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Phytochemicals and Biological Activity Studies of *Anacardium occidentale*

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GRAPHICAL ABSTRACT

Plant of *A. occidentale*

Stearic acid **(1)**
β-sitosterol **(2)**

ABSTRACT

Anacardium occidentale, locally known as "*pokok gajus*" is known for its medicinal purposes. The objectives of this study are to isolate compounds from the stem barks extract of *A. occidentale* and to study the antioxidant properties of the extracts. Cold maceration method was performed to extract the compounds present in the samples. Purification of the crude extracts using column chromatography had successfully isolated two compounds. The isolated compounds were elucidated using spectroscopic methods which were Fourier Transform Infrared (FTIR) and ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopies. These compounds were identified as stearic acid and β-sitosterol. The antioxidant potential of the crude extracts were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The results revealed that methanol extract was most effective in the capability to scavenge free radical as it exhibited the highest radical scavenging activity towards DPPH with an IC_{50} value of 87.67 µg/mL. Total phenolic content and total flavonoid content of the extracts were measured. The total phenolic and flavonoid contents showed significant correlations with the antioxidant activity indicated that phenolic and flavonoid compounds were the major contributors to the antioxidant capacity of the extracts. Based on the findings, *A. occidentale* can be considered as an effective source of natural antioxidant.

Keywords: Anacardium occidentale, stearic acid, β-sitosterol, antioxidant activity

1. INTRODUCTION

The enormous variety of Malaysian flora with diverse range of chemicals is one of the major aspects that essentially make natural products as excellent sources in every screening method. Natural products are among the most prominent sources of leading substances in drug development and have been regarded as an important source of medicinal agents and bioactive compounds since the dawn of historical times. The presence of over 200,000 natural metabolites containing a variety of unique bioactive properties emphasizes the importance of natural resources in the discovery of new drugs [1]. Medicinal plants have been the most striking sources of natural products. Medicinal plants are one of the major elements for the discovery of natural products in pharmaceutical industry as the natural products isolated from medicinal plants display an immense potential in the treatment of various diseases.

Anacardium occidentale is one of the medicinal plant species found in the tropical regions of Malaysia. *A. occidentale*, commonly known as cashew, belongs to the family Anacardiaceae. The Anacardiaceae consists of about 76 genera and more than 600 species of trees, shrubs, and woody vines are distributed pantropically around the world, while 17 of these genera with 77 species are found in Peninsular Malaysia [2]. Cashew, *A. occidentale* is an evergreen tree native from northeast part of Brazil, which has grown naturally throughout the South American countries. Cashew tree was introduced by Portuguese to India and Africa during the 16th century and has spread across southeast Asia. This plant is considered as economic importance worldwide as a source of two beneficial products which are the cashew apple (with an enlarged and fleshy pedicel) and the cashew nut (botanically representing the true fruit) [3].

Cashew tree is a small-sized tree with a dome-shaped crown that can grow at an altitude of 1000 m from sea level. It produces a shiny, soft, and juicy fruit, known as a cashew apple, which carries a single-seeded nut in its bottom coated with a hard gray shell. The fruit of a cashew tree is an accessory fruit that is often called a false fruit or a pseudo carp which has an oval or pear-shaped structure. The cashew apple is a hypocarpium that formed by a pedicel and a receptacle of a cashew flower, which ripens into a red or yellow structure about 5–11 cm long [3].

A. occidentale is popular for its medicinal purposes and being traditionally used for the treatment of many diseases, as it is well recognized as a major source of bioactive compounds that capable of alleviating various lifestyle-related diseases. Phytochemical studies on this species revealed the presence of various secondary metabolites. Secondary plant metabolites refer to various chemical constituents generated by the plant cell via metabolic pathways that composed of flavonoids, alkaloids, phenolics, terpenoids, carotenoids, fatty acids, and tannins which are the potential of medicinal uses. Studies have found that some of these compounds extracted from *A. occidentale* exhibit biological activities such as antioxidant, anti-inflammatory, antibacterial, antimicrobial, antitumor, anti-mutagenic, and anti-allergic [4].

