**Garcinia mangostana** L. fruit rind extract in ethyl acetate, n-butanol and water fractions: phytochemical analysis, antioxidant assay and cytotoxicity assay

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**Abstract.** The aim of the study is to investigate the relationship between the phytochemical content of the fruit rind of *Garcinia mangostana* Linn with antioxidant activity and cytotoxicity of different fractions (ethyl acetate, n-butanol, water) of the xanthones extract. The ethyl acetate fraction showed the highest total xanthones as quercetin equivalent and α-mangostin equivalent, total xanthones as α-mangostin, and total polyphenol content. High antioxidant activity and cytotoxicity were also shown from the ethyl acetate fraction with 55.8 μg/ml and 0.0029 μg/ml for IC₅₀ and LC₅₀ values, respectively. The linear correlations were confirmed between the total phenolic content and antioxidant activity as well as cytotoxicity of the xanthones extracts. The results of bioactivity assays and photochemical analysis of different fractions of xanthones extract showed that the ethyl acetate fraction of xanthones extract can be considered as a promising source of anticancer bioactives to be used in controlled release formulations.

1. Introduction

Mangosteen (*Garcinia mangostana* L.) is a tropical tree that has been utilized as a source of bioactive compounds with therapeutic properties, used as traditional medicine for many kinds of illnesses. Mangosteen contains plant polyphenols such as tannins, flavonoids, xanthones, among other bioactive compounds. Xanthones belong to the class of phenolic compounds that cannot be produced in the human body, but has an advantageous impact on human health. As antioxidants, these compounds protect the body against oxygen free radicals, which are produced in large amounts in a pathological condition and are related to the development of degenerative diseases, such as cancer [1-3]. A variety of prenylated and oxygenated xanthones are found in the fruit rind of mangosteen. There are 50 types of xanthones found in *G. mangostana* fruit rind [4]. More than 90% (w/w) of the xanthones present in the mangosteen fruit rind extract are α-mangosteen, β-mangosteen and γ-mangosteen [5].

The colorimetric assay using AlCl₃ as the complexing agent is a typical assay to determine the total content of flavonoids. AlCl₃ forms specific color due to the complex formation with the keto group or hydroxyl groups in flavonoids. Quercetin is one of the most abundant dietary flavonoids, a group of polyphenols. It is reported that the same colorimetric assay can be used to determine the total content of xanthonoids or xanthones [6].

Another method to assay xanthones is using the UV-spectrometry method. Since α-mangosteen is the dominant xanthone compound in the extract from fruit rind, the UV absorption peak of α-mangosteen is strongly related to the total amount of xanthones in this extract. The difference between flavonoids and xanthones is their backbone structure, shown Figure 1.
Figure 1. Different back bone structure of flavonols such as quercetin (left) and xanthonoid such as α-mangosteen (right).

In the application of xanthones for cancer treatment, the bioactive compounds were first extracted using ethanol and then the extract was fractionated into ethyl acetate, n-butanol, and water fractions. Therefore, it is important to evaluate which fraction is rich in xanthones to be used in a controlled release formulation. The objective of this study was to screen the fractions from the fruit rind of extract that has the highest content of xanthones as well as to evaluate whether the bioactivity correlates with the total content of xanthones and polyphenols in each fraction.

2. Materials and methods

2.1. Plant materials

G. mangostana fruit was purchased from a local market in Jakarta, Indonesia. The fruit rind was separated from edible part and peeled the hard purple skin. It was chopped and dried in open air for 72 hours.

2.2. Chemicals

Ethanol, methanol, ethyl acetate, n-butanol, n-hexane were purchased from Merck. Standards of phenolic acid (gallic acid) and of flavonoids (quercetin), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu’s phenol reagent 3-tert-butyl-4-hydroxyanisole (BHA) and aluminium chloride (AlCl₃) were from Sigma Aldrich. Brine shrimp (Artemia Salina Linn) larvae was purchased from Red Top

2.3. Extraction and fractionation of xanthones from Garcinia Mangostana L.

The dried and ground fruit rind of G. mangostana (0.25 kg) was extracted by maceration with ethanol (750 ml) at room temperature for 7 days. After filtration and evaporation of the solvent under reduced pressure using an evaporator, the crude ethanolic extract (50 g) was suspended in distilled water (100 ml) [7].

2.4. Total xanthone as quercetin equivalent and α-mangosteen equivalent

The total xanthone as quercetin equivalent and α-mangostin equivalent was determined using visible spectrophotometry and UV spectrophotometry, respectively. The aluminum chloride colorimetric method for the determination of total xanthones was modified from the procedure reported by Chang and Pothitirat [8-9] for flavonoids content. A standard solution of 250 µg/ml quercetin was prepared by dissolving 6.25 milligrams of quercetin in 25 ml of 96% ethanol, and diluted to 5, 10, 20, 50, and 100 µg/ml quercetin solution. The diluted standard solutions as much as 0.5 ml were separately mixed with 2.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 400-500 nm with vis-spectrophotometer UNICO 1100 RS (USA). For the blank, the 10% aluminum chloride solution was substituted by distilled water.

