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Enhanced Harnessing of the Graviola Bioactive Components Using a Neoteric Sonication Cum Microwave Coadjuvant Extraction Protocol

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Abstract: Graviola is one of the most accomplished natural anticancer therapeutics gaining popularity in recent times. Harnessing the full benefit from tapping all of its rich bioactive reservoirs is absolutely worthy and mandatory. It is in this regard that a well optimized extraction methodology gains paramount importance. In case of Graviola, no sophistication in terms of extraction methods is reported. A neoteric sonication cum microwave combined extraction technology was introduced that maximized the extraction process and minimized (7 min) the extraction time. The extraction efficiency was validated based on the significant enrichment of bioactive ingredients in Graviola extracts following the sonication cum microwave combined protocol.

Keywords: Graviola; bioactivity; microwave; sonication; acoustic cavitation

1. Introduction

Graviola, commonly called soursop belongs to the *Annonaceae* family, whose scientific name is *Annona muricata*. Graviola plays a crucial role in various traditional and alternative medicines. All parts of this evergreen tree which inhabits tropical and sub-tropical areas are used in natural medicine. The bark, leaves, roots, fruit and fruit seeds have their own respective use and specific properties and uses are attributed to different parts of the tree. The Graviola fruit is heart shaped and edible and together with its leaves, root and seeds contributes significantly in alternative medicine [1]. Generally, the fruit and fruit juice are used against worms and parasites in the body, against fever, to enhance mother's milk after childbirth and as an astringent for diarrhea and dysentery. The seeds are crushed and used against internal and external parasites, head lice and worms. The bark, leaves and roots are considered antispasmodic, hypotensive and sedative. The soursop tea prepared from the leaves and stem is consumed to provide relief for various disorders towards those effects. The fruits of Graviola are extensively used to make candies, syrups, ice creams, shakes and beverages. The fruit is also consumed as such. In the Brazilian Amazon soursop leaf tea is used to solve liver related problems. The oil extracted from the leaves and unripe fruit is mixed with olive oil and is used externally for neuralgia, rheumatism and arthritis pain.

Ethano-medicinal activities in Africa and South America employ this plant source in their conventional medicine. Researchers have established the anticonvulsant, antiparasitic, anti-arthritic, antimalarial, antidiabetic hepato protective and anticancer properties of this plant. Biological and biochemical characterization indicated that annonaceous acetogenins are the main ingredients of Graviola [2–4]. Predominantly, *Annona muricata* or Graviola plays a key role in promoting anticancer activity. *A. muricata* leaves have been subjected to investigation for a large number of human diseases, including cancer [5]. In the United States and Europe, Graviola is sold as a popular adjunctive natural therapy for cancer.

The key biomolecules orchestrating the bioactive properties of Graviola are the acetogenins. These acetogenins have demonstrated selective toxicity to tumor cells at even trace dosages (1 part per million). Further the chemicals and acetogenins in Graviola have been demonstrated to also show strong anticancerous, antitumorous and antiviral properties too [6–17]. With the huge back up and evidence regarding the medicinal aspects of Graviola, the soursop tea extracts prepared from the leaves has become a vouched-for commodity. Commercial sellers have commercialized products involving soursop leaf powders and tea bags for tea extracts. There is also the other product of selling dried leaves in traditional medicine shops. These leaves are also meant for soursop tea preparations.

In the present work, we have attempted validation of one such dried soursop leaf product. We have prepared soursop tea using the conventional boil and add leaf procedure. We have also tried to push the extraction efficiency to the maximum using various others methods such as prolonged boiling, sonication, microwave extraction and a neoteric sono-microwave extraction methodology. We have attempted to reduce the extraction time using this introduced integrated technology. The enhanced bioactive properties as shown by total phenolics, flavanoids and antioxidant ability were validated for the five extraction methodologies compared. This is the first attempt in testing the efficacy and claim of a commercial soursop product.