2. EXPERIMENTAL

The main idea of this study is based on four primary steps which were extraction, purification, characterization, and evaluation of its biological activity. Cold maceration method was applied to the dried samples of *A. occidentale* using *n*-hexane, chloroform ($CHCl_3$), and methanol (MeOH) as solvents to obtain the crude extracts. The solvents were then evaporated off using rotary evaporator. Thin layer chromatography (TLC) analysis was performed using 0.20 mm pre-coated silica gel aluminium sheets (Merck Kieselgel 60 F254) with *n*-hexane and diethyl ether as solvent system. The spots on the TLC were visualized under UV light (254 and 365 nm) and the TLC plate were then sprayed with vanillin sulphuric acid reagent. Gravity column chromatography (CC) was performed using Merck silica gel 60 (70-230 mesh) for the purification of compounds. The structure of pure compounds were characterized using spectroscopic methods which were Fourier Transform Infrared (FTIR) and ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopies.

2.1 Plant Material

The stem barks of *A. occidentale* were collected from Kelantan in September 2019. The stem barks were dried and chopped into small pieces before grinded into fine powdered sample.

2.2 Extraction of Stem Barks of *A. occidentale*

The extraction of stem barks of *A. occidentale* was carried out using cold maceration technique. The grinded stem barks of *A. occidentale* (900 g) were sequentially extracted using *n*-hexane, chloroform, and methanol (2.5 L each) at room temperature for 72 hours. The crude extracts were then filtered out and the filtrate was then concentrated under reduced pressure using rotary evaporator, yielded a green sticky of *n*-hexane crude extract (0.92 g, 0.10%), brown gummy of chloroform (1.29 g, 0.14%), and methanol (3.30 g, 0.37%) crude extracts.

2.3 Thin Layer Chromatography (TLC) Analysis of Crude Extracts

The crude extracts of *A. occidentale* were analyzed using thin layer chromatography (TLC) for the identification of chemical constituents present in the samples. This TLC analysis was carried out by separating the components of a mixture using a thin stationary phase supported by an inert backing. Pre-coated silica gel aluminium sheets (Merck Kieselgel 60 F254) with the thickness of 0.20 mm were used as the stationary phase for the analysis in order to analyze the crude extracts. The eluting solvents used were *n*-hexane and diethyl ether with different polarities of ratios as the sample mixture would separate according to the polarity of the solvents used. The spots appeared on the TLC plate were observed under UV light at both short and long wavelengths (254 nm and 365 nm, respectively). The TLC plate was then sprayed with vanillin sulphuric acid reagent before heated at 110°C in order to visualize the spots of separated chemical compounds.

2.4 Isolation of Chemical Constituents of *A. occidentale*

Purification of the crude extract was carried out by gravity column chromatography (CC) using Merck silica gel 60 (70-230 mesh) to isolate the compounds present in the sample. The components were eluted initially with *n*-hexane and the polarity of the eluent was gradually increased by addition of higher percentage of diethyl ether. Eluents were then collected and analyzed using TLC. Fractions with similar TLC profile were pooled together and combined. The combined fractions with a single spot on the TLC were labelled as AO61 and AO93.

