Antiglycation and Antioxidant Activity from Methanol Extract and Fraction of Xylocarpus granatum Stem

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Abstract

The objective of this research was to determine the antioxidant and antiglycation activity of methanol crude extract of Xylocarpus granatum stem and its fraction. The methanol crude extract was fractioned by liquid–liquid extraction to obtain three fractions, which were n-hexane fraction, ethyl acetate fraction, and methanol fraction. Antioxidant activity was performed using scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH). The antiaging activity was identified based on the ability to inhibit the formation of advanced glycation end products (AGEs). The results of the study showed that methanol crude extract and all fractions had an antioxidant and antiglycation activity that significantly different from each other (P<0.05). The results show that the most active antiglycation and antioxidant activity was the methanol fraction with IC50 were 71.55 ppm and 8.52 ppm, respectively. The methanol fraction contained an alkaloid, flavonoid, phenolic, and triterpenoid compounds. Subsequent fractionation of the methanol fraction guided by bioassay showed that sub–fraction 1 had the best antiglycation and antioxidant activity. Based on the color of the chromatogram obtained at UV 366 nm, the chemical component contained in the most active fraction was the flavonoid group.

1. Introduction

Aging is a physiological process that occurs in all living things in all organs of the body. Aging occurs because of free radicals and advanced glycation end products (AGEs), which is the final product of the protein glycation reaction between amino acids and reducing sugars in the body [1]. AGEs contribute to aging through 3 primary mechanisms: 1) AGEs cross–link with the major connective tissue matrices (such as elastin and collagen) found in the dermis and keratin layers in the epidermis layer of the skin resulting in a decrease in elasticity, dryness, and shrinkage of the skin [2, 3]; 2) AGEs can block the entry of proteasome nuclei in the Ubiquitin–Proteasome System (UPS). The proteasomes consist of two main parts, two outer α-rings for binding to the substrate and two inner β-rings that are responsible for proteolytic activity [4]. Structured AGEs can inhibit the entry of the proteasome nucleus, which is responsible for the degradation of short–lived and not folded oxidized proteins, consequently, an increase in oxidized and damaged proteins, triggering protein modification [4, 5] and; 3) AGEs will also interact with cellular receptors (RAGE). RAGE regulates several essential cellular processes such as inflammation, apoptosis, ROS signaling, proliferation, autophagy, and aging. The interaction between AGE and RAGE changes the function of cells and organs mainly through inflammatory molecules, which cause aging [5, 6]. AGEs produce free radicals upon free radicals, AGEs and other factors accelerate the reaction of protein glycation so that it will
accelerate aging [2, 7]. To overcome aging, especially premature aging, antiaging compounds are needed.

Antiaging compounds are expected to be compounds that can neutralize free radicals such as antioxidants and compounds that can inhibit glycation reactions. Antioxidants can ward off free radicals and stop the chain reactions that can damage essential macromolecules in the body [8]—one source of antioxidation and antioxidants contained in plants.

_Xylocarpus granatum_ is a type of mangrove plant that has been used traditionally by coastal women as a traditional powder for skincare [9]. This plant is reported to have strong antioxidant activity that can counteract the free radicals. The methanol extract of _X. granatum_ rind has shown to inhibit tyrosinase activity and had potential as an antioxidant agent [9]. The skin of _X. granatum_ has secondary metabolites in the form of phenolic, alkaloids, steroids, tannins, triterpenoids, and flavonoids [9, 10, 11, 12]. Not only the skin of the fruit, but the stem also has the potential to be a whiterener, because it can inhibit tyrosinase that plays a role in melanin synthesis in melanocyte burning. It is also a source of antioxidants, and the result is that the stem section of _X. granatum_ has the highest antioxidant and tyrosinase inhibitors [9, 10, 11]. Therefore, this research is focused on the stem of _X. granatum_. Natural antioxidant compounds from plant phenolic groups can inhibit premature aging of skin [13]. However, there have been no reports of antiaging activity on _X. granatum_ stems. The purpose of this study was to determine the antioxidant and antiglycation activities of _X. granatum_ crude methanol extracts and their fractions; the fraction was obtained from coarse methanol fraction of _X. granatum_ stem using liquid–liquid extraction to obtain three portions, which were n–hexane fraction, ethyl acetate fraction, and methanol fraction. In addition, this study also aims to identify secondary metabolites that act as antioxidants and antiglycation.

