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Research Article

Isolation of saponins from the leaves of *Psidium guajava* and *Garcinia quaesita* and antioxidative capacities

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Abstract

This study investigates the saponin content of *Garcinia quaesita* (Red-Garcinia) and *Psidium guajava* (Common-guava) leaves, which are known for their health benefits. The leaves were sonicated with 80% ethanol, and the obtained crude powders underwent sequential extraction/ fractionation with organic solvents. Crude saponins were obtained from the butanol and final aqueous layers. The functional characteristics of the isolated saponins were determined using FTIR spectroscopy. Antioxidant potency was evaluated through DPPH radical scavenging assay and the FRAP assay. Interestingly, both crude and isolated saponins exhibited higher antioxidant potential. *P. guajava* saponins, GuSBF3 showed the highest total antioxidant capacity (1753.86 ± 1.52 mg Trolox Eq.g^{-1}) and radical scavenging capability (IC_{50} value: 78.47 ± 0.02 ppm). The FRAP analysis of *G. quaesita* saponins indicated that the crude saponin exhibited the greatest total antioxidant capacity, measuring at 1284.56 ± 1.52 mg Trolox Eq.g^{-1}. Additionally, the DPPH assay demonstrated that a particular saponin, GaSBF4, possessed a superior radical scavenging capacity with an IC_{50} value of 102.94 ± 0.11 ppm. In this pioneering study, saponins were extracted for the first time from the Sri Lankan endemic plant *G. quaesita* and the widely cultivated guava species, *P. guajava*. Seven saponins were identified from each plant, though further purification is needed to determine their chemical structures. Notably, both crude and isolated saponins demonstrate considerable antioxidative activity.

Keywords
antioxidants, chromatographic techniques, *Garcinia quaesita*, *Psidium guajava*, saponins

Abbreviations

DPPH – 2,2-diphenyl-1-picrylhydrazyl; FRAP – Ferric Reducing Antioxidant Power; FTIR – Fourier-transform infrared spectroscopy; GaSBF – Garcinia saponin butanol fraction; GaSLF – Garcinia saponin last fraction; GuSBF – Guava saponin butanol fraction; GuSLF – Guava saponin last fraction

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Introduction

Botanical specimens play a significant role in the pharmaceutical domain, wherein global research endeavors are actively pursued to enhance their potential contributions to human society. *Psidium guajava* L., commonly referred to as guava, is a petite medicinal tree belonging to the Myrtaceae family. Indigenous to tropical America, it has been widely cultivated in tropical regions worldwide. *Garcinia quaesita*, also identified as Garcinia, is a medicinal tree within the Clusiaceae family, exhibiting endemism to the region of Sri Lanka (Nimanthika and Kaththriarachchi 2010).

Throughout antiquity, *P. guajava* has been employed in the treatment of diverse diseases. Beyond the fruit, guava leaves harbor a spectrum of chemical constituents possessing noteworthy pharmacological properties, including antioxidant activity (Ashraf et al. 2016; Bulugahapitiya et al. 2021), anticough activity (Jaiarj et al. 1999), antimicrobial activity (Biswas et al. 2013), antidiabetic activity (Mazumdar et al. 2015), antitumor activity (Ashraf et al. 2016), anticancer effect (Sato et al. 2010), anti-inflammatory activity (Jang et al. 2014), Anti-allergic effects (Han et al. 2011), etc. Conversely, the status of *G. quaesita* as an endemic plant in Sri Lanka has led to a dearth of investigations into the pharmacological properties of its leaves or the isolation of active compounds.

Saponins represent intricate compounds composed of non-sugar aglycones intricately linked to sugar chain units. Those saponins characterized by the presence of triterpene or steroidal aglycones, also known as sapogenins, fall within the aglycone classification (Oleszek and Bialy 2006). The separation of saponins poses challenges due to their tendency to exist in plants as a mixture of structurally related forms exhibiting highly similar polarities (Oleszek and Bialy 2006). Saponins have been associated with a diverse array of pharmacological effects, including but not limited to adaptogenic, antimicrobial, adjuvant, antimutagenic, analgesic, antiobesity, antiallergic, antioxidant, antiedematous, antiparasitic, antiexudative, antiphlogistic, antifeedant, antiprotozoal, antifungal, antipsoriatic, antigenotoxic, antipyretic, anti-inflammatory, antithrombotic, antispasmodic, antitussive, antiviral, antiulcer, cytotoxic, chemopreventive, diuretic, hepaprotective, haemolytic, hypoglycemic, hypocholesterolemic, neuroprotective, and other effects (Güçlü-Üstündağ and Mazza 2007).

