Abstract
Alteration of radical scavenging capacity (RSC), phenol (TPC) and flavonoid contents (FC) of pomegranate fruit parts were extracted using Boiling, Sonication, Microwave, Sonication followed by microwave and compared. RSC was determined by DPPH activity and FRAP assay while TPC and FC were measured by Folin-Ciocalteu method and Aluminium chloride colourimetric assay, respectively. Sonicated peel extract exhibited maximum RSC (IC50 3.3 μg/mL), PC (64.0 W/W% Gallic Acid Equivalent) and FC (19.7 W/W% Quercetin Equivalent). Fermented juice showed higher RSC (243 fold less IC50), TPC (4 folds) and FC (1.5 folds) than the fresh juice. Seeds had minimum phytochemical content; RSC (IC50 11698 μg/mL), PC (2.0 W/W% Gallic Acid Equivalent) and FC (2.2 W/W% Quercetin Equivalent). GC-MS enable to identify some functional groups (Furfural, Benzoic acid, and n-Hexadecanoic acid) in sonicated peel water extract. Thus, this study revealed Sri Lankan pomegranate peel presented with excellent TPC, FC and RSC than other parts of pomegranate fruit apart from extraction method.

Total phenol Content
Keywords: Pomegranate, Polyphenols, Radical Scavenging Capacity, Sonication, DPPH

Introduction
Natural phytochemicals play a major role in maintaining a healthy life in humans. These phytochemicals can be consumed generally during diets. Phytochemicals, the bioactive plant compounds in fruits, vegetables, grains, and other plant foods, have been linked to reductions in the risk of major chronic diseases such as; cancer, diabetes, cardiovascular diseases, inflammation, and neurologic disorders. Fruits and vegetables contain a wide variety of phytochemicals with antioxidant properties such as polyphenols, flavonoids, isoflavones and carotenoids that may help to protect cellular systems from oxidative damage and lower the risk of chronic diseases (1).

Pomegranate (Punica granatum L.) is a medicinal herb belongs to Lythraceae family. Pomegranate in Sri Lankan home gardens has been revealed to have nutritional benefits (2). Even though in Indian ayurveda and siddha medicine have been used pomegranate as an anti-tumor remedy it was not based on scientific experiments (3). It is reported that pomegranate peel, pericarp (mesocarp), juice, seeds, leaves, bark have shown several health benefits such as antibacterial, antiviral, antioxidant, anti-inflammatory activities (4)(5)(6). Punicic acid, oleic acid, palmitic acid, stearic acid, linoleic acid, sterols, tocopherols were identified as the major constituents of the pomegranate seeds (7)(8). Pomegranate fruit is rich in tannins, phenolics (ellagic tannins, ellagic acid and gallic acid), which are potent antioxidants (9). Pomegranate peel which is inedible, constitutes about 50% of the total fruit weight and...
it contains higher amounts of polyphenols than the juice, which is discarded as a waste (10). Department of Agriculture, Sri Lanka has been introduced three varieties of Sri Lankan pomegranate; *Nimali*, *Daya* and *Nayana* as Sri Lankan varieties. Health benefits of *Nimali* were investigated, as the most popular variety of pomegranate among three, due to its high yielding ability, soft seeded and sweet taste (11).

Efficiency of emerging extraction methods compared with its phytochemical analysis with selected five notable methods. SN considered as an emerging and efficient method which can intensely reduce extraction time while increasing extraction yields and antioxidant properties of extracts (12)(13). This highlighted the importance of extraction step for yield of total phenolic, flavonoid contents and antioxidant activities. Analysis of phenolic compounds and evaluation of anti-oxidant activity in different parts of Sri Lankan pomegranate fruit is not elucidated, hence the present investigation gains importance towards understanding its potential health benefits.

**Materials and Methods**

**Materials**
Gallic acid, Folin-Ciocalteu reagent, Trichloroacetic acid, Sodium carbonate (Na₂CO₃), Aluminum chloride (AlCl₃), Sodium nitrite (NaNO₂), Sodium hydroxide (NaOH) were purchased from Sigma Chemicals Co. (P.O.Box 14508, St. Louis, MO 63178 USA). 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), polyvinyl-polypyrrolidone (PVPP) and Quercetin (≥95%) were purchased from Fluka (Flukachemie GmbH, CH-9471 Buchs). Other chemicals were obtained from Sigma-Aldrich Co (St Louis, MO, USA) unless mentioned. All chemicals used were in analytical grade.

