Anti-Diabetic Activity and Nuclear Magnetic Resonance (NMR) Characterization of Natural Esters from Hexane and Dichloromethane Crude Extracts from Calyces of *Hibiscus sabdariffa* Linn

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Abstract: Non-polar crude extracts obtained from the calyces of *Hibiscus sabdariffa* Linn. (Roselle) and their α-glucosidase inhibitory activity has been investigated. Roselle extracts potentially act as an anti-diabetic activity. However, most of the previous studies on Roselle were just focused on the polar crude extracts. Therefore, hexane and dichloromethane crude extracts were selected for these study. 3 kg of samples were air dried at room temperature, ground and serially extracted by solid-liquid extraction technique using hexane and dichloromethane. Compounds were isolated and purified by various chromatographic techniques. Their structures were elucidated with 1D and 2D-NMR, and other spectroscopic methods including MS, IR and UV as well as comparison with data reported in the literature. The phytochemical study has led to the isolation of three compounds namely squalene, triglyceride fatty acids (consist of ethyl oleate, ethyl linoleate and γ-ethyl linolenate) and ethyl stearate. Based on the literature, this is the first reported squalene isolated from the calyces of Roselle. The α-glucosidase inhibitory activity on the crude extracts was conducted and showed moderate inhibitory activity was detected on hexane crude extract. Thus, results from this study can be used as future references for the discovery of natural esters and the potential of Roselle as an anti-diabetic source.


I. INTRODUCTION

*Hibiscus sabdariffa* Linn. or Roselle is also known as Jamaican sorrel, red sorrel, Indian sorrel, Rozella hemp and *B10*.

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that usually grows in tropical and subtropical climates countries [2].

Roselle grows up to 5 meters tall with the robust stem. It has oval, simple and three lobes leave that grow alternately. Besides, the flower is borne singly and has yellow color or buff petals with a rose or maroon eye [3]. The color turn pink when the flower matured and can reach up to 12.5 cm. The red calyx consists of five large sepals with epicalyx, crisp but juicy and contains five valves which are each valve contains 3-4 kidneys shaped with light brown seeds [4]. The calyx begins to enlarge at the end of the day with 3.2 to 5.7 cm long [5].

The calyces of Roselle contain rich sources of dietary fiber, vitamins, minerals and bioactive compounds such as polyphenols, phytosterols and organic acid [6].

Recently, some researches that carried out study on Roselle found that Roselle were used to preventing chronic disease such as a cardiovascular disease [7]-[8] atherosclerosis [9] reducing hepatic disease [10] reducing high cholesterol [11] and hypertension [12]-[13]. The flower was used to decrease the viscosity of blood and reducing blood pressure because of the presence of gossypetin, anthocyanin and glycocide hibiscin. It was used in folk medicine for the treatment of constipation, heart ailment, high blood pressure, urinary tract infection, cancer, diabetes and nerve disorder [14].

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces [15]. Based on a report by the World Health Organization (WHO), WHO estimates that, globally, 422 million (8.5%) adults aged over 18 years were living with diabetes in 2014. It showed an increase of 3.8% compared to 1980. Based on the report also showed that in 2016, diabetes was the direct cause of 1.6 million deaths and in 2012 high blood glucose was the cause of another 2.2 million deaths.

Therefore, this paper reports the isolation and identification of chemical constituents from the non-polar crude extract which is hexane and dichloromethane crude extracts of calyces of *Hibiscus sabdariffa* Linn. and its anti-diabetic activity. The structure elucidation was...
performed with the aid of spectroscopic methods whereas anti-diabetic activity was performed according to the method reported by Chen in 2013 with minor modification.

II. MATERIALS AND METHODS

A. Collection and Preparation of Plant Materials

The calyces of Hibiscus sabdariffa Linn. were collected from Pulau Pinang. The calyces were air dried and ground into small pieces, weighed (3 kg) and stored at room temperature.

B. Extraction and Isolation

3 kg of dried plant materials were extracted using ethanol (Denatured ethanol 95%). The solvent was replenished every 72 hours three times to ensure that all possible compounds could be extracted. The resulting extracts were filtered and evaporated under reduced pressure by using a rotary evaporator at approximately 40 °C to provide gummy concentrated of the crude extracts. 300 g of the ethanol crude extract was mixed with celite and allowed to dry. Then, hexane was added in the celite and filtered to obtain the hexane crude extract and the filtrate was then evaporated under reduced pressure to provide gummy concentrated of the hexane crude extract. The celite was reused to produce dichloromethane crude extract. 17.54 g (0.58%) hexane crude extracts and 60.35 g (2.01%) dichloromethane crude extracts were obtained from the extraction process.

