Isolation of a High Antioxidant Non-Toxic Polar Fraction from *Garcinia mangostana* Fruit Pericarp by Reverse Phase Column Chromatography

Wahyuningsih\(^a,b\), Miranti\(^a\), Hilwan Yuda Teruna\(^a\), Titania Tjandrawati Nugroho\(^a,\*\)

\(^a\) Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Riau, Pekanbaru, Indonesia  
\(^b\) UPT PSMB, Dinas Perindagkop UKM, Riau Province, Pekanbaru, Indonesia

*Corresponding author: titania.nugroho@lecturer.unri.ac.id

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**Abstract**

The crude polar extract of mangosteen fruit pericarp not only has a moderate antioxidant activity of (55±4 μg/mL) but also has high cytotoxicity (16±0.5 μg/mL). The high cytotoxicity presumably is caused by the presence of complex cytotoxic compounds from the mangosteen pericarp. To obtain a non-toxic extract preparation with high antioxidant activity, polar crude 50% ethanol extracts of mangosteen pericarp were partially purified using reverse-phase column chromatography with Silica C18 as the stationary phase and acetonitrile-water gradient elution. Six of the ten fractions collected had high antioxidant activities, with IC\(_{50}\) 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging antioxidant levels <50 μg/mL. Three fractions (fractions 3, 5, and 7) with the highest antioxidant activities of (16.4 ± 0.6 μg/mL), (17.8 ± 2 μg/mL) and (17.4 ± 1.8 μg/mL) respectively, were chosen for further cytotoxicity, phenolic content and High-Performance Liquid Chromatography (HPLC) analysis. The cytotoxic tests were conducted with the Brine Shrimp Lethality Assay. Fraction 3 had low cytotoxicity (LC\(_{50}\) 485 ± 96 μg/mL) and fraction 5 was non-toxic (LC\(_{50}\) ≥ 1000 μg/mL), while fraction 7 still had high cytotoxicity (LC\(_{50}\) 2.8 ± 0.8 μg/mL). The chromatogram profiles of HPLC showed that fractions 3 and 5 contained more polar compounds than the compounds present in fraction 7. It can be concluded that the reverse phase method succeeded in the isolation of a non-toxic polar fraction, that is, fraction 5, with a significantly higher (p<0.05) antioxidant activity than in the original crude polar extracts. This fraction had a high total phenolic content of 43.3 ± 0.3 g GAE per 100 g extract.

1. Introduction

Mangosteen (*Garcia mangostana* Linn.) is a member of the Clusiaceae family and cultivates in South-East Asia’s tropical climate. The fruit pericarp of mangosteen is used as herbal medicine in South-East Asia to treat dermatitis, wound inflammation, diarrhea, cholera, and dysentery [1]. Mangosteen pericarp contains a wide variety of compounds with biological activities. The most studied to date have been the non-polar compounds from the xanthone family, such as the α-mangostin [2], β-, and γ-mangostin [3]. These xanthone compounds have been shown to exhibit anti-cancer [4], anti-diabetic [5], and anti-rheumatoid arthritis properties [1]. Apart from the xanthones, mangosteen pericarp also contains other phenolic and polyphenolic compounds such as anthocyanin [6], vanillic acid, quercetin, rutin, protocatechuic acid, catechins, and epicatechins [7]. Other biological activities attributed to mangosteen pericarp phenolic extracts have been antibacterial agents [8] and antioxidants [7].

Recently, polar extracts from mangosteen fruit pericarp have attracted attention due to the growing data on polar phenolics and polyphenolics' medicinal properties. Like the non-polar α-mangostin, polar extracts of mangosteen fruit pericarp also have anti-oxidative properties [9]. Pothititrat et al. [10] showed that...
phenolic compounds and tannin contributed major antioxidant activities in mangosteen fruit pericarp polar fractions. Furthermore, the free radical scavenging activity of polar phenolic compounds from mangosteen fruit pericarp was significantly higher than xanthones’ activity [9]. Polar phenolic compounds extracted from mangosteen fruit pericarp have also been shown to alleviate exaggerated vasoconstriction in metabolic syndrome–induced rats [11]. A diet of polar phenolics extracted from mangosteen fruit pericarp given to rats in conjunction with a high-fat diet also could prevent thickening of the rats’ blood vessel walls, thus preventing atherosclerosis [12].