SHIMADZU UV 1601 UV/Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used to measure absorbance. Deionized water was obtained from Labconco Water Pro-PS UV ultra-filtered water system (Labconco Corporation, Kansas City, Missouri). Labconco Free Zone Legacy 2.5 L Benchtop Vaccum Freeze Drier was used to obtain the lyophilized plant samples (Model No: 7670530, -54° with SS interior, 220V). Sonication done with NEY ULTRAsonic cleaner controller 300 (The J M N ey Company Barkmeyer Division 13553 Calimesa Blvd, Yucaipa, CA, 50 kHz, 135 W). Memmert WNB 22 water bath (Memmert GmbH + Co. KG, Aeussee Rittersbacher Strasse, 38, D-91126, Schwabach) used for heating of samples.

**Plant materials**
Sri Lankan *Punica granatum* L. (*Nimali*) obtained from Fruit Research Institute, Department of Agriculture, Kalpitiya, Sri Lanka (January 2017). Plant species was taxonomically identified and voucher specimen was deposited in Botany Department, Bandaranayake Memorial Ayurveda Research Institute, Nawinna, Colombo, Sri Lanka under number 2025). Pomegranates were handpicked, washed and stored in 4°C refrigerators (14). The fruit was separated to its parts (pericarp, peel, seeds, juice) manually.

**Sample Preparation**: Freeze-drying retains higher levels of phenolic content in herbal samples compared to air-drying (15). Pomegranate peel, pericarp, fresh juice, fermented juice and seed samples freeze dried at -40°C until a constant weight acquired.

**Preparation of Pomegranate peel and pericarp extracts**
Pomegranate peel and pericarp extracts were prepared by five different methods; boiling, sonication, heating in water bath, microwave and sonication followed by microwave. Lyophilized peel samples were grounded to obtain a powder and sieved to remove coarse particles. The fine powder was subjected to the following extraction methods. 1) Boiling water (45 minutes) 2) Sonication (50 kHz, 135 W, 30 minutes) 3) Sonication (50 kHz, 135 W, 30 minutes) followed by microwave (2450MHz, 1050W, 3 minutes) 4) Microwave (2450MHz, 1050W, 3 minutes) 5) Water bath (50°C, 20 minutes). Two grams of peel (n=6) / pericarp powder (n=6) was dissolved in 100mL deionized water for each extraction method. Extract was centrifuged (3000 rpm, 10 min) and filtered through Whatman No. 1 filter paper. Resulting filtrate of five extractions were freeze dried until obtained a constant weight and stored in -20°C freezer.
Lyophilized samples of peel and pericarp were prepared and the yield was calculated as a percentage of dry weight.

**Preparation of Juice extracts** Juice was separated into two portions. One portion was allowed to ferment with wine yeast (*Saccharomyces bayanus* – Lalvin EC-1118, from Lallemend, Montreal, Canada) (1g for 3.8L) for one week and filtered extract was freeze dried until a constant weight obtained. Other portion was allowed to remain as fresh juice and freeze dried until a constant weight obtained.

**Preparation of Seed Extract:** Seeds were cleaned with deionized water and freeze dried until a constant weight obtained. Dried seeds were grounded and refluxed for three hours. Resulting extract was centrifuged (3000rpm, 10 mins) and filtered through Whatman No. 1 filter paper. Resulting filtrate was freeze dried until obtained a constant weight and stored in -20°C freezer.

**Total Phenolic Content (TPC)**: TPC of Pomegranate peel, pericarp, juice and seed extracts was determined by Folin-Ciocalteu's method (16). Calibration curve was plotted with gallic acid as the standard. TPC was expressed as W/W% Gallic acid equivalents.

**Flavonoid content (FC)** FC was measured by aluminium chloride colorimetric assay (17). Calibration curve plotted with quercetin as the standard and FC was expressed as w/w% Quercetin equivalents.

