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.

Index Terms*: *Anti-diabetic activity, Carboxilic acid ester, Hibiscus sabdariffa Linn, Trigliceride.

I. INTRODUCTION

Hibiscus sabdariffa Linn. or Roselle is also known as Jamaican sorrel, red sorrel, Indian sorrel, Rozella hemp and natal sorrel in English-speaking countries. Among Malaysian, Roselle is called as ‘asam susur’ or ‘asam paya’[1]. It is the most important plant of *Malvaceae* family 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 glycoside 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

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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 $CDCl_3$ 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 (Na_2CO_3) in each well. The experiment was performed in triplicate by using 96 well microplates. Quercetin and acarbose were used as a positive control and IC_{50} 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):

$$Inhibition = 1 - \left[\frac{Sample\_Absorbence}{Control\_Sample\_Absorbance} \times 100\% \right] \quad (1)$$

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 as hexane 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): 7.7mg (0.0005%). ^{1}H NMR (500 MHz, $CDCl_3$), 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-29and H-30). ^{13}C NMR (125 MHz, $CDCl_3$), 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): 4.9mg (0.0001%). ^{1}H NMR (500 MHz, $CDCl_3$), 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-1a'), 4.29 (2H, *dd*, *J*=4.0 and 7.5 Hz, H-1a and H-1a"), 4.14 (2H, *dd*, *J*=5.7 and 6.3 Hz, H-1a and H-1a"), 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"), 1.24 (22H, *m*, H-6, H-7, H-6', H-7', H-15', H-6", H-7", H-11", H-12", H-14" and H-16"), 0.97 (3H, *t*, *J*=7.45 Hz, H-18), 0.88 (6H, *t*, *J*=6.9 Hz, H-18' and H-18"). ^{13}C NMR (125 MHz, $CDCl_3$), 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

(C-13), 128.1 (C-15), 127.9 (C-10 and C-10'), 127.8 (C-12'), 68.9 (C-1a'), 62.2 (C-1a and C-1a"), 34.3 (C-2'), 34.1 (C-2 and C-2"), 32.0 (C-16'), 31.6 (C-16"), 29.8 (C-7 and C-7'), 29.7 (C-6, C-6', C-15', C-6", C-7", C-12" and C-14"), 29.6 (C-11"), 29.3 (C-15"), 29.4 (C-5, C-5', C-5" and C-13"), 29.1 (C-4, C-4' and C-4"), 27.3 (C-8, C-8′, C-14' and C-8"), 25.7 (C-11 and C-14), 25.6 (C-11'), 25.0 (C-3, C-3' and C-3"), 22.8(C-17'), 22.7 (C-17"), 20.6 (C-17), 14.4 (C-18), 14.2 (C-18'), 14.1 (C-18″).

Ethyl stearate (Fig. 3): 30.1mg (0.002%). ^{1}H NMR (500 MHz, $CDCl_3$), δ:4.12 (2H, *dd*, *J*=6.85 and 7.45 Hz, H-2), 2.28 (2H, *t*, *J*=8.05 Hz, H-4), 1.61 (2H, *m*, H-5), 1.28 (2H, *m*, H-19), 1.25 (3H, *t*, *J*=9.15 Hz, H-1), 1.25 (26H, *m*, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17 and H-18), 0.88 (3H, *t*, *J*=6.85 Hz, H-20). ^{13}C NMR (125 MHz, $CDCl_3$), δ:60.3 (C-2), 174.1 (C-3), 34.5 (C-4), 32.0 (C-18), 29.7 (C-8, C-9, C-10, C-11, C-12, C-13, C-14 and C-15), 29.6 (C-16), 29.5 (C-7), 29.4 (C-6), 29.2 (C-17), 25.1 (C-5), 22.8 (C-19), 14.3 (C-1), 14.2 (C-20).

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, HMQC, 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 $C_{30}H_{50}$. The UV spectrum showed absorption bands at 214.0 nm.