While saponins exhibit diverse pharmacological characteristics, there is a current dearth of studies reporting on the presence of saponins in the leaves of Sri Lankan endemic plants *G. quaesita* and *P. guajava* cultivated in Sri Lanka, as well as their antioxidative potential. The absence of scientific research specifically focused on *G. quaesita* leaves underscores the significant responsibility to establish their botanical identity within the Sri Lankan context. Despite the challenging and time-consuming nature of saponin extraction, earlier research has provided evidence that *G. quaesita* and *P. guajava* leaves do indeed contain saponins (Kokilananthan et al. 2022a; Kokilananthan et al. 2020; Kokilananthan et al. 2022c), and considering saponins have a wide range of actions, starting the saponins isolation process is a great approach for future researchers. The objective of this study was to seperate saponin-entriched extracts from the leaves of Sri Lankan endemic plants, specifically *G. quaesita*, and *P. guajava*, which were cultivated in Sri Lanka. Additionally, the study aimed to evaluate the antioxidant activity of the seperated saponin fractions.

Materials and Methods

Plants materials and chemicals. The leaves of *P. guajava* and *G. quaesita* were collected in Matara, Sri Lanka, situated at a latitude of 5.9428ºN and a longitude of 80.5685ºE. The specimen of *P. guajava* was authenticated and deposited in the National Herbarium, Department of National Botanic Gardens, Peradeniya, Sri Lanka (Voucher Number AHEAD/DOR 05/C4). The specimen of *G. quaesita* was authenticated and deposited in the Bandaranaike Memorial Ayurvedic Research Institute, Nawinna, Maharagama, Sri Lanka (Voucher Number 3006).

Absolute ethanol (100%), n-butanol (99.9%), dichloromethane, diethyl ether (99.8%), dimethyl sulfoxide (99.5%), distilled water, ethyl acetate (99.5%), glacial acetic acid (99.8%), sulfuric acid (98%), and vanillin (99%) in AR grade, acetone (99.5%), hexane (99%), and methanol (99.85%), in GC grade, and isopropyl alcohol (99.9%), methanol (99.9%), and ultra-pure deionized water in HPLC

grade were utilized for the isolation and characterization of targeted compounds. All the chemicals were purchased from Merck, Sigma Aldrich.

Extraction of crude saponins. The healthy *P. guajava* and *G. quaesita* leaves were washed, allowed to air dry for three weeks, and then powdered. For two hours at 30-35℃, the powdered plant leaves were sonicated with 80% ethanol-water (Okubo et al. 2021). The extract was filtered using cotton wool, then Whatman No-01 filter paper, and the residue was sonicated with ethanol-water afresh (this process was triplicated). All the filtrates were collected and concentrated utilizing the rotary evaporator at 45℃, and the moisture was eliminated using the freeze dryer. Finally, crude extracts were collected and kept at -30℃ until it was needed.

Obtained crude powder from *P. guajava* leaves was re-extracted with distilled water by the method of indirect sonication (Time: two hours, Temperature: 30-35℃, Frequency: 40 kHz). The filtered, concentrated, and freeze-dried powder was defatted with diethyl ether by sonicating them. The diethyl ether contain with extracted fat was discarded and the remaining crude was used further extraction after concentrating them. The continuous extraction process was continued with dichloromethane, ethyl acetate and butanol (Güçlü-Üstündağ and Mazza 2007; Majinda 2012). Both butanol fraction (GuSBF) and the last water fraction (GuSLF), known as crude saponins, were stored at -30℃ for further analysis after concentrating and freeze-dried them. Saponins were extracted from *G. quaesita* leaves using the identical solvent system employed for guava saponin extraction, incorporating a process of fractionation (Güçlü-Üstündağ and Mazza 2007; Majinda 2012). All the butanol fractions (GaSBF) and the lastly obtained aqueous layer (GaSLF) were separately concentrated and both freeze-dried crude powders were used for further isolation process.