Fraction A061 was obtained from the purification of *n*-hexane extract using CC had afforded white solid of stearic acid **(1)** (0.01 g, 1.09%); m.p. 65-68°C (lit. [5] 70-72°C; IR (ATR) ν_{max} cm^{-1} : 3500-2500 (O-H), 2915 (sp^3 C-H), 1701 (C=O); ^{1}H NMR ($CDCl_3$, 300 MHz) : δ 2.35 (2H, t, *J* = 7.5 Hz, H-2), 1.64 (2H, m, H-3), 1.29 (14 × 2H, m, H-4 - H-17), 0.88 (3H, t, *J* = 6.6 Hz, H-18). Fraction AO93 was yielded from the purification of *n*-hexane extract using CC had yielded white solid of β-sitosterol **(2)** (0.02 g, 2.17%); m.p. 130-132°C (lit. [6] 134-135°C; IR (ATR) ν_{max} cm^{-1} : 3413 (OH), 2934 (sp^3 C-H), 1642 (C=C alkene), 1052 (C-O); ^{1}H NMR ($CDCl_3$, 300 MHz) : δ 3.54 (1H, m, H-3), 5.37 (1H, m, H-6), 0.70 (3H, s, H-18), 1.03 (3H, s, H-19), 0.94 (3H, d, *J* = 6.0 Hz, H-21), 0.85 (3H, d, *J* = 6.9 Hz, H-26). **Figure 1** showed the molecular structure of stearic acid **(1)** and β-sitosterol **(2)**.

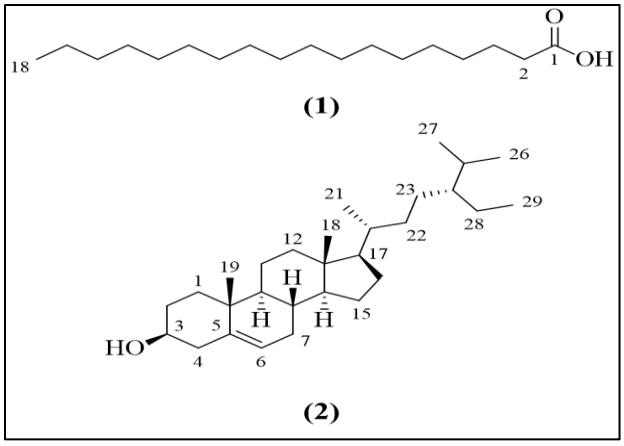

Figure 1. Molecular structure of stearic acid **(1)** and β-sitosterol **(2)**

2.5 Total Phenolic Content and Total Flavonoid Content

Total phenolic contents of *A. occidentale* bark extracts were measured using the Folin-Ciocalteu method with slight modifications while total flavonoid contents of *A. occidentale* bark extracts were measured using aluminium chloride colorimetric method. Both experiments were performed in triplicate.

2.6 Antioxidant Activity

Free radical scavenging activity of crude extracts of *A. occidentale* stem barks were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Johari and Khong [7] with some modifications. The absorbance was measured at 517 nm by using microplate reader and a commercial vitamin C was used as a positive control. All measurements were carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1 Characterization of Stearic Acid **(1)**

Extraction and purification of the *n*-hexane crude extracts of have led to the isolation and characterization of two compounds, which were identified as stearic acid **(1)**, and β-sitosterol **(2)**.

Compound **(1)** was characterized and the IR spectrum showed a broad absorption band at 3500-2500 cm^{-1} indicated the presence of O-H group of an acid. A strong absorption band at 2915 cm^{-1} represented the stretching vibration of sp^3 C-H group, while a strong stretching absorption at 1701 cm^{-1} was assigned to the C=O stretching of an acid as shown in **Figure 2**. The ^1H NMR spectrum in **Figure 3** showed a triplet signal at δ 0.88 with *J* value of 6.6 Hz indicated the presence of H-18 methyl proton. A broad multiplet peak was observed at δ 1.29 represented a long chain of methylene protons comprising 14 overlapping methylene groups. A multiplet signal at δ 1.64 indicated the two protons of H-3 which attached to methylene adjacent to carboxyl group. A triplet peak at δ 2.35 with *J* value of 7.5 Hz corresponded to the methylene protons adjacent to carboxyl group.