C. Spectroscopic Characterization

Different spectroscopic methods were used to elucidate the structure of the isolated compound, including IR, 1D and 2-D NMR, and GC-MS were carried out. IR spectra were recorded using Nicolet 6700 FTIR spectrophotometer and methanol as solvent whereas NMR spectra were recorded using JEOL ECX 500 and CDCl3 as a solvent.

D. α-glucosidase Inhibition Activity

The α-glucosidase inhibition activity was performed according to the method reported by Chen in 2013 with minor modification. 20 µL of the sample with varying concentration and 40 µL of α-glucosidase solution were incubated together for 10 minutes at temperature 37 °C. The enzyme reaction was started by adding 40 µL of 0.5 mM 4-nitrophenyl-α-D-glucopyranoside (pNPG) and further incubated at 37 °C for 30 minutes. The reaction was terminated by adding 100 µL of 0.2 M sodium carbonate (Na2CO3) in each well. The experiment was performed in triplicate by using 96 well microplates. Quercetin and acarbose were used as a positive control and IC50 values were calculated by the graphics method. The absorption was read at 405 nm. The inhibitions of the test sample on α-glucosidase could be calculated as formula (1):

\[
Inhibition = 1 - \frac{Sample\_Absorbence}{Control\_Sample\_Absorbence} \times 100\%
\]

II. RESULT

A. Chromatographic Separation

The column was packed with fine TLC grade silica gel 230-400 mesh ASTM (Merck 1.09385.5000) and 70-230 mesh ASTM (Merck 1.07734.2500) using n-hexane solvent. For the hexane crude, 310 g fine silica gel was compacted to the column (15.5 cm diameter x 55 cm length) and washed with n-hexane to facilitate compact packing. 10.31 g hexane crude extract was subjected to the column chromatography. The column was then eluted with n-hexane followed by mixtures of n-hexane: dichloromethane and then mixtures of dichloromethane: methanol. The polarity was gradually increased by adding increasing proportions of dichloromethane and methanol. 25 fractions were collected. Fraction 4 and fraction 16 were further purified using the preparative thin layer chromatography (PTLC) and yielded squalene (Fig. 1) and triglyceride that consist of acids which are linolenic acid, linoleic acid and oleic acid (Fig. 2). For the dichloromethane crude extract, 900 g fine silica gel was compacted to the column (23.5 cm diameter x 57.5 cm length) and washed with n-hexane to facilitate compact packing. 30 g dichloromethane crude extract was subjected to the column chromatography. The solvent used for elution was same ashexane crude extract. A total 23 fractions were collected and fraction 1 was further purified using the preparative thin layer chromatography (PTLC) and yielded ethyl stearate (Fig. 3).

B. Spectral Data of Isolation Compounds

Squalene (Fig. 1): 7.7 mg (0.0005%). ¹H NMR (500 MHz, CDCl3), δ:5.15-5.08 (6H, m, H-3, H-7, H-11, H-14, H-18 and H-22), 2.07 (4H, m, H-4 and H-21), 2.01 (4H, dd, J=2.85 and 3.45 Hz, H-12 and H-13), 1.99 (12H, t, J=7.45 Hz, H-5, H-8, H-9, H-16, H-17 and H-20), 1.68 (6H, s, H-1 and H-24), 1.60 (18H, s, H-25, H-26, H-27, H-28, H-29 and H-30). ¹³C NMR (125 MHz, CDCl3), δ:135.2 (C-10 and C-15), 135.0 (C-6 and C-19), 131.4 (C-2 and C-23), 124.5 (C-11 and C-14), 124.4 (C-7 and C-18), 124.3 (C-3 and C-22), 39.8 (C-5, C-9, C-16 and C-20), 28.3 (C-12 and C-13), 26.8 (C-8 and C-17), 26.7 (C-4 and C-21), 25.8 (C-25 and C-30), 17.8 (C-1 and C-24), 16.1 (C-26, C-27, C-28 and C-29).