Conformation test for saponins.

Foam test. Extracted crude saponins (GuSBF, GuSLF, GaSBF, and GaSLF) were placed in a separate test tube which contained water and vigorously shaken; the production of stable foam was used to confirm the characteristics of saponins. In addition, the Olive oil test was carried out to confirm the existence of saponins (Biswas et al. 2013; Cangussu et al. 2021; Mohamed et al. 2021).

Vanillin-sulfuric acid test. Extracted crude saponins (GuSBF, GuSLF, GaSBF, and GaSLF) were treated with thin layer chromatography and the prepared vanillin-sulfuric acid reagent was sprayed, the red/pink color formation confirmed the saponins present (James and Dubery 2011). These qualitative assays indicated the proper separation of saponins from plant extracts.

Isolation of saponins using size exclusion chromatographic technique. Size exclusion chromatographic technique was used to seperate the saponin fractions from crude saponins where Sephadex LH-20 was utilized as the stationary phase (Clochard et al. 2020). At a flow rate of 24 drops per min, the compounds were eluted using a gradient of distilled water, methanol, and acetone as the mobile phase. Seven individual fractions obtained from each plant (*P. guajava*: GuSBF1, GuSBF2, GuSBF3, GuSBF4, GuSLF1, GuSLF2, and GuSLF3, and *G. quaesita*: GaSBF1, GaSBF2, GaSBF3, GaSBF4, GaSLF1, GaSLF2, and GaSLF3) were subjected to rotary evaporator and freeze dryer to concentrate the mobile phase used.

Identification of saponins using HPLC technique. To further identification, each seperated saponin fraction was subjected to analytical HPLC techniques (Liu et al. 2021). HPLC analyses were performed with SHIMADZU LC-20AP liquid chromatograph (Japan) with four solvent delivery system quaternary pumps (FCV-200AL) including a photodiode array detector (SPD-M40). The SHIMADZU LC was comprised with Degasser (DGU-10B) and the analytical line was especially interconnected with Autosampler (SIL-10AP), and Column oven (CTO-20AC). The purity and the compounds identification were analyzed by analytical HPLC technique (Anal column: Shim-pack GIST C18-AQ μm, 4.6 I.D. × 150 mm). 10.0 μL of sample was injected into the column by the auto sampler. Analytical methods were developed by changing the solvents polarity and flow rate where ultra-pure distilled water and methanol were used as the mobile phase. By changing the mobile phase polarity and solvent flow rate, HPLC method was developed to run all theseperated saponin fractions (Mobile phase; Water: Methanol = 1:1, and solvent flow rate; 1 $mL.min^{-1}$).

Fourier transform infrared spectroscopy (FTIR) analysis. Freeze-dried powders of the crude andseperated saponin fractions obtained from the leaves of *P. guajava* and *G. quaesita* and saponins standards were subjected to FTIR analysis. Infrared spectra were acquired using the FTIR spectrophotometer (Software; Spectrum IR), operating in ATR mode. Spectra were recorded between 4000-550 cm^{-1} at a resolution of 16 cm^{-1} and cumulating 32 scans (Almutairi and Ali 2015; Schreiner et al. 2021).

Antioxidant analysis. DPPH Radical Scavenging Assay, was employed to test the free radical scavenging activity of crude and seperated saponin fractions obtained from the leaves of *P. guajava* and *G. quaesita* (Kokilananthan et al. 2022a; Kokilananthan et al. 2022c). The DPPH solution (0.06 mM, 3.9 µL) was well mixed with different concentrations of extracts (100 µL). The absorbance at 517 nm was measured after 30 min in the dark. A percentage of scavenging effect vs. concentration plot was used to get the IC_{50} value for free radical scavenging activity.

Ferric Reducing Antioxidant Power (FRAP) Assay, to study the FRAP value of crude and seperated saponin fractions obtained from the leaves of *P. guajava* and *G. quaesita*, a standard technique described in the literature was used (Kokilananthan et al. 2022b; Kokilananthan et al. 2023). The test sample (100 µL) was mixed with freshly prepared FRAP solution (3 mL). The absorbance at 593 nm was measured after 30 min of incubation at 37℃. The standard Trolox was used for calibration.