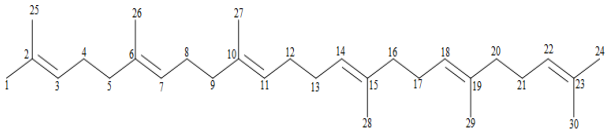

Fig. 1 Molecular structure of squalene

The ^{1}H NMR resonance between δ_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_2 region is characterized by three clusters of proton resonances centered near δ_H 1.99 (*t*, *J*=7.45 Hz, 8H), δ_H 2.01 (*dd*, *J*=2.85 and 3.45, 4H) and δ_H 2.07 (*m*, 8H). Methyl groups proton appeared at δ_H 1.60 (*s*, 18H), and δ_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}C NMR and DEPT spectrum showed the presence of thirty carbon signals. There were eight methyl carbons at δ_C 16.0 to 25.8, ten methylene carbons at δ_C 26.7 to 39.8 and six olefinic carbons at δ_C 124.3 to 124.5. Signals for quaternary carbons appeared at δ_C 131.4 to 135.

Triglyceride (30.1 mg, 0.17 %) was isolated from hexane crude extract of the calyces as a yellow oil with a molecular formula of $C_{57}H_{98}O_6$. On subjection 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.

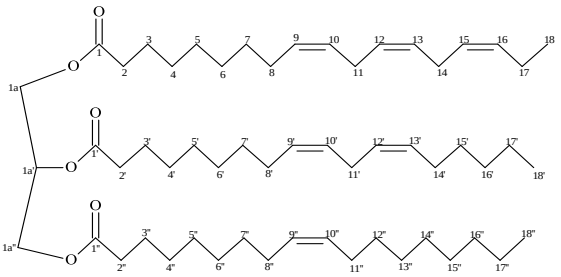

Fig. 2 Molecular structure of triglyceride

Based on the ^{1}H NMR, the olefinic protons –CH=CH- of unsaturated fatty acids resonated at δ_H 5.26-5.35 (*m*). Proton signal at δ_H 5.26 (*m*) was assigned to H-1a' of the glycerol backbone. The H-1a and H-1a" protons of glycerol resonate at δ_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 δ_H 2.75 with coupling constant 6.3 Hz. Acyl moieties in triacylglycerols resonated at δ_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 polyunsaturated acids were shifted at a higher frequency at δ_H 0.97 and 0.88 with the coupling constant 7.45 Hz and 6.9 Hz, respectively. The ^{13}C NMR spectrum revealed the presence of carbonyl carbons at δ_C 173.4 (C-1 and C-1') and 173.0 that assigned to C-1" carbon. Unsaturated carbons were resonating in the range from δ_C 127.8 to 132.0 and aliphatic carbons from δ_C 20.6 to 34.4. The spectrum also showed recognizable signals at δ_C 62.2 and 68.9 belonged to glycerol moiety at C-1a, C-1a' and C-1a". Three methyl carbons appeared at δ_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 $C_{20}H_{40}O_2$ based on the HRESIMS which showed an ion peak at *m/z* 312.5304.

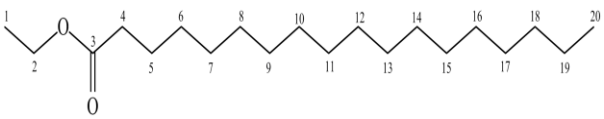

Fig. 3 Molecular structure of ethyl stearate

The ^{1}H NMR spectrum showed two triplet signals at δ_H 0.88 (3H, *t*, *J*=6.85 Hz) and 1.25 (3H, *t*, *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) present 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 at H-2. The ^{13}C 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 IC_{50} value. The concentration of the samples and control used for the crude extracts and isolated compounds range from1.12 to 71.43µg/mL. Hexane crude extract exerted the highest inhibitory effect against α-glucosidase with IC_{50} of 0.86 µg/mL. It showed stronger inhibition activity in comparison to quercetin (IC_{50} = 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 I. The *α*-glucosidase inhibitory activity of crude extracts and isolated compounds

Sample	*α*-glucosidase inhibitory activity
	IC_{50} Value (µg/mL)
Hexane crude extract	0.86
Dichloromethane crude extract	NA
Triglyceride (2)	NA
*Quercetin	4.35

*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 IC_{50} of 0.86 µg/mL while dichloromethane crude extract and triglyceride were not active for the *α*-glucosidase inhibitory activity.