2. Materials and Methods

2.1. Preparation of Extracts

Dried Graviola leaves (origin: Indonesia), were procured from Daesung International, Ojosan-r 57 bean-gil, Gyeyang-gu, Incheon, Republic of Korea. 2 g of the leaves (~4 leaves) were cut into small 1cm sized strips along the breadth of the leaves and used for the extraction procedures. Five different extraction methodologies were attempted. The first and second methodologies are that which are traditionally practiced. The preboiled water extraction (PB-WE) method included adding the 2 g leaves in 50 mL of boiling water (water brought to rolling boil) and keeping it aside from the heater for 15 min allowing for the extraction. After the specified time the extracts were filtered and stored away and labeled as PB-WE. The co-boiled water extraction (CB-WE) procedure, which is the second method, involves adding the 2 g leaves to 50 mL water and then allowing the contents to boil till evaporated to half the original volume. The final volumes were made up to 50 mL in all cases. This second extraction method was labeled CB-WE. The third extraction method involved sonicating 2 g Graviola leaves in 50 mL water at 40 °C using a JAC-2010 bath sonicator (JAC-2010, AC220 V, 300 W /60 Hz), purchased from KODO Technical Research Co., Ltd., Korea (Hwaseong, Korea) for 15 min. The time of sonication lasted 15 min, after which the extracts were filtered and stored away as sonicated water extracts (S-WE). The same bath sonicator was used for all the experiments; probe sonicator was not employed at all. The fourth method used a microwave (Samsung Model No. RE MC20T) (Samsung, Seoul, Gangnam-gu, Nonhyeon 1(il)-dong, 122-2. Republic of Korea), having specifications as follows: 220 V, 60 HZ, 1100 W, 700 W output, 2450 MHz oscillation frequency. Following the microwave based extraction for 2 min, the contents were filtered and stored away as microwave water extracts (MW-WE). The last method, involved subjecting 2 g Graviola leaves in 50 mL water to S-WE for 5 min followed by 2 min MW-WE, this sample was labeled sono-microwave water extraction (SMW-WE). Thus, the neoteric sono-microwave extraction had an initial 5 min bath sonication followed by a 2 min

microwave extraction. The sonicator and the microwave used were the same as above. All samples were stored in the refrigerator until further use.

2.2. Characterization of Extracts

The phenolic compounds, flavonoid content and antioxidant activity of the Graviola extracts obtained from the five different extraction methods was evaluated. Phenolic compounds in the PB-WE, CB-WE, S-WE, MW-WE and SMW-WE of the Graviola leaves were analyzed based on the spectrophotometric method described by Loots et al., 2007 [18]. Briefly, 50 µL of the respective extract was mixed with 1150 µL of distilled water and 200 µL of dilute Folin–Ciocalteu reagent was added. The mixture was thoroughly shaken and allowed to stand for 7 min at room temperature. 600 µL of 20% sodium carbonate aqueous solution was added to this mixture and incubated for 60 min and the absorbance measured at 765 nm against the blank using a Shimadzu UV-1700 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Different concentrations of gallic acid (0.0325–0.5 mg/mL) were used to construct the calibration curve. The results were expressed as mg of gallic acid equivalents per gram dry weight (mg GAE/g DW).

The total flavonoid content in the different Graviola extractions was estimated by a colorimetric assay according to Chang et al., 2002 [19] with slight modifications. Briefly, 0.5 mL of the respective extract was mixed with 0.1 mL of 10% aluminum chloride and 4.3 mL of distilled water. The mixture was incubated for 30 min at room temperature and the absorbance measured at 415 nm using a Shimadzu UV-1700 spectrophotometer. Rutin was used as the standard (15.15–500 µg/mL) and the results were expressed as µg rutin equivalent per gram dry weight (µg RE/g DW).

The antioxidant activity of the five different Graviola extracts was determined following Chung et al., 2000 [20]. 0.25 mL of each extract was mixed with 2.5 mL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) and made up to 3 mL with distilled water. The mixture was vortexed and incubated for 80 min at room temperature in a dark place. The absorbance was measured at 517 nm using a Shimadzu UV-1700 spectrophotometer. The extract replaced with distilled water served as the control. The scavenging activity was calculated using the following equation:

$$\text{Free radical scavenging effect\%} = (1 - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

The absorbance of the 5 different Graviola extracts was estimated using a NanodropND-1000 v 3.3.1 spectrophotometer, (Nanodrop Technologies, Inc., Wilmington, DE, USA), by scanning from 220 to 700 nm.