Polar phenolic compounds from mangosteen fruit pericarp are easily extracted by 50% to 70% ethanol. Suttirak and Manurakchinkorn [13] suggested that 50% ethanol extracts are the preferred solvent for extracting mangosteen fruit pericarp antioxidants. However, our preliminary study of a 50% ethanol mangosteen fruit pericarp extract showed that it was toxic when applied to keratinocyte cells in vivo, with an IC₅₀ of 5.3 µg/mL [14]. Fractionation of this crude extract by gel filtration chromatography and its subsequent analysis revealed that the crude extract’s cytotoxicity activity was not caused by low molecular weight salts but rather by higher molecular weight compounds originating from the mangosteen fruit pericarp. Furthermore, no fraction from the gel filtration method had the three combined desired target properties of having high antioxidant activity, high phenolic content, and being a non-toxic fraction [15].

Several other methods can be employed to fractionate polar phenolic compounds from a crude extract. One method is reverse-phase liquid chromatography, commonly used to separate and analyze polyphenols and other phenolic compounds in plant extracts [16]. This paper presents our results of fractionation of a crude polar extract from *Garcinia mangostana* fruit pericarp by reverse-phase liquid column chromatography, intending to obtain a non-toxic fraction having high phenolic content and high antioxidant activity.

2. Methodology

This research was initiated by preparing crude ethanol 50% (pH 5.5) extracts of mangosteen pericarp. The 50% ethanol mangosteen dry crude extracts were then fractionated by reverse-phase liquid column chromatography (RP-LC), using Silica C₈ as the stationary phase and a water-acetonitrile gradient as the mobile phase. Each fraction obtained from the RP-LC process was analyzed for its antioxidant activity using the free-radical scavenging method. Its total phenolic content was using the Folin–Ciocalteu, its cytotoxicity was using the Brine Shrimp Lethality Assay (BSLA) method. Fractions with the highest antioxidant activities were also analyzed using analytical High-Performance Liquid Chromatography (HPLC).

2.1. Equipment and Materials

The equipment used in this study was a spectrophotometer microplate reader (Berthold Technology TriStar LB 941, Germany); Thermo Scientific Genesys 10S UV–Vis spectrophotometer; High-Performance Liquid Chromatography (HPLC) Agilent 1200 Series. Other laboratory standard tools used were according to the experimental procedures.

Materials used in this study was mangosteen ripe fruits purchased from local markets in Pekanbaru, Riau Province, Indonesia; 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma–Aldrich Cat. No. D91323–IG), Silica C₈ (Chromatorex Cat. No. CRX-002–000–E); gallic acid (Sigma–Aldrich Cat. No.G7384–100G), Folin–Ciocalteau reagent (Merck Cat.No.10046.0100); and Great Salt Lake Artemia Cysts was from Sanders Brine Shrimp Co.(Utah, USA). All other reagents and organic solvents were of analytical grade, as described in the experimental procedures.

2.2. Preparation of Polar Mangosteen Fruit Pericarp Crude Extracts

Preparation of 50% ethanol (pH 5.5) crude extracts of mangosteen pericarp followed the procedure described by Miranti [17] Briefly, 2.5 grams of powdered dry mangosteen pericarp was macerated in 25 mL 50% ethanol in a 0.05 M Na-acetic buffer (pH 5.5) for eight days with shaking at 100 rpm in a 40°C incubator. The 50% ethanol extracts were then separated from the powdered residue by filtration, and the extracts were concentrated by vacuum rotary evaporation at 50°C. The resulting concentrated extracts were further dried in a 70°C water bath for 2 hours, followed by air-drying at 50°C until constant weight.