The UV spectrometry method for determination of total xanthones as α-mangosteen equivalent was carried out by collecting spectra data of α-mangosteen standards (4-20 mg/L) in the wavelength range
of 500-200 nm. The total xanthenes in each fraction was calculated by applying the linear regression equations of α-mangosteen calibration curves.

2.5. Total polyphenol content
The total polyphenol content of the *G. mangostana* fruit rind extract in each fraction was determined using the Folin-Ciocalteu reagent [3,6,10-12]. Gallic acid was used as standard to make the calibration curve. Certain amounts of gallic acid were dissolved in ethanol (96%) to make standard solutions of 10, 50, 100, 150, 200 and 250 ppm. The standard solutions of gallic acid as much as 0.5 ml were separately mixed with 5 ml of 10% Folin-Ciocalteu reagent solution and left for 5 min. Each solution was added by 4 ml of 1M sodium carbonate solution and kept in dark at room temperature for 15 min to complete the reaction. The absorbance was measured at 738 nm. As a blank, 0.5 ml ethanol was used to substitute the gallic acid solution. Sample solutions were prepared by dissolving extract in ethanol to form a solution with a concentration of 1 mg/ml with similar treatment as that for standard solutions. The result was expressed as mg gallic acid equivalent (GAE)/g of *G. mangostana* extract.

2.6. Total xanthone as α-mangostin using liquid chromatography-mass spectrometry (LC-MS)
The total xanthone in each fraction was analyzed by a Shimadzu Separation LC system, connected with a Micro TOF-Q mass spectrometer. Chromatographic separation was performed using a reverse-phase C18 column (250 x 4.6 mm, 5 μm) maintained at 30°C. The mobile phase consisted of 95% acetonitrile and 5% of 0.1% formic acid in water, was used with a flow rate set at 0.5 ml/min for 15 min. Spectral data were collected at 244 nm. Mass analysis was performed in the range 50–1000 m/z, under the negative ion mode, and the nebulizer was set at 3.0 bar and heated to 150°C. The capillary voltage was set at 3000 V using a nitrogen dry gas at 8.0 L/min. The total xanthone is reported as α-mangosteen equivalent.

2.7. Bioactivity assay

**Cytotoxicity assay using Brine Shrimp Test (BST)**
In vitro lethality assay of *A. Salina* was used to detect cytotoxicity of *G. mangostana* extract in each fraction. Brine shrimp eggs were placed in seawater and aerated at room temperature (24-28°C). Eggs were hatched within 72 h giving a large number of larvae. Ten larvae were placed in vials containing 1 ml of mixture solution of seawater and extract that have concentration start from 1 ppm to 1000 ppm. As a control, the same volume of ethanol 96% was added to the sea water without the addition of mangosteen extract. Total larvae alive were counted after 24 h left in front of the lamp and the lethal concentration (LC50) was calculated [13-15].

**Antioxidant activity assay using DPPH reagent**
Total antioxidant activity was evaluated by spectrometric method based on the scavenging of the free radical DPPH (2,2 diphenyl-1-pycrilhidrazyl) as reported by other researchers [8,10,16] with some modifications. The stock solution of extract was prepared in methanol to obtain a concentration of 1000 ppm. Dilutions of this extract solution were made to obtain concentrations from 15 ppm to 480 ppm. 5 ml of each extract sample solution was mixed with 5 ml 0.2 M DPPH solution. After incubation in the dark at room temperature for 30 min, the absorbance was measured at 517 nm. As control, all reagents were mixed except the extract solution. Ethanol was used as blank to calibrate the spectrophotometer. Percentage inhibition was calculated using equation (1), whilst inhibition concentration at 50% (IC<sub>50</sub>) values were estimated from the % inhibition versus concentration plot. The data were presented as a mean value ± standard deviation of triplicate analysis for each sample.

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\text{% Inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]
3. Results and discussion

Dried ethanolic extract of mangosteen fruit rind was suspended in water and partitioned in turn with several solvents. The yield after partitioning 50 grams of dried ethanolic extract in each solvent fraction was measured. Table 1 showed the percentage of the dried solid obtained from each solvent fraction including a commercial mangostin supplement product (SM).