3. Results

The total phenolics, flavonoids and antioxidant activity of the Graviola extracts prepared using the five different methodologies was determined. These parameters are reported to signify the bioactivity of the tested sample. Of the five different methodologies used, the first two, PB-WE and CB-WE were the commonly practiced extraction methods, while the sonication based (S-WE), microwave based (MW-WE) and the dual SMW-WE methods were those that were applied and experimented on for the first time in this report. The successful extraction of the bioactive compounds following the application of the various extraction methods validates the efficiency of the applied extraction protocol. Figure 1A presents the comparative results of the five different protocols on the extraction of bioactive total phenolic substances from Graviola leaves. As observed from the figure, significantly higher phenolic compounds were observed in SMW-WE, MW-WE and CB-WE while PB-WE and S-WE were almost similar. Phenolics are major bioactive compounds known for their health benefits reported to exhibit multiple biological effects, including antioxidant activity. Their scavenging activity is mainly due to their redox properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [21]. The efficient extraction of higher phenolics signifies the efficacy of

the extraction technique in leading to enhanced harnessing of the bioactivity of the Graviola leaves. The SMW-WE method is considered superior to the others in this aspect.

Flavonoids belong to a large group of polyphenolic compounds having a benzo- γ -pyrone structure and are ubiquitously present in plants. It is reported that secondary metabolites of phenolic nature including flavonoids are indulging in a variety of pharmacological activities [22–24]. Phenolic acids are secondary metabolites widely distributed in the plant kingdom and are second only to flavonoids in terms of their dominance. So saying, flavonoids play an equilateral role in the bioactivity and are reported to occur in abundance in case of Graviola extracts and participate in the so called ‘sour sop properties’ [25]. The efficient extraction of these vital bioactive components from the Graviola leaves was evaluated in order to rate the efficiency of the five different extraction methods employed. In case of flavonoids (Figure 1B), an almost similar trend with marginal variation was observed. SMW-WE lead to the highest extraction of flavonoids from the Graviola leaves, followed by MW-WE > CB-WE. S-WE was more effective compared to PB-WE in terms of extraction of flavonoids. A recent study demonstrated the importance of flavonoid content in Graviola, where the synergistic interactions among flavonoids and acetogenins in Graviola (*Annona muricata*) leaves conferred protection against prostate cancer [25]. Hence, in this aspect the fact that the SMW-WE method led to enhanced extraction of flavonoids stands highly significant in terms of enhancing its anticancer properties.

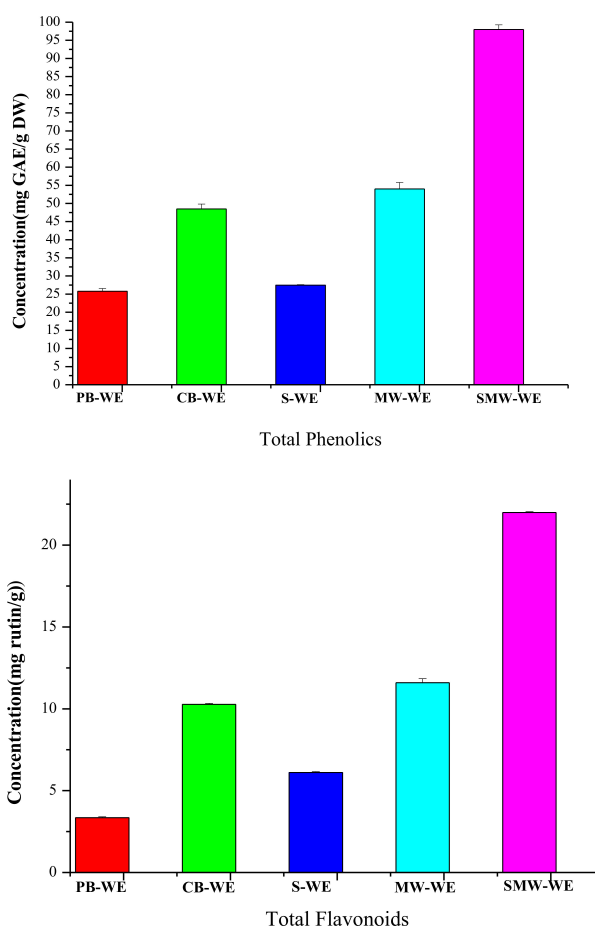


Figure 1. (A) Graph showing total phenolics extracted using the five extraction methods; (B) Graph showing flavonoid contents from Graviola leaf following extraction using the five different methods. preboiled water extraction (PB-WE); co-boiled water extraction (CB-WE); sonicated water extracts (S-WE); microwave water extracts (MW-WE); sono-microwave water extraction (SMW-WE).