Triglyceride fatty acid (Fig. 2): 4.9 mg (0.0001%). ¹H NMR (500 MHz, CDCl3), δ:5.35 (6H, m, H-10, H-12, H-13, H-15, H-10', H-12'), 5.34 (6H, m, H-9, H-16, H-9', H-13', H-9'' and H-10''), 5.26 (1H, m, H-1'a), 4.29 (2H, dd, J=4.0 and 7.5 Hz, H-1a and H-1'a''), 4.14 (2H, dd, J=5.7 and 6.3 Hz, H-1a and H-1'a''), 2.75 (6H, t, J=6.3 Hz, H-11, H-14, H-11'), 2.30 (6H, m, H-2', H-2'' and H-2'''), 2.05 (2H, m, H-17), 2.04 (8H, m, H-8, H-8', H-14' and H-8''), 1.58 (6H, m, H-3, H-3' and H-3''), 1.30 (22H, m, H-4, H-5, H-4', H-5', H-16', H-17', H-4', H-5', H-13', H-15' and H-17'). 1C NMR (125 MHz, CDCl3), δ:173.4 (C-1 and C-1'), 173.0 (C-1'), 132.0 (C-16), 130.3 (C-9' and C-10''), 130.1 (C-9, C-9' and C-13'), 128.4 (C-12), 128.3...
Triglyceride (30.1 mg, 0.17 %) was isolated from hexane crude extract of the calyces as a yellow oil with a molecular formula of $\text{C}_{30}\text{H}_{48}$. On subject to IR spectroscopic analysis, the presence of absorption band was 1025.47 cm$^{-1}$ which a characteristic of C=O, ester. Absorption at 1695.88 cm$^{-1}$ was due to C=O, ester. Mass spectrometry revealed a parent molecular ion peak at [M]$^+$/m/z 879.3844.

### III. DISCUSSION

The research work through systematic chemical investigation has determined and identified the presence of squalene (Fig. 1), triglyceride (Fig. 2) and ethyl stearate (Fig. 3) from the calyces of Hibiscus sabdariffa Linn. The work was carried out using chromatographic techniques. The assignments were supported by 2D-NMR (COSY, HMOC, and HMBC) spectroscopic analysis. The structures of the compounds were also elucidated by comparison with the literature.

Squalene (7.7 mg, 0.0439 %) was isolated as a light yellow oil from fraction 4 of hexane crude extracts. The IR spectrum revealed the presence of a double bond at 1670.95 cm$^{-1}$ whereas the mass spectrometry revealed an [M]$^+$/m/z 410.4913 gave the possible molecular formula of $\text{C}_{30}\text{H}_{48}$. The UV spectrum showed absorption bands at 214.0 nm.

**Fig. 1 Molecular structure of squalene**

The $^1\text{H}$ NMR resonance between $\delta_{\text{H}}$ 5.12 and 5.15, characteristic of the internal vinylic signals assigned to H-3, H-7, H-11, H-14, H-18 and H-22. The CH$\text{H}$ region is characterized by three clusters of proton resonances centered near $\delta_{\text{H}}$ 1.99 (t, $J=7.45$ Hz, 8H), $\delta_{\text{H}}$ 2.01 (dd, $J=2.85$ and 3.45, 4H) and $\delta_{\text{H}}$ 2.07 (m, 8H). Methyl groups proton appeared at $\delta_{\text{H}}$ 1.60 (s, 18H), and $\delta_{\text{H}}$ 1.68 (s, 6H). The former was attributed to H-25, H-26, H-27, H-28, H-29 and H-30 and the later assigned to H-1 and H-24. The $^{13}\text{C}$ NMR and DEPT spectrum showed the presence of thirty carbon signals. There were eight methyl carbons at $\delta_{\text{C}}$ 16.0 to 25.8, ten methylene carbons at $\delta_{\text{C}}$ 26.7 to 39.8 and six olefinic carbons at $\delta_{\text{C}}$ 124.3 to 124.5. Signals for quaternary carbons appeared at $\delta_{\text{C}}$ 131.4 to 135.

Based on the $^1\text{H}$ NMR, the olefinic protons –CH=CH- of unsaturated fatty acids resonated at $\delta_{\text{H}}$ 5.26-5.35 (m). Proton signal at $\delta_{\text{H}}$ 5.26 (m) was assigned to H-1a' of the glycerol backbone. The H-1a and H-1a'' protons of glycerol resonate at $\delta_{\text{H}}$ 4.14 (dd, 5.7, 6.3 Hz) and 4.29 (dd, 4.0, 7.5 Hz) while the methylene proton between unsaturated acyl chains appeared with triplet signal at $\delta_{\text{H}}$ 0.75 with coupling constant 6.3 Hz. Acyl moieties in triacylglycerols resonated at $\delta_{\text{H}}$ 2.30 and 1.58 for position H-2, H-2', H-2'' and H-3, H-3', H-3'', respectively. The methyl protons of the polysaturated acids were shifted at a higher frequency at $\delta_{\text{H}}$ 0.97 and 0.88 with the coupling constant 7.45 Hz and 6.9 Hz, respectively. The $^{13}\text{C}$ NMR spectrum revealed the presence of carboxyl carbons at $\delta_{\text{C}}$ 137.4 (C-1 and C-1') and 173.0 that assigned to C-1'' carbon. Unsaturated carbons were resonating in the range from $\delta_{\text{C}}$ 127.8 to 132.0 and aliphatic carbons from $\delta_{\text{C}}$ 20.6 to 34.4. The spectrum also showed recognizables signals at $\delta_{\text{C}}$ 62.2 and 68.9 belonged to glycerol moiety at C-1a, C-1a' and C-1a''. Three methyl carbons appeared at $\delta_{\text{C}}$ 14.4, 14.2 and 14.1 corresponding to C-18, C-18' and C-18'', respectively. The total of carbon present was supported by DEPT NMR analysis which showed the presence of three quaternary carbons, three methyl carbons, 13 methine carbons and 38 methylene carbons.