2. Methodology

2.1. Materials and Tools

The materials used in this study were _X. granatum_, ethyl acetate, chloroform, methanol, tolune, n–hexane, DPPH, ascorbic acid, BSA (Bovine serum albumin) (Merck), phosphate buffer 0.2 M pH 7.4, glucose (Merck), fructose (Merck), and aminoguanidine (Sigma Aldrich). The instruments used in this study were oven, fluorometry (FluoroSTAR BMG LABTECH), _multispan reader_ (Iwaki), TLC vessels, chromatographic columns, G60F254 silica gel plates from Merck and commonly used glass tools.

2.2. Simplicia Preparation

_X. granatum_ samples were from Bengkagi Village, Tojo Una–Una, Togean Islands, Southeast Sulawesi. The samples were determined in the LIPI Cibinong laboratory. The stems are cut into pieces, put into the oven for two days at 40°C to dry, then mashed to a powder (simplicia). The simplicia was analyzed for water content and ashed using the AOAC method.

2.3. Maceration Extraction

A total of 3 kg of Simplicia was macerated with methanol (10 L) for three times of 24 hours. The macerate was then filtered and concentrated with a rotary evaporator at 30°C.

2.4. Phytochemical Test

Phytochemical tests on samples qualitatively include the identification of alkaloids, saponins, triterpenoids/steroids, flavonoids, and phenolics, according to Harborne [14]. The standard used refers to Adawiah [15].

2.5. Multilevel Fractionation

The antiglycation method referred to Povichit et al. [16] with a slight modification. The reaction consisted of a solution containing 0.2 M phosphate buffer (pH7.4), BSA (20 mg/mL), glucose (235 mM), fructose (235 mM), and samples mixed in a test tube. As for the correct solution, distilled water was added as a substitute for glucose and fructose. The control solution was added with distilled water as a substitute for the sample and aminoguanidine as a positive control. The whole solution was incubated for 40 hours at 60°C and then the fluorescent intensity was measured using a fluorimeter with an excitation wavelength of 330 nm and an emission of 440 nm. Antiglycation activity was measured using the following equation:

\[
\text{Inhibition} \% = 1 - \frac{A_{1} - A_{0}}{B_{1} - B_{0}} \times 100\%
\]

Note:

\(A_{1}\) = intensity of fluorescence of sample solution
\(A_{0}\) = intensity of fluorescence of correction sample solution
\(B_{1}\) = intensity of fluorescence of control solution
\(B_{0}\) = Intensity of fluorescence of correction control solution

A regression curve of inhibitory activity calculated the percentage of inhibition of 50% (IC_{50}) to the fluorescence of AGEs.

2.6. Antioxidant Activity Test

A total of 100 µL of 125 µmol/L DPPH solution was put into a 96 well plate in which there were 100 µL of extract/fraction sample. The solution was incubated for 30 minutes at room temperature. The absorbance of the solution was measured at a wavelength of 517 nm using an _multi–well plate reader_. The positive control used was ascorbic acid. The percentage of inhibition was determined by measuring the sample absorption and calculated with the formula:

\[
\text{Inhibition} \% = 1 - \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{standard}}}{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{standard}}} \times 100\%\]

A regression curve of inhibitory activity calculated the percentage of inhibition of 50% (IC_{50}) of antioxidants.

2.7. Data Analysis

All data expressed were as mean ± SD Data, analyzed using Analysis of Variance (ANOVA), and further tested
with Duncan's multiple range test (DMRT) using SPSS version 16.0.