The antioxidant activities of extracts obtained from employing the five different extraction methodologies were also evaluated using the DPPH and FRAP assays. Figure 2A,B give results of these

assays. DPPH and FRAP assays showed a similar trend where SMW-WE extraction method yielded extracts exhibiting the highest antioxidant activity, followed by PB-WE extracts. It was interesting to observe that the antioxidant activity of the extracts, varied from the total phenolics and flavonoids where PB-WE recorded the least. S-WE and MW-WE showed high antioxidant activity too. The least antioxidant activity was observed in CB-WE. Earlier authors have reported the relationships between phenolic content and antioxidant activity; while some authors found a high correlation between the phenolic content and the antioxidant activity [26–30], others found no relationship [31,32]. In our study we found a strong relationship between the phenolic contents and antioxidant activity. These results signify that in terms of enhanced bioactivity of the Graviola leaf extraction, the most ideal extraction method was the SMW-WE method, which uses sonowaves combined with the microwave technology. This dual extraction method lasted only 7 min, hence less time consuming compared to the conventional extraction methods which required PB-WE = 30 min and CB-WE, about 20 min. The standalone microwave based extraction (MW-WE) was also significantly less time consuming (2 min) and more efficient, while the sonication method (S-WE) required 15 min and was less efficient. Thus as shown by these results, the neoteric sono-microwave conjunct methodology was optimized as the most ideal and successful protocol for enhanced extraction of bioactive components from Graviola leaves.

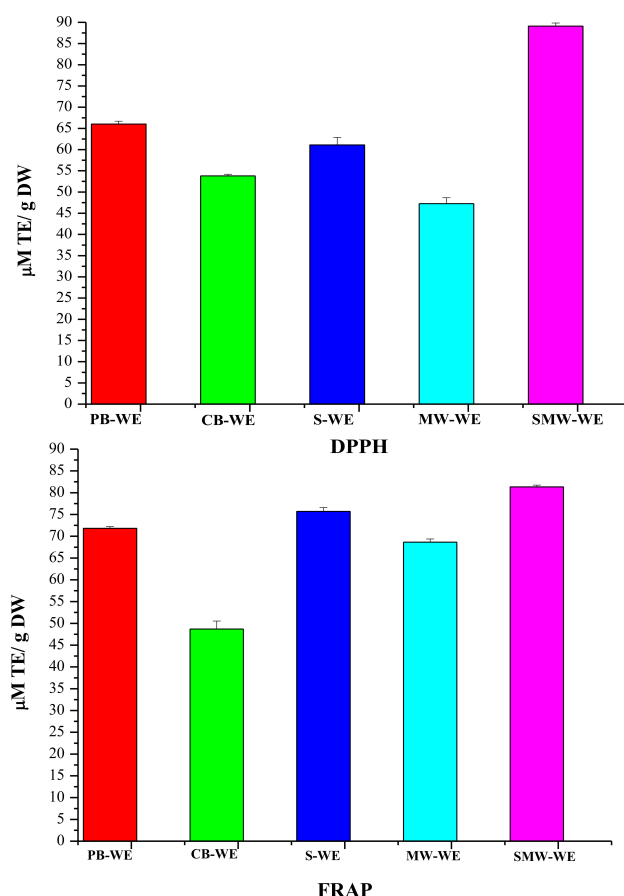


Figure 2. Comparative antioxidant properties of the Graviola leaf extracts using (A) DPPH assay and (B) FRAP assay.