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REFERENCES

1. Z. Aziz, S. Y. Wong and N. J Chong, "Effects of *Hibiscus sabdariffa L*. on serum lipids: A systematic review and meta-analysis," *Journal of Ethnopharmacology*, vol. 150, no. 2, October 2013, pp. 442-450.
2. A. H. Gendy, H. A. H. Said-Al Ahl and A. A. Mahmoud, "Growth, productivity and chemical constituents of Roselle (*Hibiscus sabdariffa L*.) plants as influenced by cattle manure and biofertilizers treatments," *Australian Journal of Basic and Applied Sciences*, vol. 6, no. 5, 2012, pp. 1-12.
3. H. A. Sindi, L. J. Marshall and M. R. A. Morgan, "Comparative chemical and biochemical analysis of extracts of *Hibiscus sabdariffa*," *Food Chemistry*, vol. 164, December 2014, pp. 23-29.
4. I. Da-Costa-Rocha, B. Bonnlaender, H. Sievers, I. Pischel and M. Heinrich, "*Hibiscus sabdariffa L* a phytochemical and pharmacological review," *Food Chemistry*, vol. 165, December 2014, pp. 424-443.
5. N. Mahadevan, Shivali and P. Kamboj, "*Hibiscus sabdariffa* Linn. - An overview," *Natural Product Radiance*, vol. 8, no. 1, February 2009, pp. 77-83.
6. A. Azza, M. Ferial, and A. Esmat, "Physico-chemical properties of natural pigments (anthocyanin) extracted from roselle calyces (*Hibiscus sabdariffa*)," *Journal of American Science*, vol. 7, no. 7, 2011, pp. 445-456.
7. C. C. Chen, F. P. Chou, Y. C. Ho, W. L. Lin, C. P. Wang, E. S. Kao, A.C. Huang and C. J. Wang, "Inhibitory effects of *Hibiscus sabdariffa L* extract on low-density lipoprotein oxidation and anti-hyperlipidemia in fructose-fed and cholesterol-fed rats," *Journal of the Science of Food and Agriculture*, vol. 84, no. 15, September 2004, pp. 1989-1996.
8. E. Prenesti, S. Berto, P. G. Daniele and S. Toso, "Antioxidant power quantification of decoction and cold infusions of *Hibiscus sabdariffa* flowers," *Food Chemistry*, vol. 100, no. 2, 2007, pp. 433-438.
9. H. A. Sindi, L. J. Marshall and M. R. A. Morgan, "Comparative chemical and biochemical analysis of extracts of *Hibiscus sabdariffa*." *Food Chemistry*,vol. 164, December 2014, pp. 23-29.
10. B. H. Ali, N. Al-Wabel and G. Blunden, "Phytochemical, pharmacological and toxicological aspects of *Hibiscus sabdariffa L*.: A review," *Phytotherapy Research*, vol. 19, no. 5, August 2005, pp. 369-375.
11. E. Hainida, A. Ismail, N. Hashim, N. Mohd-Esa and A. Zakiah, "Effects of defatted dried Roselle (*Hibiscus sabdariffa L*.) seed powder on lipid profiles of hypercholesterolemia rats," *Journal of the Science of Food and Agriculture*, vol. 88, no. 6, March 2008, pp. 1043-1050.
12. E. G. Maganha, R. D. Halmenschlager, R. M. Rosa, J. A. P. Henriques, A. Ramos and J. Saffi, "Pharmacological evidences for the extracts and secondary metabolites from plants of the genus Hibiscus," *Food Chemistry*, vol. 118, no. 1, January 2010, pp. 1-10.
13. D. L. McKay, C. Y. O. Chen, E. Saltzman and J. B. Blumberg, "*Hibiscus sabdariffa* L. tea (Tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults," *Journal of Nutrition*, vol. 140, no. 2, February 2010, pp. 298-303.
14. S. Patel, "*Hibiscus sabdariffa*: An ideal yet under-exploited candidate for nutraceutical applications," *Biomedicine & Preventive Nutrition*, vol. 4, 2014, pp. 23-27.
15. Geneva, 1999, "Definition, diagnosis and classification of Diabetes Mellitus and its complications. Part 1: Diagnosis and classification of Diabetes Mellitus" World Health Organization, Available: https://www.who.int/diabetes/en/