**Determination of antioxidant activity** Free radical scavenging capacity was assessed by the DPPH Radical Scavenging Method (18) with slight modifications and compared with IC$_{50}$ of ascorbic acid. The scavenging activity of each concentration (n = 6) were quantified by the decolorization of DPPH in solution at 517nm.

\[
\% \text{ Inhibition capacity} = \frac{(Ab_{\text{control}} - Ab_{\text{sample}})}{Ab_{\text{control}}} \times 100
\]

The Ferric Reducing Antioxidant Power (FRAP) was determined according to Sharma and Kumar, 2011 (19). The test samples (100 ll) with different concentrations were mixed with phosphate buffer (0.2 M, pH 6.6, 250ii) and Potassium ferricyanide (1%, 250i). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%, 250ii) was added and the samples were centrifuged at 6500rpm for 10 min. The supernatant was mixed with deionized water and Ferric chloride (0.1%) at a ratio of 1:1:2 respectively. The samples were vortexed and absorbance was measured at 700nm in UV-Visible spectrophotometer. The blank contained deionized water instead of test sample. L-ascorbic acid was used as the standard. Increased absorbance of the reaction mixture indicates stronger reducing power.

**Removal of Polyphenols from Pomegranate juice**: Polyphenols were removed using Polyvinylpolypyrrolidone (PVPP) column as described previously (20). A cotton wool plug was placed inside a 5cm$^3$ syringe after removing the plunger and the needle. Syringe was filled with PVPP (1.0g) and juice samples (3mL each) were layered over the PVPP column. The PVPP column was placed in a 15 mL falcon tube and centrifuged at 2,000g for 10 min. Centrifugation was repeated for 6 times with the same column adding 1mL of the extract and each fraction was collected to separate tubes. First fraction was discarded and remaining fractions were analyzed for the presence of polyphenols. The Absorbance of fermented juice before and after PVPP treatment were scanned for wave lengths using a UV/Visible spectrophotometer.

**Gas Chromatography/ Mass Spectrometry (GC/MS) Analysis:** GC-MS analysis was performed for the freeze dried Sonicated peel water extract using an Agilent 6890 series instrument equipped with an Agilent 5973 N series mass selective detector. The sample was injected into the GC-MS on a 5% Phenyl methyl Siloxane glass capillary column with a film thickness of 0.25 μm (30 m × 0.25 mm) with helium as carrier gas at 0.9 mL/min constant flow mode. GC temperature programme was 60ÚC - 280ÚC at 15ÚC/min. The mass spectra were recorded in splitless mode. The scan repetition was 10 sec over a mass range of 15 - 550 atomic mass units.
Statistical analysis Results were presented as mean values of six replicates ± standard deviation. The IC\textsubscript{50} values were calculated from either linear or logarithmic dose response curves. A one way analysis of variance (ANOVA) was performed and the significant differences between mean values (p value) were determined.

Results and Discussion The effectiveness of the extraction method with reference to the antioxidant activity, total phenol content, and flavonoid content were determined by this study. Furthermore, the relationship between free radical scavenging activity with polyphenol and flavonoid contents were monitored. Several studies have been carried out to detect antioxidant activity, total phenol content, and flavonoid content for Sri Lankan variety of \textit{Punica granatum} L. fruit.

Sonication or Ultrasound-Assisted Extraction (UAE) is a low cost and non-complex instrument, which is used for small and large scale extractions. With the acoustic waves generated by the shear force, can be disrupted the biological membranes which facilitate the release of endogenous substances (21). Faster kinetics, high extraction yield and safer method for thermolabile compounds are some benefits of UAE method (22) (23).

With microwaves a pressure generates inside cell and it causes the rupturing of the cell membrane, which will facilitate the release of active substances (23). Extraction yield depends on the microwave power, extraction time and type of solvent used. Microwaves can cause tremendous temperature elevation of the sample. Due to elevation of temperature in microwaves, thermolabile compounds may destroyed in the sample (24).

Boiling is the traditional extraction method used in ayurveda medicine for preparation of most of drugs. Therefore, in this study open boiling was selected as an extraction method, which is only suitable for extracting heat-stable compounds, unbreakable plant materials (25). As UAE method is protective for thermolabile compounds, it may have given higher values for total phenol and flavonoid contents in this study(21) (22).

Extraction yield Extraction yield for parts of the pomegranate fruit extracts are illustrated in Table 1 as a percentage of dry weight obtained after freeze drying. UAE extracts of peel and pericarp obtained the highest extraction yield of 38%, 24% respectively.

