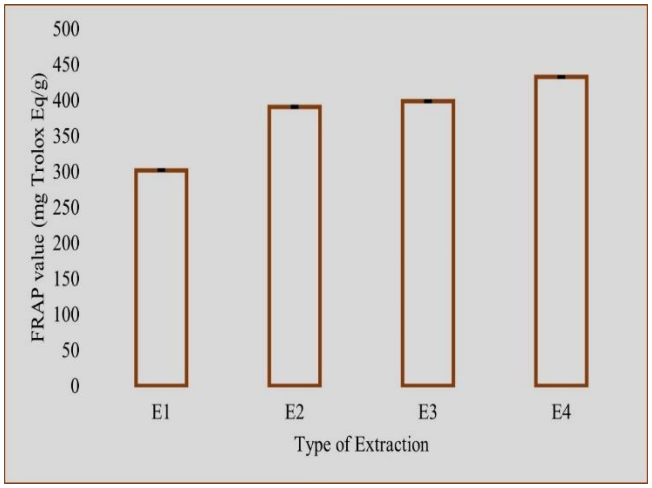

Figure 3: The total antioxidant capacity (in mg Trolox Eq/g) of crude aqueous extracts of PGCG extracted by four different extraction techniques.

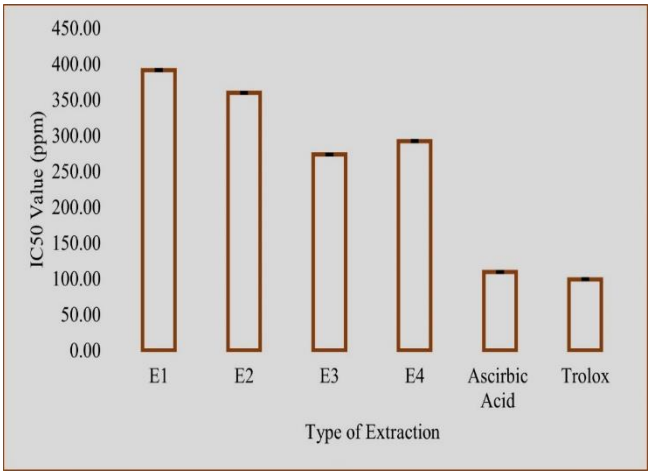

Figure 4: DPPH scavenging activity of crude aqueous extracts of PGCG extracted by four different extraction techniques and standards.

Conclusion

The following conclusion can be made on extraction of phytoconstituents from guava leaves; the extraction technique of maceration with agitation upon heat, is the most suitable extraction method to extract polyphenolics, tannins, and terpenoids. The Soxhlet extraction approach is good for extracting flavonoids and saponins where water is the solvent. The extraction method, maceration with agitation is suitable for extracting alkaloids. Finally, the selection of appropriate extraction techniques is highly important in the natural product isolation process from the plants in general.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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