Table 1. Yield of dried extract and phenolics in various fractions

| Fraction        | %-dried solid (g/g) ethanolic extract | % phenolics (g/g) as GAE/dry solid sample |
|-----------------|---------------------------------------|------------------------------------------|
| ethyl acetate   | 40                                    | 18.2                                     |
| n-butanol       | 14                                    | 6.7                                      |
| water           | 4                                     | 4.1                                      |
| ‘SM’            | -                                     | 12.6                                     |

3.1. Total polyphenol content

The total polyphenol content of the solid obtained from each fraction and a commercial sample is given in Table 1, expressed as % (g/g) of gallic acid equivalent/solid sample, in the range of 4.1-18.2% (g/g) GAE/dry sample. The highest concentration of polyphenols in mangosteen fruit rind was found in the ethyl acetate fraction while the lowest concentration in the water fraction. Partitioning the dried ethanolic extract of mangosteen fruit rind seemed to separate the polyphenols, mostly in less polar solvent such as ethyl acetate.

3.2. Total xanthones as quercetin equivalent

The principle of aluminum chloride colorimetric method is based on the fact that aluminum chloride forms acid stable yellow complexes with the C4 keto group and either C3 or C5 hydroxyl group of flavonols. When there is no C5 or C3 hydroxyl group, no complexes will form after the addition of AlCl3 solution [6, 9, 17]. Determination of flavonoids using colorimetric method usually used quercetin as standard [8, 17, 18]. The results of this colorimetry method are given in Table 2.

Table 2. Xanthones contained in extract fractions as the equivalent of α-mangostin and quercetin.

| Fraction | % (w/w) xanthones as α-mangostin/dry sample* | % (w/w) xanthones as quercetin/dry sample** |
|----------|---------------------------------------------|-------------------------------------------|
| ethyl acetate | 53.1                                    | 1.6                                        |
| n-butanol  | 0.6                                       | 0.3                                        |
| water      | 0.4                                       | 0.2                                        |
| ‘SM’       | 39.4                                      | 0.6                                        |

* UV spectrophotometry, ** visible spectrophotometry

3.3. Total xanthones as α-mangostin equivalent

Total xanthones in each fraction of the mangosteen fruit rind extract was also measured as α-mangostin equivalent, using the UV spectrometry method. The UV-spectrum of pure α-mangostin is very similar to the UV spectrum of fraction samples. The total xanthones was determined by measuring the peak area at the wavelength of the highest peak in the UV spectrum (316 nm). Table 2 showed the total xanthones as α-mangostin in water fraction is the lowest, while the ethyl acetate fraction has the highest total xanthones.

The magnitude of total xanthones using UV spectrometry of α-mangosteen, are different from that of colorimetry result using quercetin as standard. However, the ratio between total xanthones or xanthonoid in ethyl acetate fraction and in commercial product ‘SM’ is relatively the same. Based on this result, xanthones content in GM fruit rind extract can be determined by both methods because the result will be the same, relative to the standard used. The high content of xanthones in ethyl acetate
fraction indicated that bioactive compounds in GM fruit rind are soluble in a less polar solvent such as ethyl acetate compared to a polar solvent such as water.

Xanthones in GM fruit rind extract has mostly been α-mangosteen, seemed to have a higher solubility in ethyl acetate than in water. These results were contradicting with public opinion which states that GM fruit rind juice in water could provide high xanthones. The commercial product ‘SM’ showed also lower content of xanthones than in ethyl acetate using this UV spectrometry. This result indicated that fractionation of ethanolic GM fruit rind extract produces higher purity of xanthones in ethyl acetate fraction rather in regular ethanolic extract (SM sample). The high content of xanthones in this fraction leads to the application of this fraction as a potential source of active compounds in controlled release formulations.

3.4. Total xanthones/mangosteen content using HPLC
The analysis of samples using HPLC showed a strong peak at retention time 5.6 minutes representing the presence of α-mangosteen in all fraction samples [5, 6]. The α-mangosteen content in each fraction is shown in Table 3. Ethyl acetate fraction contains more than 16% of α-mangosteen per dry extract, whereas in water fraction it is only around 1% α-mangosteen per dry extract. The percentage of α-mangosteen content in each fraction was calculated based on the peak area of standard α-mangosteen with 98% purity. The α-mangosteen content in the ethyl acetate fraction is higher around sixteen times than α-mangosteen in the water and n-butanol fractions. This result indicates that this active component in GM fruit rind is very slightly soluble in water or n-butanol solvent. Compare it to the commercial product ‘SM’, the α-mangosteen content in ethyl acetate is higher more than eight times. This result supports the use of ethyl acetate fraction of GM fruit rind extract as an anti-tumor substance in controlled drug release because of its high content of α-mangosteen. The value of α-mangosteen content in fraction samples was slightly different from the data obtained using UV-Vis analysis. It is understandable since the value using UV-Vis analysis is based on the absorbance of the total xanthones in the samples not only α-mangosteen.