3. Result and Discussion

3.1. Extract Yield and Stem Fraction of X. granatum

Initial extraction of the X. granatum stem was carried out using the maceration method and using methanol as a solvent. The maceration method was chosen so that the sample did not undergo heating, which can damage compounds which could not stand the heat. Methanol was used as an initial solvent because it could dissolve almost all organic compounds, both polar and nonpolar. Methanol molecular structure is CH₃OH, which consists of a polar and nonpolar part. It is polar as it has a hydroxyl group (-OH) and also nonpolar as it has a methyl group (-CH₃). Methanol has a low boiling point, which makes it easier to evaporate [17]. The moisture content of stem powder of X. granatum was 5.80 ± 0.02%. The moisture value was used to correct the yield obtained; hence the yield could be determined based on the dry weight. The yield of methanol extract from the stem of X. granatum was 10.66%, this value was obtained from the maceration of 2.8 kg dried simplicia. The fractionation was continued through liquid-liquid extraction from X. granatum stem methanol extract using n-hexane solvent obtained by the 10.60 g fraction, with a green color and a yield of 7.35% fraction from initial dry weight simplicia (141.3 g). The ethyl acetate fraction was dark red, and the methanol fraction was reddish-brown with the yield shown in Table 1.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Extracts Yield Weight (%)</th>
<th>Fraction Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane</td>
<td>7.35</td>
<td>Green</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>21.30</td>
<td>Dark Red</td>
</tr>
<tr>
<td>Methanol</td>
<td>70.91</td>
<td>Brownish Red</td>
</tr>
</tbody>
</table>

Based on the results of the fractionation, the results obtained varied. This happens due to the different levels of the polarity of each solvent. The higher the extract yield, the higher the substance contained in the raw material.

3.2. Phytochemical Content

The principle of the phytochemical content test is the analysis of plant chemical groups with specific testing reagents that provide specific information on certain chemical groups [18]. The more intense color intensity indicates that the extract has a higher level of secondary metabolites.

Table 2. Phytochemical content of n-hexane, ethyl acetate, methanol, and crude methanol extract

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Reagent</th>
<th>Crude methanol</th>
<th>n-hexane fraction</th>
<th>Ethyl acetate fraction</th>
<th>Methanol fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Dragendorf</td>
<td>+++ -</td>
<td>++ + +</td>
<td>+++ -</td>
<td>+++</td>
</tr>
<tr>
<td>Mayer</td>
<td>+</td>
<td>+</td>
<td>++ + +</td>
<td>++ + +</td>
<td>++ + +</td>
</tr>
<tr>
<td>Wagner</td>
<td>+</td>
<td>+</td>
<td>++ + +</td>
<td>++ + +</td>
<td>++ + +</td>
</tr>
<tr>
<td>Fenolik</td>
<td>Reagent FeCl₂</td>
<td>++</td>
<td>++ + +</td>
<td>++ + +</td>
<td>++ + +</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>powder Mg + HCl</td>
<td>++</td>
<td>-</td>
<td>++ + +</td>
<td>++ + +</td>
</tr>
<tr>
<td>Saponin</td>
<td>Hot distilled water + HCl</td>
<td>-</td>
<td>-</td>
<td>++ + +</td>
<td>++ + +</td>
</tr>
<tr>
<td>Triterpenoids/</td>
<td>Reagent Liebermann</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Steroid</td>
<td>Bouchard</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Steroid</td>
<td>Triterpenoids</td>
<td>+ +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Steroid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: (-): not detected, (+): detected. Amount of (+) shows the color intensity

Phytochemical content testing showed that only the n-hexane fraction was identified with steroids, and no flavonoids were detected. Flavonoids and triterpenoids are relatively higher in the methanol fraction, which was characterized by a more intense orange color than extracts and ethyl acetate (Table 2).