UV-Visible spectrometric analysis (Figure 3) of the extracts obtained using the five different methodologies indicated the presence of extensive peaks in the SMW-WE, CB-WE, MW-WE extracts compared to the PB-WE and S-WE methods. As revealed from the earlier results pertaining to the total phenolics, flavonoids and antioxidant activity, where SMW-WE, CB-WE, MW-WE recorded higher values, these UV results also confirm that enhanced extraction was enabled by these methodologies

compared to PB-WE and S-WE methods. The fruits, leaves, stems and roots of Graviola are known to be rich in flavonoids, isoquinoline alkaloids and annonaceous acetogenins [2,33–40]. It is reported [41,42] that absorption peaks from 200 to 230 nm are characteristic of acetogenins. In case of PB-WE and S-WE extracts we find that specifically a broad band peak in this wavelength dominating. While the other extracts too show peaks within this range, other peaks possibly belonging to other flavonoids, alkaloids are also observed.

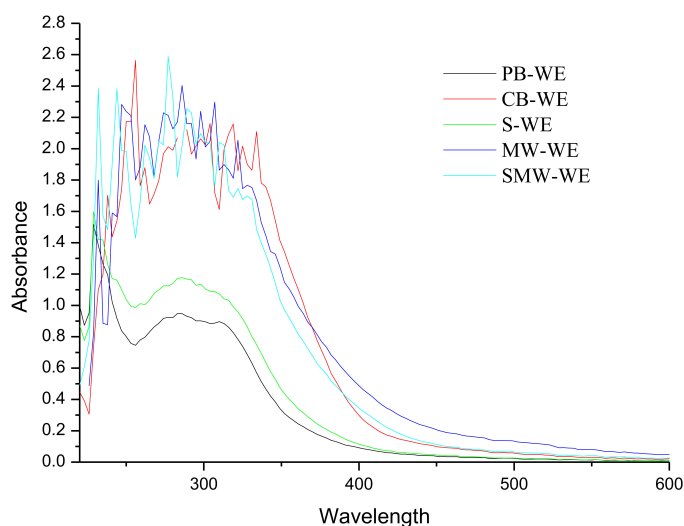


Figure 3. UV-Visible spectra of the extracts showing peaks pertaining to the flavonoids and phenolics present in Graviola.

Phytochemical screening conducted on leaves extracts of *A. muricata* revealed the presence of following classes of compounds: alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols, phytosterols, and saponins [43]. These are reported from both the ethanolic and water extracts of Graviola leaves. Generally, it is known that the presence of alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiacglycosides, phenols, phytosterols, and saponins validate the economic importance of *A. muricata* leaves extracts for extensive use in medicine both traditionally and pharmaceutically [33]. These compounds were reported for their antimicrobial, antifouling, antioxidant, hypo-cholesterolemic, anti-tuberculosis, insecticidal, anti-inflammatory, nematocidal, pesticidal, anti-androgenic, hemolytic 5-alpha reductase inhibitory, anticancer, anti-diuretic, immune-stimulatory, anti-diabetic, antihistaminic, insectifugal, anti-eczemic and anti-acne properties [33].

4. Discussion

Speculating the reason for the success behind the SMW-WE methodology, previous authors have described that microwave-assisted extraction was a more effective technique compared to the maceration method, reflux method, Soxhlet method and ultrasonic methods for the extraction of bioactive compounds from *Urtica dioica* leaves and stems. Conventional extraction methods have been associated with high solvent requirements of toxic organic solvents, usually required long extraction time which increased risk of degradation of thermo-labile constituents thus resulting in lower extraction yields [44–46]. In the present study we have limited our extraction solvent to water, in order to rid the use of toxic organic solvents. When previous authors [46] used microwave extraction, they have reported that the extraction time was reduced, less solvent was used and the amounts of extracted compounds were increased. During microwave extraction, the solvent penetrates into the solid matrix by diffusion (effective), and the solute is dissolved until a concentration limited by the characteristics of the solid is attained. Then the solution containing the solute diffuses to the surface