### Table 3. α-mangosteen content in extract samples based on HPLC data.

| Compound          | % α-mangosteen in dry extract |
|-------------------|-------------------------------|
| F002 (ethyl acetate fraction) | 16.8                          |
| F004 (n-butanol fraction)      | 0.9                           |
| F005 (water fraction)          | 0.9                           |
| Commercial Product ‘SM’        | 1.9                           |

3.5. Antioxidant activity
Antioxidant activity measurement using spectrophotometric method and DPPH solution was based on the free radicals scavenging activities performed by the active compounds in the sample. Blue color of DPPH solution changed to yellow as the extract samples were added into the solution. Antioxidant activity expressed IC$_{50}$ values that indicating the minimum concentration of extract sample that can inhibit free radical activity as much as 50% [19].

The total antioxidant activity measurements for all solvent fractions are shown in Table 4, where the ethyl acetate fraction has the lowest IC$_{50}$. Lower values of IC$_{50}$ indicated higher antioxidant activity for this extract sample. The LC$_{50}$ value for water fraction is higher than that in ethyl acetate. This means that active compounds in water fractions have lower antioxidant activity compare to the extract in ethyl acetate. This low antioxidant activity might be due to low total flavonoid /xanthones content as well as to low total phenolic content in water fraction.

3.6. In vitro cytotoxicity
Cytotoxicity parameter in BST method is expressed as LC50, where a sample has active cytotoxicity or can be used as anti-cancer drug if the LC50 value is lower than 1000 ppm [20, 21]. This cytotoxicity assay using BST is the simple and easy way to predict the cytotoxicity of an active compound in samples. Kim
[22] used this BST method to screen the fractions that are active cytotoxicity for further isolation to obtain acetogenins. The results in Table 4 showed that dried extract in ethyl acetate fraction has very low LC$_{50}$ value which indicated that bioactive compound in this fraction is very active cytotoxicity and has the potential to be used as anticancer drug. The dried extract obtained from water fraction showed very high LC$_{50}$ which means has no cytotoxicity and cannot be considered as anti cancer drug. This result of cytotoxicity in water fraction also contradicts the people opinion that water extract of GM fruit rind can be used as anti-cancer drug.

Table 4. Cytotoxicity assay and antioxidant activity of measurement.

| Fraction   | Cytotoxicity as LC$_{50}$ (μg/ml) | Antioxidant activity as IC$_{50}$ (μg/ml) |
|------------|----------------------------------|------------------------------------------|
| ethyl acetate | 0.0029                          | 55.7 ± 0.1                                |
| n-butanol   | 0.0258                          | 84.9 ± 0.1                                |
| water      | > 50000*                         | 165.9 ± 0.1                               |
| ‘SM’       | 0.0034                          | 219.0 ± 0.1                               |

* not active

3.7. Relationships between antioxidant activity and phenolic content

Figure 2 shows that a linear correlation was found between the values of total phenolic content and the antioxidant activity of the GM extract in various fractions ($R^2$ value of 0.901). Stankovic [10] also reported about the linear correlation between the values of phenol content and antioxidant activity. Xanthones or xanthonoids is one of various compounds belong to polyphenolic compounds contained in GM extract.

Figure 2. Relationships between polyphenolic content and antioxidant activity of GM extracts in various fractions.

Figure 3 shows the relationships between polyphenolics, xanthones and α-mangosteen of the GM extract contained in various fractions. GM extract in ethyl acetate fraction has the highest amount of xanthones, α-mangosteen and polyphenolic compounds, compared to the other fractions and the commercial product. The order from large to small content of xanthones, α-mangosteen, polyphenols in these fractions was ethyl acetate, SM, n-butanol and water, respectively. The trend of total polyphenolic content is almost similar to the trend of α-mangosteen in each fraction. High content of xanthones and % inhibition of GM extract in ethyl acetate fraction indicated that xanthones, as active compounds in GM fruit rind, might contribute strongly to the strong antioxidant activity. Further studies of this extract of GM fruit should be directed by carrying out in-vitro medicinal assay prior preparing a controlled drug release material for anti-cancer drug with GM extract as active compounds.
Figure 3. The relative amount of xanthones, mangostins, and polyphenolics of the GM fruit rind extract present in various fractions.

4. Conclusions
Result from this investigation on the extract of GM fruit rind in various fractions suggests that the ethyl acetate fraction is a good selection to be used as medicinal active component sources in preparation of controlled drug release material. All tests on this fraction showed that it has the highest content of polyphenolic compounds, xanthonoid, mangosteen, antioxidant activity and cytotoxicity compare to extract in other fractions. Based on this information, introduction of GM fruit rind in ethyl acetate fraction is a potential source for anti-cancer drug.

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