2.3. Reverse-Phase Liquid Chromatography (RP-LC) Fractionation

50 mg of dried crude polar fruit pericarp extracts were applied to a chromatography column (1.5 cm diameter x 30 cm height), filled with Silica C₈ as the stationary phase. The packed column was conditioned by washing twice with methanol and letting stand overnight before use. The column was eluted with a gradient water-acetonitrile solvent, starting with an initial ratio of 85% water:15% acetonitrile and ending with a ratio of 5% water:95% acetonitrile. The mobile phase gradient composition was changed every 25 mL by 5% for each solvent. Fractions of 25 mL each were collected. The fractions were evaporated under vacuum at 50°C until concentrated and further air-dried at 50°C in an oven to constant weight.

2.4. Antioxidant Activity Analysis by the Free Radical Scavenging Assay

Each fraction’s antioxidant activities were determined based on the 2,2 diphenyl–1-picrylhydrazyl (DPPH) radical scavenging activity, using 96 well microplate format according to the method as described by Zhanget et al. [18]. Briefly, 10 µL of DPPH solution (80 µg/mL in methanol) was added to microplate wells containing 90 µL dried extract dissolved in methanol.
with various concentrations (1000; 500; 250; 125; 62.5; 31.25 µg/mL). 10 µL of DPPH solution (80 µg/mL) added to 90 µL methanol was used as a blank. Ascorbic acid was used as the positive control. The mixtures were incubated for 30 minutes at room temperature in the dark. Absorbance was measured at a wavelength of 520 nm using a Berthold Technology Tristar LB 941 microplate reader. Measurements of antioxidant activities were repeated in triplicate experiments. Inhibition concentration 50% (IC50) was determined by regression analysis of the data. Following the equation, the percentages of DPPH inhibition were calculated using the following equation:

\[ \text{%DPPH inhibition} = \frac{\text{Absorbance}_{blank} - \text{Absorbance}_{sample}}{\text{Absorbance}_{blank}} \times 100\% \]

2.5. Total Phenolic Content Analysis

Total phenolic content (TPC) of dried extracts and fractions were determined using an optimized Folin-Ciocalteu method [19, 20]. In this method 0.1 mL of sample (1 mg/mL) was mixed with 0.5 mL 0.25 N Folin-Ciocalteu reagent and 0.9 mL deionized water. The mixture was incubated at room temperature in the dark for 5 minutes before adding 2.5 mL of 7.5% NaCO3. The mixture was further incubated in the dark, at room temperature, for another 20 minutes. The absorbance of the mixture was measured at 765 nm with a Thermo Scientific Genesys 10S UV-Vis spectrophotometer. A standard curve was generated by using various concentrations (50 to 400 ppm) of gallic acid. Total phenolic content was expressed as gallic acid equivalent (GAE) per 100 g of sample.

2.6. Cytotoxicity Analysis

Cytotoxicities of selected fractions were determined by The Brine Shrimp Lethality Assay (BLSA) [21]. Dried brine shrimp's eggs were hatched in a hatching chamber adequately aerated, having a dark and bright zone, and filled with seawater. The dark zone was filled with brine shrimp eggs to be hatched, while in the bright zone, a lamp was placed as a lighting source for incubation and nauplii separation. The chamber was incubated at room temperature (±30°C) for 48 hours. Active nauplii that hatched from its shell moved to the chamber’s bright side after 48 hours of incubation. Active nauplii were picked using a pipette and added to fresh, clear seawater, ready for the BLSA. Dried fractionated mangoes extract to be tested was dissolved in methanol with the concentration of 10, 100, and 1000 µg/mL. 500 µL of each concentration of the extract was added to test vials, and the methanol was allowed to evaporate at room temperature to dryness. Dried extract in each vial was then re-dissolved by adding 50 µL LDMSO and 4.95 mL seawater. Ten nauplii were added to each vial. The vials were incubated at room temperature (±30°C) for 24 hours. After 24 hours, the number of dead and live nauplii were enumerated using a magnifying glass and a black background. The percentage of dead nauplii was corrected to the percentage of dead nauplii in simultaneously control experiments. The control experiments consisted of vials to which ten nauplii from the same batched hatch as test experiments were added to 50 µL of DMSO and 4.95 mL of seawater and incubated at room temperature. All experiments were repeated three times. The mortality percentage was calculated using the following equation:

\[ \text{Mortality} = \frac{\text{The numbers of dead test larva} - \text{the numbers of dead control larva}}{\text{The numbers of control larva}} \times 100\% \]

Mortality percentages were plotted against the logarithmic value of the concentration of extracts. The value of LC50 was estimated using Finney’s probit regression analysis statistical method [22].