3.3. Antiglycation and Antioxidant Activity

The ability of X. granatum extract to inhibit the formation of AGEs was evaluated using antiglycation tests in which the bovine serum albumin acted as a protein, while glucose and fructose as reducing sugars [3]. Antiglycation was determined by determining the amount of AGEs produced by the reaction of proteins (Bovine serum albumin, BSA) with glucose and fructose using fluorimeter [19]. Antiglycation activity was reported with concentrations that could inhibit 50% of the glycation reaction known as IC₅₀. The smaller the concentration needed to inhibit 50% of the glycation reaction, the more active the material. The order of antiglycation ability from the strongest was methanol fraction> methanol extract> ethyl acetate fraction> n-hexane fraction (Figure 1). All fractions have possible anti-glycation activity because all fractions contained phenolic compounds. Phenolic compound group derivatives (quercetin, curcumin, ferulic acid, ellagic acid, and iridoids) have significant antiglycation and antioxidant activity [20]. Based on the literature, X. granatum has a phenolic group that is dihydrokafeic acid-(3 → 8) -dicatechin, catechin-(4 → 8) -catechin, kaempferol-3-O-beta-D-glucoside [21, 22, 23, 24, 25, 26, 27]. However, the antiglycation activity in the methanol fraction was higher than in the others (Figure 1).
The value of this antiglycation activity is better when compared to the IC₅₀ amount of antiglycation activity in other plants extracted using methanol, such as Zingiber officinale 203.86 ppm, Curcuma xanthorrhiza 274.14 ppm, Elettaria cardamomum 285.58 ppm, Boesenbergia rotunda 274.14 ppm and Curcuma xanthorrhiza 274.14 ppm, Elettaria cardamomum 285.58 ppm, Boesenbergia rotunda 274.14 ppm and Bulbocastanumium 132.88 ppm [20, 28, 29]. The best antiglycation activity is in the methanol fraction because, in the methanol fraction, more flavonoids and triterpenoids are detected than other fractions. This is marked by a change in color to a tighter orange and red. Amino guanidine as a positive control has a lower IC₅₀ than that of the methanol fraction because it is a single compound (Figure 1) however, amino guanidine is not approved for commercial production due to side effects associated with the process of absorption of vitamin B6, drug resistance and hepatotoxicity [3, 30].

The presence and activity of antioxidants can be determined through oxidation and reduction reactions using reagents that have chromophores so that they are easily detected using a UV-Vis spectrophotometer. The direct method for detecting antioxidants is 2,2 diphenyl-1-pircylhydrazine (DPPH). The principle of the DPPH method is that DPPH captures hydrogen from the reaction of antioxidant compounds and converts it to 2,2 diphenyl-1-pircylhydrazine. DPPH is a stable radical that contains organic nitrogen, with a dark purple color that will turn yellow when reacting with antioxidants, causing a decrease in absorbance value. Testing antioxidant activity using the DPPH method has many advantages because it is stable, does not form dimers caused by delocalization of free electrons in all molecules, does not require substrate so that it is easier and faster analysis time so that the measurement of antioxidant activity can be done accurately [31].

Figure 1 shows that the IC₅₀ antioxidant activity starts from 14.3 ppm to 65.35 ppm. The best antioxidant ability is in line with its anti-glycemic ability. The same was also reported in methanol extracts from the stems and roots of Scutellaria alpina L and Scutellaria altissima L [3]. The most active antioxidant and antiglycation activity are in the methanol fraction because the methanol fraction contains more flavonoids than the ethyl acetate fraction and the n-hexane fraction. Even in the n-hexane fraction, flavonoids are not detected. Flavonoids inhibit DPPH free radical activity because they can donate proton radicals, which will reduce the formation of non-vertical DPPH [32].

Flavonoids are polar compounds because they have several unsubstituted hydroxyl groups. Therefore, flavonoids are more detectable in more polar solvents. As reported by Haslina and Eva [33], Haslina, the number of flavonoids in corn silk methanol extract was significantly different (p < 0.05) compared in ethanol and ethyl acetate extracts. In addition, Maimulyanti and Prihadi [34] states that the antioxidant activity in the methanol extract of Acmella uliginosa is better than ethyl acetate and n-hexane extracts.

### 3.4. Antiglycation and Antioxidant Methanol Fraction Activity

The continued fractionation of the methanol fraction using the chromatographic column produced a methanol sub-fraction with the results, as shown in Table 3. There was a reduction in DPPH free radical scavenging activity among the sub-fractions compared with methanol extract with significantly different values (p < 0.05). The reduction of DPPH free radical scavenging activity by X. granatum extract was better than methanol sub-fraction 2–5 (F2–F5) (Table 3).