Total phenolic and flavonoid contents UAE method has exhibited the highest mean value of total phenolic content in both peel (64.0±11.5 W/W% GAE) and pericarp (43.5±6.5 W/W% GAE) (Fig 1). A significant difference (p<0.005) was observed between extraction methods for both peel and pericarp. However, peel had the higher level of total phenolic content irrespective of the extraction method. Catechin, epicatechin, epigallocatechin-3-gallate, flavan-3-ol, kaempferol, luteolin, luteolin 7-O-glucoside, naringin, and quercetin available as flavonoids in peel (26). Total phenolic content of fermented juice (2.7±0.3 W/W% GAE) is higher than fresh juice (0.7±0.05 W/W% GAE) and seed (2.0 ±0.3 W/W% GAE) extracts. But it was lower than peel. Gallic acid was considered as the standard phenolic compound and expressed as Gallic Acid Equivalent (W/W% GAE).

The flavonoid content was higher in peel than pericarp. UAE method demonstrated the highest value of flavonoid content for both peel (19.7±2.9 W/W% QCE) and pericarp (15.2±1.5 W/W% QCE) (p<0.005). Fermented juice (6.7±0.2 W/W% QCE) had a higher value than fresh juice (4.6±0.7 W/W% QCE) and seed (2.2± 0.4 W/W% QCE) extracts. Folin-Ciocalteu (F-C) assay is based on the reduction of Folin-Ciocalteu reagent from phenolic compounds, which is a mixture of tungsten and molybdenum oxides (16). Vinson et al reported that F-C method can be used for fruits (27). Total phenol content in pomegranate...
peel is ten folds higher than the pulp extract (10). A higher TPC was observed with 100% water extract of pomegranate peel, while 30% water: 70% ethanol extracts show the lowest. Though some reports suggest that there is a correlation between total phenol content and antioxidant activity, no correlation has been found in some studies (28). In this study, UAE method showed the highest TPC and antioxidant activity. Therefore, it revealed that there is a correlation between antioxidant activity and TPC.

**Antioxidant activity**

According to Gil, 2000 DPPH and FRAP methods were suggested as easy and precise methods for the detection of antioxidant activity of fruit and vegetable extracts (29). It is further reported that DPPH radical scavenging assay is easy, sensitive and rapid method to follow and 90% of antioxidant activity can be measured (30). It is revealed that the pomegranate juice consists with gallic acid, organic acids, simple sugars, ellagic acid, quinic acid, flavonols, amino acids, minerals, epigallocatechin-3-gallate (6) (31). In addition to that punicalagine, hydrolyzable tannins, ellagittannins, anthocyanins, anthocyanidins can be found in pomegranate juice, as the main compounds which play a major role in antioxidant activity (29). Tezcan, 2009 has revealed that glucose and fructose as the main sugar types present in juice (32). According to Rice-Evans et al. Glucose has been identified as the commonest sugar involved in the glycoside formation. As well as galactose, rhamnose, xylose and rutose are involved. Some compounds show a different antioxidant capacities with different solvents, depending on their structure and different binding affinities to the solvent. Further he says, if number of hydroxyl groups is higher, the free radical scavenging capacity is high due to the high conjugation ability (33).

DPPH free radical scavenging capacity was significantly higher \((p<0.005)\) in UAE method both peel \((IC_{50} 3.3\pm1.1 \mu g/mL)\) and pericarp \((IC_{50} 6.2\pm0.1 \mu g/mL)\) extracts comparable to ascorbic acid standard than the other extraction methods. Pomegranate peel is composed with tannins, flavonoids, organic acids and alkaloids. With the presence of variety of tannins; punicalagin, punicalin, gallic acid and casuarinin, peel shows the antioxidant activity (29) (34). As tannins form strong complexes with proteins, they are less disposed to degradation even in the digestive tract, which is transported to other tissues. According to the suggestion from Marshall, 1990 tannins have the ability of replacing other antioxidants, therefore they prevent oxidative damage of proteins, carbohydrates and lipids during digestion.
(35). Lowest DPPH free radical scavenging capacity was present with seed extract (IC<sub>50</sub> 11698 μg/mL) and value for fermented juice extract was 243 fold higher than the fresh juice (Table 2).

PVPP is a polymer which is highly cross-linked with a high affinity for polyphenols (36) and used to remove polyphenols from pomegranate fresh and fermented juice to investigate the effects of polyphenols on DPPH radical scavenging capacity. With the presence of PVPP, fresh juice and fermented juice have shown a very low % radical scavenging capacity at a concentration of 100 μg/mL in DPPH assay. We performed the spectrum analysis especially for juice, as it showed a vast difference in % scavenging capacity between two types of juice. According to photometric scanning spectrum, broadness of peaks and wave length were reduced after treatment with PVPP (Fig 3). The evidence of presence of polyphenols in fermented juice and fresh juice was represented with the peaks obtained between 190nm to 600nm. Phenol, nitrous compounds, carotenoids and other unidentified compounds can act as natural antioxidants other than polyphenols.