2.7. High-Performance Liquid Chromatography Analysis

High-Performance Liquid Chromatography (HPLC) analysis was carried out using a Zorbax Eclipse C-18 column (4.6 x 100 mm, 5 µm) attached to an Agilent HPLC 1200 Series (Agilent, Santa Clara, California, USA), with a Diode Array Detector–UV monitored at 240 nm. The mobile phase was a gradient of water–acetonitrile (4:1, v/v), eluted for 20 minutes with a 1 mL/minute flow velocity. The mobile phase was a gradient of water and acetonitrile with the following gradient elution 0–20 minutes 10% to 80% of acetonitrile in water and 2 minutes for equilibrium before the next injection. The flow rate of the mobile phase was adjusted to 1 mL/minute.

2.8. Data Analysis

The test results for antioxidant activities, total phenolic content, toxicity, and HPLC analysis are presented in tables and figures. The antioxidant activities, total phenolic content, and BSLA data were analyzed statistically, expressing averages as mean ± SD. Data were analyzed by the one-way analysis of variance (ANOVA) followed by Duncan’s New Multiple Range (DNMR) Test [23, 24].

3. Results and Discussion

The 50% ethanol crude extract of mangoes fruit pericarp before fractionation had a DPPH free radical scavenging activity (55.4 ± 4.1 µg/mL) and phenolic content of 37.3 ± 1.3 g GAE per 100 g extract (Table 1). This crude extract was prepared in 50% ethanol and pH 5.5 Na-acetate buffer to obtain high extract’s high phenolic content. This was due to our preliminary study showing that extraction of polar phenolics from mangoes fruit pericarp in 50% ethanol in a pH 7.0 buffer resulted in a significantly lower phenolic content (p<0.05) of 6.8 ± 0.5 g GAE per 100 g extract [25]. The 50% ethanol crude extract (pH 5.5) of mangoes fruit pericarp was fractionated using a Silica C18 column by RP–LC as described in the Methodology section. The ten dried fractions collected from this method were further analyzed for antioxidant activity, total phenolic content, cytotoxicity, and analytical HPLC profiles.

3.1. Antioxidant Activity and Total Phenolic Content of RP–LC Fractions

Antioxidant activity was measured as the ability of the sample solution to scavenge free radicals of DPPH. The antioxidant activity and total phenolic content of each fraction after RP–LC and the crude extract before RP–LC are shown in Table 1. According to Phongpaichit et
al. [26] the strength of antioxidant compound according to its DPPH radical scavenging activity can be categorized as very high (<10 µg/mL), high (10–50 µg/mL), moderate (>50–100 µg/mL), low (100–250 µg/mL), and has no significant amount of antioxidant activities (>250 µg/mL). According to these criteria, six fractions can be categorized as having high antioxidant activities from the ten collected fractions. This is a significant increase (p<0.05) compared to the original crude extract’s antioxidant activity, which is categorized in the moderate antioxidant level.

The highest increase in antioxidant activity is seen in fractions 3, 5, and 7, having antioxidant activity levels ranging from 16 to 18 µg/mL. This increase in antioxidant activity is due to the significantly higher (p<0.05) Total Phenolic Content (TPC) observed in fractions 3 and 5, compared to that in the original crude extract. However, not all increases in higher antioxidant activity in the collected fractions are due to increased TPC, as demonstrated in fractions 2 and 4. Fraction 7, which has a high antioxidant activity, also had a significantly lower (p<0.05) TPC than the crude extract. Although having lower TPC content than the original crude extract, fractions 2, 4, and 7 still contain TPC levels between 18 to 32 g GAE per 100 g samples. Therefore, they can still be considered to contain phenolic compounds with antioxidant activity. Nevertheless, the presence of a non-phenolic substance in these fractions cannot be ruled out yet to increase the antioxidant activity compared to the crude extract since the analytical HPLC profile of fraction 7 (figure 1, section 3.3.) showed that this fraction still contained several compounds. A candidate non–phenolic polar antioxidant compound present in G. mangostana pericarp is vitamin C (ascorbic acid). Although the presence of ascorbic acid in mangosteen fruit pericarp has not been published, the fruit itself contains a high content of ascorbic acid [27].