#### Table 3. Antioxidant activity, antiglycation in methanol extract, methanol fraction, and each sub-fraction (F1, F2, F3, F4, F5, F1.1, F1.2, F1.3) and the yield of X. granatum stem.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (%)</th>
<th>Antioxidant activity IC₅₀ (ppm)</th>
<th>Antiglycation activity IC₅₀ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Extract</td>
<td>10.66 ± 0.20</td>
<td>14.13 ± 0.16</td>
<td>83.23 ± 0.41</td>
</tr>
<tr>
<td>Methanol Fraction</td>
<td>70.91 ± 0.70</td>
<td>8.52 ± 0.15</td>
<td>71.55 ± 0.37</td>
</tr>
<tr>
<td>F1</td>
<td>17.83 ± 0.17</td>
<td>7.58 ± 0.11</td>
<td>67.25 ± 0.06</td>
</tr>
<tr>
<td>F2</td>
<td>5.76 ± 0.14</td>
<td>154.90 ± 0.51</td>
<td>80.03 ± 0.58</td>
</tr>
<tr>
<td>F3</td>
<td>20.20 ± 0.13</td>
<td>107.6 ± 0.52</td>
<td>89.64 ± 0.25</td>
</tr>
<tr>
<td>F4</td>
<td>13.01 ± 0.10</td>
<td>171.03 ± 0.76</td>
<td>134.24 ± 0.58</td>
</tr>
<tr>
<td>F5</td>
<td>13.27 ± 0.12</td>
<td>187.30 ± 1.03</td>
<td>114.90 ± 0.23</td>
</tr>
<tr>
<td>F1.1</td>
<td>4.54 ± 0.15</td>
<td>30.79 ± 0.17</td>
<td>70.31 ± 0.12</td>
</tr>
<tr>
<td>F1.2</td>
<td>4.76 ± 0.14</td>
<td>74.41 ± 0.34</td>
<td>95.48 ± 0.46</td>
</tr>
<tr>
<td>F1.3</td>
<td>4.76 ± 0.14</td>
<td>114.78 ± 0.36</td>
<td>63.26 ± 0.12</td>
</tr>
</tbody>
</table>

Note: The numbers in the same column followed by the same letter are not significantly different (P < 0.05), based on Duncan’s multiple range test (DMRT)

That is because the synergy effect of antioxidant compounds is defined as the effect produced by a mixture of two or more types of antioxidants. This effect has a more significant effect than the cumulative effect of the mixture of the two antioxidants. Trang et al. [35] stated that the existence of a synergistic effect that causes antioxidant activity in the ethyl acetate sub-fraction of...
the stem of *Oroxyllum indicum* Linn is no better than ethyl acetate extract.

The order of strength of the strongest antiglycation activity was F1 > F2 > F3 > F5 and> F4 (Table 3). Sub-fraction 1 of methanol (F1) has the most active antioxidant and antiglycation activity, which is characterized by a low IC₅₀ value. Therefore, F1 was separated again using preparative TLC to obtain three F1 sub-fractions, namely F1.1, F1.2, and F1.3. The separation process in F1 provides a change in activity in F1.1, F1.2, and F1.3 to be less feasible compared to F1 itself.

Prediction of compounds contained in the active band was carried out qualitatively based on luminescence under UV light at 254 and 366 nm. The F1.1 fraction has black spots at UV 254, the F1.1 and F1.2 fractions have blue spots at UV 366, while the F1.3 fraction is red (Figure 2).

![Figure 2. Preparative thin layer chromatogram in UV 254 (a) and UV 366 (b)](image)

According to Markham, the blue color indicates the presence of flavone, flavanone, or flavonol compounds, red anthocyanidin compounds, and the greenness suggests the presence of auron and flavon compounds [36]. Based on this result, the active compounds as an antioxidant and antiglycation are included in the group of flavonoid compounds. The appearance of these colors refers to the appearance of flavonoid compounds. *Xylocarpus granatum* is rich in flavonoids such as quercetin, kaempferol, catechin, and epicatechin and routine [19, 37].

4. Conclusion

The most active antiglycation and antioxidant activity in the methanol fraction with IC₅₀ were 71.55 ppm and 8.52 ppm, respectively. Antiglycation and antioxidant activity of the most active are methanol fraction > methanol extract > ethyl acetate fraction > n–hexane fraction. The methanol fraction contains an alkaloid, flavonoid, phenolic, and triterpenoid compounds. Subsequent fractionation of the methanol fraction guided by bioassay showed that sub–fraction 1 (F1) had the best antiglycation and antioxidant activity. Based on the spot chromatogram color formed at UV 366 nm, the chemical component found in the most active fraction is the flavonoid group.

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