Statistical Analysis. Analysis of variance (ANOVA), T-test (LSD; Least Significant Difference), and non-parametric statistics (Cochran's Q-test) were used to analyze and compare the data. SAS OnDemand for Academics: Studio (SAS 9.4) and R-studio software were used for the statistical analysis. The data were presented in the form of means and standard deviations.

Results and Discussion

Extraction of saponins. Owing to their distinctive structural characteristics, the extraction and seperation of saponins present a formidable challenge. This study represents the inaugural attempt to seperate saponin-entriched fractions from the leaves of Sri Lankan guava. The crude saponin content in guava leaves was quantified, yielding GuSBF at 1.08% and GuSLF at 3.18%. The respective yields of seperated saponin-entriched fractions from guava crude saponins are illustrated in Fig. 1. It demonstrates that GuSBF3 exhibits a higher yield in crude saponins among the GuSBF fractions, whereas GuSLF2 demonstrates a higher yield in crude saponins among the GuSLF fractions. Qualitative testing for saponins on the seperated fractions revealed a substantial abundance of saponins in the seperated fractions.

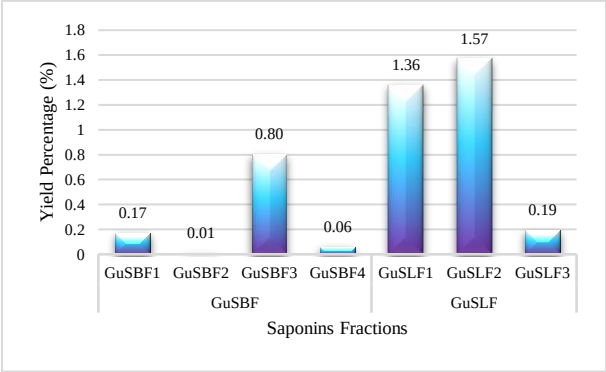


Figure 1. Yield percentage of seperated saponin fractions via size exclusion chromatography from Crude saponins extracted from *P. guajava* leaves

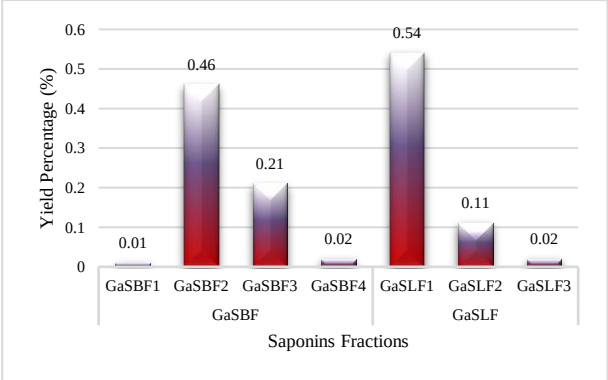


Figure 2. Yield percentage of seperatedsaponin fractions via size exclusion chromatography from crude saponins extracted from *G. quaesita* leaves.

Concerning the extraction of saponins from *G. quaesita* leaves, the crude saponin content in *G. quaesita* leaves was quantified, resulting in GaSBF at 0.76% and GaSLF at 1.79%. Qualitative saponin testing indicated a significant presence of saponins in the seperated fractions. The yield percentages of the seperated saponin fractions from the crude saponins of *G. quaesita* are presented in Fig. 2. Specifically, within the GaSBF fraction of *G.*

quaesita, GaSBF2 exhibited a notable quantity at 0.46%, while in the GaSLF fraction of *G. quaesita*, GaSLF1 demonstrated a higher quantity at 0.54%, as depicted in Fig. 2.