### Table 1. Antioxidant DPPH scavenging activity and TPC of dried extracts before and after RP–LC fractionation

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC	extsubscript{50} DPPH scavenging activity (µg/mL)</th>
<th>TPC (g GAE per 100 g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude original extract</td>
<td>55.4 ± 4.1 ± 0.6</td>
<td>37.3 ± 1.3 ± 0.8</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>66.20 ± 2.0 ± 0.6</td>
<td>4.3 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>33.0 ± 1.0 ± 0.6</td>
<td>18.3 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>16.4 ± 0.4 ± 0.6</td>
<td>45.3 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>34.1 ± 1.0 ± 0.6</td>
<td>18.9 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>17.8± 0.2 ± 0.6</td>
<td>42.3 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>27.2 ± 0.8 ± 0.4</td>
<td>35.0 ± 1.0 ± 0.8</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>17.4 ± 1.0 ± 0.6</td>
<td>31.0 ± 0.8 ± 0.8</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>46.7 ± 1.0 ± 0.6</td>
<td>32.0 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>115.0 ± 2.7 ± 0.9</td>
<td>15.1 ± 0.2 ± 0.8</td>
</tr>
<tr>
<td>Fraction 10</td>
<td>82.3 ± 3.7 ± 0.9</td>
<td>11.6 ± 1.2 ± 0.8</td>
</tr>
</tbody>
</table>

Positive control for DPPH scavenging (ascorbic acid): 7.3 ± 0.6

*) Values are means of three separate measurements. Different superscript alphabet in a column denotes significantly different means at the 5% confidence levels (p<0.05) by the DNMR test.

### 3.2. Cytotoxicity of fractions

The three fractions with the highest antioxidant activities were chosen for further cytotoxicity analysis by the BSLA method. Table 2 shows the BSLA results concerning the antioxidant activities and TPC of the selected fractions and the original crude polar mangosteen fruit pericarp extract. According to Clarkson’s Toxicity Criterion based on BSLA LC	extsubscript{50} values, herbal extracts having LC	extsubscript{50} in the range of 0–100 µg/mL are highly toxic, LC	extsubscript{50} 100–500 µg/mL are moderately toxic, LC	extsubscript{50} 500–1000 µg/mL are in the low toxic levels, and LC	extsubscript{50} > 1000 µg/mL can be considered non-toxic [21]. The original crude polar extracts before fractionation had a moderate antioxidant activity of 55.4 ± 4.1 µg/mL but also was highly toxic with a BSLA LC	extsubscript{50} of 15.6 ± 0.5 µg/mL. After RP-LC, fraction 3 still had low levels of toxicity, while fraction 5 was non-toxic. Fraction 5 is an ideal candidate for consumption. It is used as an herbal medicine for maintaining health in prolonging consumption since it is non–toxic, has high antioxidant activity, and a high TPC. Fraction 7, although it had significantly (p<0.05) higher antioxidant activity than the crude extract, was still as toxic as the crude extract.

### Table 2. Summary of antioxidant activities, TPC, and cytotoxicity from selected fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC	extsubscript{50} DPPH scavenging activity (µg/mL) *)</th>
<th>TPC (g GAE per 100 g sample) *)</th>
<th>BSLA LC	extsubscript{50} (µg/mL) *)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude original extract</td>
<td>55.4 ± 4.1 ± 0.6</td>
<td>37.3 ± 1.3 ± 0.8</td>
<td>15.6 ± 0.5</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>16.3 ± 0.6 ± 0.2</td>
<td>45.3 ± 0.2 ± 0.8</td>
<td>485.2 ± 96.2</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>17.8 ± 2.0 ± 0.2</td>
<td>42.3 ± 0.2 ± 0.8</td>
<td>118.9 ± 205.9</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>17.4 ± 1.8 ± 0.8</td>
<td>31.9 ± 0.8 ± 0.8</td>
<td>2.8 ± 0.8</td>
</tr>
</tbody>
</table>

*) Values are means of three separate measurements. Different superscript alphabet in a column denotes significantly different means at the 5% confidence levels (p<0.05) by the DNMR test.