Ethyl stearate was obtained as yellowish oil from dichloromethane crude extracts. The IR spectrum showed the presence of absorption bands at 1025.47 cm$^{-1}$ (C=O) and 1695.88 cm$^{-1}$ (C=O). It’s exhibited a molecular formula of $\text{C}_{20}\text{H}_{36}$$\text{O}_2$ based on the HRESIMS which showed an ion peak at $m/z$ 312.5304.

**Fig. 3 Molecular structure of ethyl stearate**

The $^1\text{H}$ NMR spectrum showed two triplet signals at $\delta_{\text{H}}$ 0.88 (3H, $J=6.85$ Hz) and 1.25 (3H, $J=9.15$ Hz) could be assigned to the methyl group at H-20 and H-1, respectively.
On the other hand, a triplet signal appeared at δ H 2.28 (2H, t, J=8.05 Hz) for H-4. The spectrum also displayed two multiplet signals at δ H 1.61 (2H, m) and δ H 1.25 (26H, m) that belong to H-5 and H-6 until H18, respectively. In addition, a doublet of doublet at δ H 4.12 (2H, dd, J=6.85, 7.45 Hz) could be ascribed to a methylene proton on H-2. The 13C NMR and DEPT spectrum showed twenty carbon signals. There was a quaternary carbon signal at δ C 174.1 assigned to C-3. Besides, there were seventeen methylene carbon signals at δ C 22.8 (C-19), 25.1 (C-5), 29.2 (C-17), 29.4 (C-6), 29.5 (C-7), 29.6 (C-16), 29.7 (C-8 until C-15), 32.0 (C-18), 34.5 (C-4) and 60.3 (C-2). The spectrum also revealed two methyl carbons appeared at δ C 14.2 and 14.3 assigned to C-1 and C-20, respectively.

Anti-diabetic activity of the crude extracts and isolated compounds were tested by using in-vitro α-glucosidase inhibitory assays. The effectiveness of enzymatic inhibition of the crude extracts was determined by calculating IC50 value. The concentration of the samples and control used for the crude extracts and isolated compounds range from 12 to 71.43 µg/mL. Hexane crude extract exerted the highest inhibitory effect against α-glucosidase with IC50 of 0.86 µg/mL. It showed stronger inhibition activity in comparison with quercetin (IC50 = 4.35 µg/mL) as a positive control. Dichloromethane crude extract and triglyceride were not active for the α-glucosidase inhibitory activity as shown in Table 1 while squalene and ethyl stearate were not tested for their α-glucosidase inhibitory activity due to the samples do not dissolve in phosphate buffered saline (PBS) solvent.

### Table 1. The α-glucosidase inhibitory activity of crude extracts and isolated compounds

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-glucosidase inhibitory activity (IC50 Value (µg/mL))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane crude extract</td>
<td>0.86</td>
</tr>
<tr>
<td>Dichloromethane crude extract</td>
<td>NA</td>
</tr>
<tr>
<td>Triglyceride (2)</td>
<td>NA</td>
</tr>
<tr>
<td>*Quercetin</td>
<td>4.35</td>
</tr>
</tbody>
</table>

*NA: Not active, *Positive control

### IV. CONCLUSION

The research work through systematic chemical investigation has determined and identified the presence of squalene (Fig. 1), triglyceride (Fig. 2) and ethyl stearate (Fig. 3) from the calyces of Hibiscus sabdariffa Linn. Hexane crude extract exerted the highest inhibitory effect against α-glucosidase with IC50 of 0.86 µg/mL while dichloromethane crude extract and triglyceride were not active for the α-glucosidase inhibitory activity.

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