Percentage radical scavenging capacity of fresh juice and fermented juice were 11.0±0.0 % and 42.5±0.0 % before removal of polyphenols and 0.03±0.0 % and 0.2±0.0 % after removal of polyphenols consecutively at a concentration of

![Fig 2. Flavonoid content (W/W % QCE) of peel, pericarp extracts with respect to extraction method (A) and fresh juice, fermented Juice, seed extracts (B).](image)

![Fig 3. The photometric scanning spectrum for fermented juice (A) and fresh juice (B) before and after treatment with PVPP (PFFRJ – Polyphenol free fermented juice, PFFJ – Polyphenol free fresh juice)](image)
100μg/mL which evidences polyphenols as the major component responsible for the antioxidant activity of Sri Lankan pomegranate.

Capacity of reduction of ferric ions (Fe³⁺) to ferrous ions (Fe²⁺) was greater in UAE method both peel and pericarp (Fig 4). Fermented juice has shown a significantly higher (p<0.005) value for Fe³⁺ reduction capacity comparable to fresh juice. Least reduction was obtained for seed extract comparing to ascorbic acid standard which is negligible.

Gas Chromatography/ Mass Spectrometry (GC/MS) Analysis: The GC/MS analysis of *Punica granatum* L. Sonicated peel powder extract exhibited five distinct peaks at retention times of 3.470, 6.716, 12.993, 13.943 and 14.260 minutes with 21.8%, 32.5% 35.2% 2.1% and 4.2% values respectively as area percent report (Fig 5).

It has been revealed that sonicated peel extract composed with furfural ring, benzoic acid, n-Hexadecanoic acid as functional groups.

![Fig 4. Ferric ion reducing power of peel (A), pericarp (B) with respect to extraction methods compared to ascorbic acid standard.](image)

![Fig 5. Total Ion Chromatogram of Volatile Compounds in the Sonicated Peel powder of Punica granatum L. fruit Analyzed by GC/MS](image)
Gunasena 2017, revealed that 2, 5-dimethyl furan, Methyl beta-d-galactopyranoside have anticancer activity. Presence of Decanoic acid derivatives such as tridecane, 3-methylhexadecanoic acid, 9,12-octadecadienoic acid, 7-tetradecyne, 1-hexadecanol have antioxidant activity. Gallic acid which belongs to Hydroxybenzoic acids is a potent antioxidant component (37).

The polyphenols are the major compounds which are responsible for antioxidant activity according to the spectrum analysis of pomegranate fresh juice and fermented juice. Water extract of pomegranate peel showed the highest total phenolic and flavonoid contents among the other components of the fruit. Fermented juice has a higher antioxidant activity than fresh juice. UAE method shows the high total phenol and flavonoid contents in peel and pericarp extracts of pomegranate fruits.

**Conclusion** This study revealed that Sri Lankan *Punica granatum* L. peel extract indicated the highest total phenolic, flavonoid contents and antioxidant capacities compared to other extracts irrespective of the extraction method. As well as

<table>
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<tr>
<th>Component</th>
<th>Extraction yield (W/W %)</th>
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<td>b. Microwave</td>
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<td>c. Sonication &amp; Microwave</td>
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<tr>
<td>d. Boiling</td>
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<td>e. Waterbath</td>
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<td>c. Sonication &amp; Microwave</td>
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<tr>
<td>e. Waterbath</td>
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<td>Juice</td>
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<td>a. Fresh juice</td>
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<tr>
<td>b. Fermented juice</td>
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<th>IC₅₀ value (µg/mL)</th>
<th>Method of extraction</th>
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<td>4.2±0.4*</td>
<td>9.1±1.3*</td>
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</table>

*IC₅₀ values of peel and pericarp extracts with respect to extraction methods and fresh juice, fermented juice, seed extracts. *All values are mean±SD of 3 replicates;
UAE extraction method presented the highest value for all the evolutionary criteria mentioned above. The polyphenols in pomegranate fruit parts are mainly contributed to antioxidant activity.

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Conflict of Interest
The authors declare no conflict of interest.

Abbreviations:
IC50half maximal inhibitory concentration
HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
DPPH 2,2-diphenyl-1-picrylhydrazyl
PVPP Polyvinylpolypyrrolidone

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