### 3.3. Analytical HPLC analysis of selected fractions

HPLC analysis chromatogram profiles of fractions 3, 5, and 7 showed different constituent profiles present in each fraction, with fewer polar compounds appearing progressively from fractions 3 to 7 (Figure 1). Most of the crude extracts’ cytotoxicity appears to have eluted at retention time peaks higher than 1.2 minutes. These are the same peaks found in fraction 7 but not present in fraction 3 or 5. Fraction 5 still contained a compound present in fraction 3, eluting at the retention time of 0.5 minutes. The compound represented by the 0.5 minutes retention time had lower concentration levels in fraction 5 than in fraction 3, denoted by peak height and area. The high non–toxic polar antioxidant activity observed in fraction 5 may be contributed by the three compounds represented by elution peaks having retention times of 0.8, 0.9 dan 1.0 minutes. Peaks with retention times 0.8 and 0.9 minutes were also present in fractions 3 and 7 but with much–diminished concentration compared to fraction 5.

Without comparison to pure known compounds, compounds present in each fraction cannot be identified yet based solely on the HPLC chromatogram profiles. At
this stage, it can only be concluded that each fraction’s composition is markedly different and still contain several compounds. From the chromatogram profiles in fraction 5, at least three compounds may contribute to the high non-toxic polar antioxidant present. The literature search suggests several candidate compounds that may be present in fraction 5. Zarena and Sankar [28] reported several compounds as polar phenolic acids present as free phenolic acids with relatively high concentrations (0.1 to 2 mg per g sample) in mangosteen pericarp are gallic acid, protocatechuic acid, caffeic acid, syringic acid, ferulic acid, and epicatechin. Abdallah et al. [11] isolated epicatechin and 2,3,4,5-6-pentahydroxy benzophenone as polar phenolic compounds from mangosteen pericarp having protective effects against vascular damage by reactive oxygen species.

Figure 1. Reverse-phase HPLC chromatogram profiles of crude mangosteen pericarp extracts and RP-LC fractions with high antioxidant activity detected at 240 nm. Numbers above peaks denote retention time in minutes of each peak identified.

Polar polyphenols from mangosteen fruit pericarp have the potential to be used as a medication or food supplement to prevent and treat neurodegenerative diseases [29] or to prevent cardiovascular diseases [11]. This is due to the high antioxidants of polar phenolics in mangosteen having the ability to reduce Reactive Oxygen Species activity, stimulate Nitric Oxide (NO) generation, and inhibit acetylcholine esterases. However, a mangosteen extract containing polar antioxidants for prolonging consumption as a nutritional supplement or as preventive medication should not contain any cell toxic elements. As shown in this study, crude polar 50% ethanol extracts of mangosteen fruit pericarp contain toxic compounds. The toxic compounds in the crude polar extracts could be easily separated from the beneficial compounds using a simple RP-LC method. The RP-LC method affords to upscale to industrial levels in a continuous process with minimal organic solvent usage. Suthammarak et al. [30] prepared a non-toxic polar fraction of mangosteen pericarp using a different method by partitioning the toxic compounds into ethyl acetate. The partitioning method employed by Suthammarak et al. [30] uses a large amount of organic solvent. In the RP-LC method, the use of organic solvent can be kept to a minimum, so less organic solvent waste. The RP-LC stationary phase can be reused by regeneration, with washing using less solvent than the partition method, so that the stationary phase’s cost can be reduced.

4. Conclusion

RP-LC of crude polar 50% ethanol extracts of mangosteen fruit pericarp is an excellent method to separate non-toxic polar phenolics from toxic compounds. A non-toxic fraction with high antioxidant activity and high TPC could be obtained using an RP-LC procedure. Toxic compounds in crude polar 50% ethanol extracts of mangosteen fruit pericarp have lower polarity than the non-toxic compounds.

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