by effective diffusion. Finally, by natural or forced convection, the solution is transferred from the surface to the bulk solution, effecting successful extraction. It is reported that in microwave extraction, the process acceleration and high extraction yield are a result of the synergistic combination of two transport phenomena namely, heat and mass gradients working in the same direction [47–49]. However, in conventional extractions the mass transfer occurs from inside to the outside, although the heat transfer occurs from the outside to inside, this largely slows down the efficiency of the conventional extractions. By increasing the extraction efficiency, we can abstain from the use of efficient yet toxic organic solvents and stick to the use of water as the solvent system. With this idea in mind we have combined two effective extraction systems to facilitate more effective extraction. Prior to microwave extraction we have rendered a 5 min pulse of sonication, this is to facilitate the release of the cages bioactive compounds prior to microwave digestion. Sonication works on acoustic cavitation [49]: the formation, growth, and implosive collapse of bubbles in a liquid leading to rupturing of cell walls and glands for releasing the bioactive compounds easily during the subsequent microwave treatment. As observed from our results standalone sonication (S-WE) or microwave (MW-WE) did not yield as much extraction efficiency as when combined (SMW-WE). Combining both these advanced and accomplished extraction systems is that which lead to the enhanced extraction of *Graviola* bioactive components as demonstrated in this study. As Figure 4 demonstrates, the mode of action following the sono-microwave method (SMW-WE) for the effective extraction of the bioactive compounds in *Graviola* in water is as follows: (i) the 5 min bath sonication process, leads to the rupturing of cell walls through localized acoustic cavitation, creating the pathway for the release of the bioactive compounds locked within and (ii) the 2 min microwave treatment, enables the interaction of the solvent with the leaf material, the sonication process paved way for the enhanced penetration of the solvent, enabling easy and effective solubilization of the caged compounds, resulting in effective extraction.

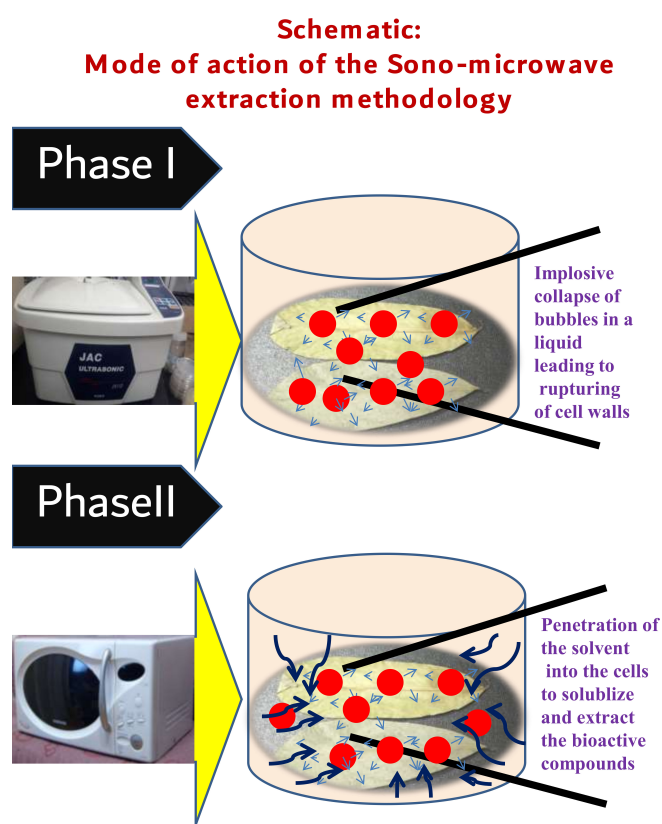


Figure 4. Schematic showing the mode of action for enhanced release of bioactive compounds by the demonstrated sono-microwave methodology for effective extraction of bioactive compounds from *Graviola*.

The importance of analyzing the crude extract in case of such medically renowned products, is for the reason of convenience and cost-effectiveness apart from the fact that it is actually the crude mixtures that are the most likely product that will be widely used. These mixtures could possess synergistic properties that would be lost in the analysis of pure compounds. And hence this study has ardently stuck to analysis of crude extracts. However, further studies for pharmaceutical applications would need to extend to purification and detailed analysis subsequent to following the proposed optimized extraction method presented here.

5. Conclusions

In conclusion this work confirms the indispensable efficiency of the SMW-WE method to lead to rapid and efficient extraction of bioactive components from Graviola leaves that are of medical and pharmaceutical relevance. The demonstrated extraction using the sono-microwave combined strategy will undoubtedly greatly influence the pharmaceutical and medical application of Graviola extracts significantly.

Author Contributions: Se Chul Chun and Judy Gopal conceived and designed the experiments; Judy Gopal, Shang Xiaomin, Shimels Tilahun Belachew and Hyejin Jung performed the experiments; Judy Gopal, Vimala Anthonydhason and Diby Paul analyzed the data and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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