Characterization of saponins. The saponin fractions obtained from the leaves of *P. guajava* and *G. quaesita* were analyzed using High-Performance Liquid Chromatography (HPLC) to assess the purity of the separated fractions derived from the crude saponins. The HPLC spectra indicated the presence of predominant peaks in the majority of the fractions, accompanied by minor impurities. Impurities in saponin fractions from *P. guajava* and *G. quaesita*, identified by HPLC, may arise from factors such as co-extracted compounds, variations in plant material, incomplete separation, contaminants in extraction solvents, and environmental influences during plant growth. The inherent complexity of plant matrices may lead to the co-isolation of structurally related compounds. To enhance purity for accurate characterization, refinement of extraction and isolation methods, along with precise chromatographic optimization, is crucial. The HPLC spectra of saponin fractions are provided as an appendix in Fig. A.1 and Fig. A.2. As a result, further purification is required before proceeding with the structural elucidation of those compounds.

Moreover, for the elucidation of functional groups, FTIR analysis was conducted on all saponin fractions, confirming their distinctive characteristics. The FTIR spectra of the saponin fractions are appended in Fig. A.3 and Fig. A.4. Notably, the spectra revealed characteristic infrared absorbances corresponding to the hydroxyl group (OH), carbon-hydrogen (C-H), C=C bonds, C=O bonds, and the unique Oligosaccharide linkage absorptions associated with sapogenins, specifically C-O-C.

Antioxidant Potential of the Saponin Fractions. The antioxidant activity of crude and seperated saponin fractions from the leaves of *P. guajava* and *G. quaesita* was assessed using the FRAP and DPPH assays. The distinct FRAP assay results for each plant are presented individually in Fig. 3, accompanied by corresponding statistical analyses in Fig. A.5. Similarly, the DPPH assay outcomes are illustrated separately in Fig. 4, with the respective statistical analyses displayed in Fig. A.6. Notably, both the crude saponins and a significant proportion of the saponin fractions exhibited noteworthy antioxidant potential, as indicated by the results of both the FRAP and DPPH assays.

The saponin fractions of *P. guajava*, seperated through size-exclusion chromatography, exhibit heightened antioxidant capacity in comparison to the crude saponins extracted from P. guajava leaves. In the assessment of the antioxidant potential via FRAP and DPPH assays, GuSBF3 emerged as the fraction with the highest antioxidant capacity, quantified at 1753.86 ± 1.52 mg Trolox Eq.g^{-1}, along with the lowest IC_{50} value of 78.47 ± 0.02 ppm, surpassing both the crude saponins and other fractions. Statistical analysis further confirms the superior antioxidant potential and radical scavenging capacity of GuSBF3 relative to other fractions, and the divergent antioxidant capacities among fractions were found to be statistically significant at the 5% significance level.

Regarding *G. quaesita* saponins, the FRAP experiment delineated that the crude saponins (GaSLF) exhibited the highest total antioxidant capacity at 1284.56 ± 1.52 mg Trolox Eq.g^{-1}. Concurrently, among the fractions, GaSBF4 demonstrated superior radical scavenging capacity with an IC_{50} value of 102.94 ± 0.11 ppm. Statistical analysis substantiated these findings, affirming the accuracy of the stated observations. Furthermore, each saponin fraction from *G. quaesita* leaves demonstrated varying degrees of antioxidant potential. Notably, in terms of radical scavenging capacity, pairs such as GuSLF and GuSBF1, as well as GaSBF4 and GaSLF3, exhibited equivalent peaks at the 5% significance level.

The remarkable antioxidant properties exhibited by the saponins of *P. guajava* and the endemic Sri Lankan plant *G. quaesita* underscore their potential as valuable compounds with significant pharmacological implications. These compounds, owing to their robust antioxidant characteristics, could serve as promising sources for various pharmacological applications. Consequently, further investigation into the pharmacological properties of these fractions is imperative. It is noteworthy that ongoing efforts involve additional purification and detailed characterization of these compounds, enhancing our understanding of their potential applications in pharmaceutical contexts.

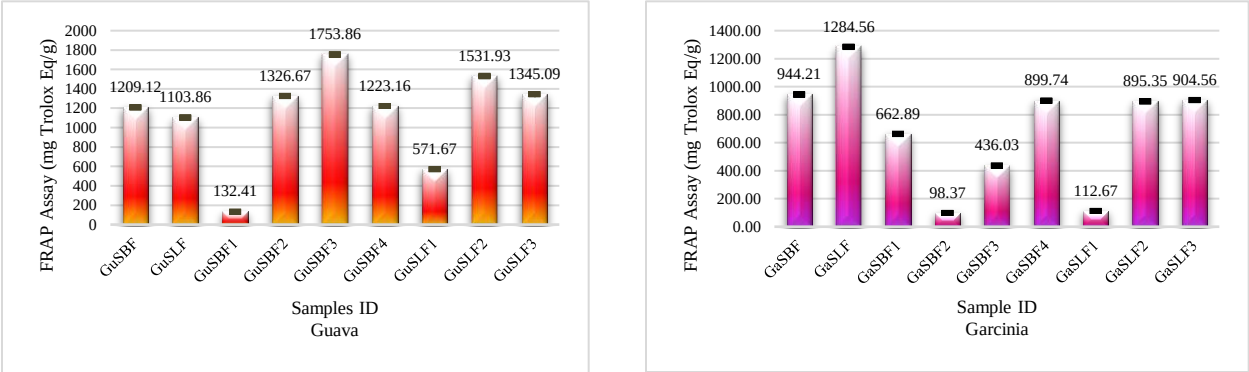


Figure 3. Total antioxidative capacity of crude and saponin fractions from the leaves of *P. guajava* and *G. quaesita*: FRAP assay

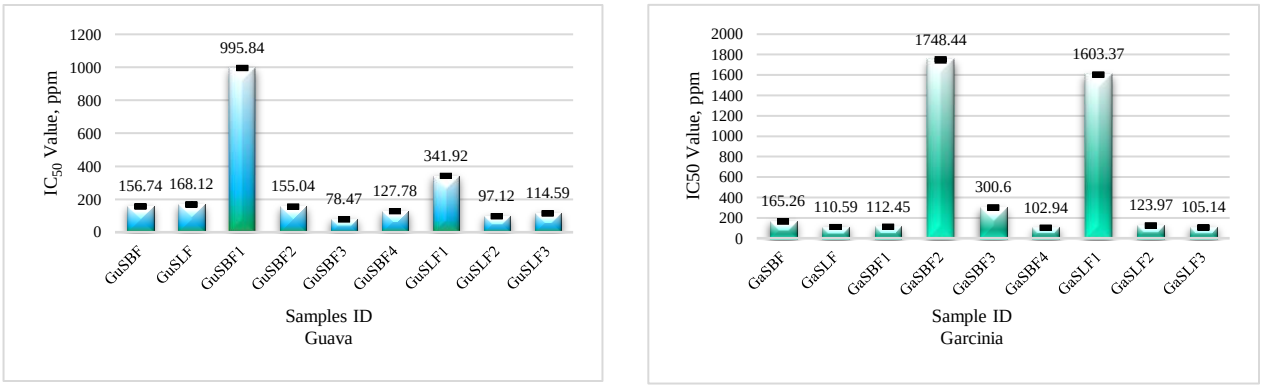


Figure 4. Radical scavenging capacity of crude and saponin fractions from the leaves of *P. guajava* and *G. quaesita*: DPPH assay.

Conclusions

In conclusion, this study represents a pioneering effort in the seperation of active saponin fractions from the leaves of Sri Lankan endemic plant, *G. quaesita*, and *P. guajava* cultivated in Sri Lanka. The extraction process resulted in the identification of seven distinct saponins from the crude saponins of each plant. Notably, allsaponin fractions exhibited significant antioxidant activity, with GuSBF3 demonstrating exceptional antioxidant capacity.

The findings suggest the considerable potential of *G. quaesita* and *P. guajava* leaves as sources of natural antioxidants. The seperated saponin fractions hold promise for application in the development of antioxidant-rich functional foods and nutraceuticals. Particularly, the outstanding antioxidant performance of GuSBF3 underscores its potential as a key component in such formulations.

As we embark on the prospect of utilizing these separated saponin fractions for nutraceutical development, it is imperative to acknowledge the necessity for further research. Ongoing investigations aim to purify these saponins and rigorously assess their toxicity profiles. This precautionary step is vital to ensure the safety and efficacy of the derived compounds before they can be integrated into antioxidant-based nutraceutical products. The outcomes of this study not only contribute to the understanding of the antioxidant potential of *G. quaesita* and *P. guajava* leaves but also set the stage for future applications in the field of functional foods and nutraceuticals.

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