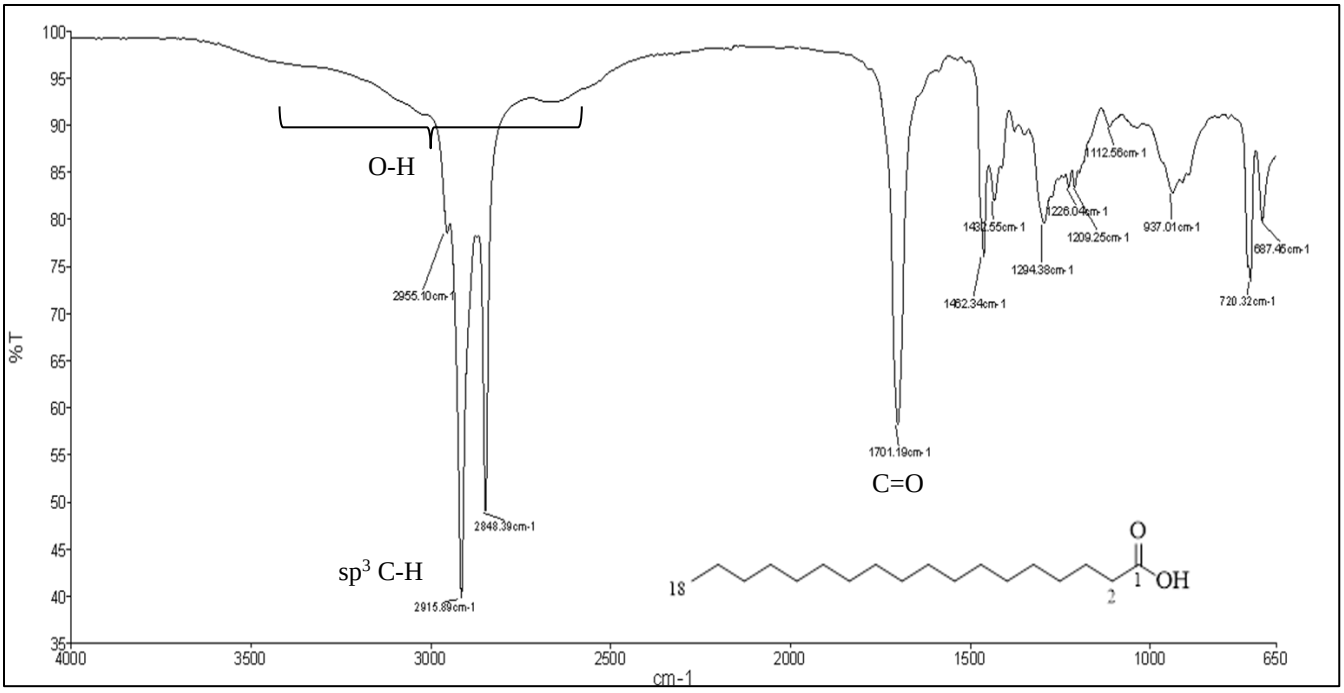

Figure 2. IR spectrum of compound **(1)**

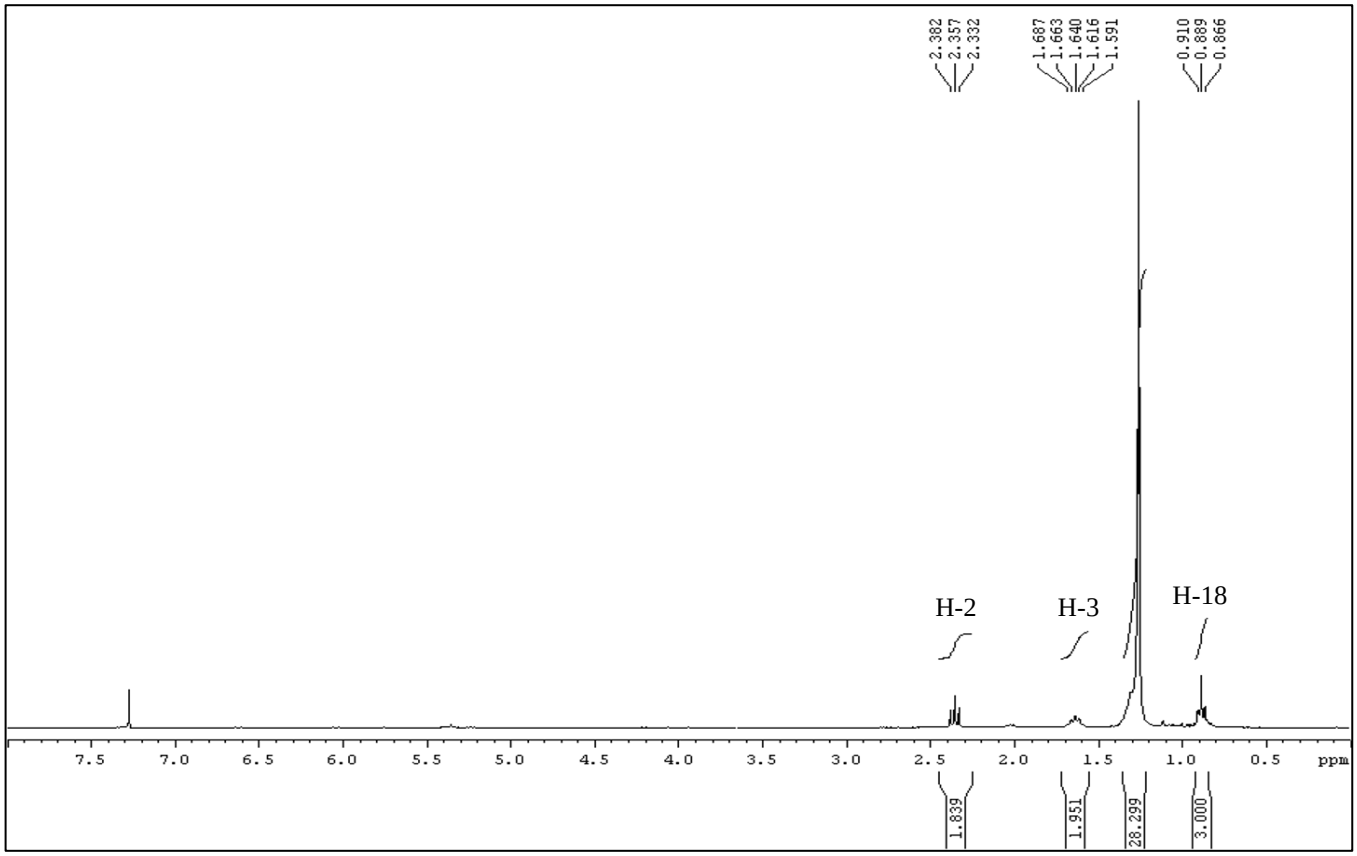

Figure 3. ^{1}H NMR spectrum of compound **(1)**

3.2 Characterization of β-Sitosterol **(2)**

Compound **(2)** was also characterized using IR and ^{1}H NMR. The IR spectrum of this compound in **Figure 4** showed a broad absorption band at 3413 cm^{-1} indicated the presence of hydroxyl group. A sharp peak appeared at 2934 cm^{-1} represented the stretching vibration of sp^3 C-H group. A peak indicated the presence of C=C alkene was observed at 1642 cm^{-1}, while a strong absorption band centered at 1052 cm^{-1} was assigned to the C-O stretching of a secondary alcohol. The ^{1}H NMR spectrum in **Figure 5** showed a multiplet signal at δ 3.54 represented the oxymethine proton of H-3, a proton corresponding to a proton attached to the hydroxy group of C-3. The presence of olefinic proton of H-6 was observed at δ 5.37. Compound **(2)** displayed four methyl signals. Two methyl singlets were appeared at δ 0.70 and δ 1.03 represented the protons of H-18 and H-19, respectively. The presence of two methyl protons of H-21 and H-26 were observed at the doublet signals centered at δ 0.94 and δ 0.85, with *J* value of 6.0 Hz and 6.9 Hz, respectively.

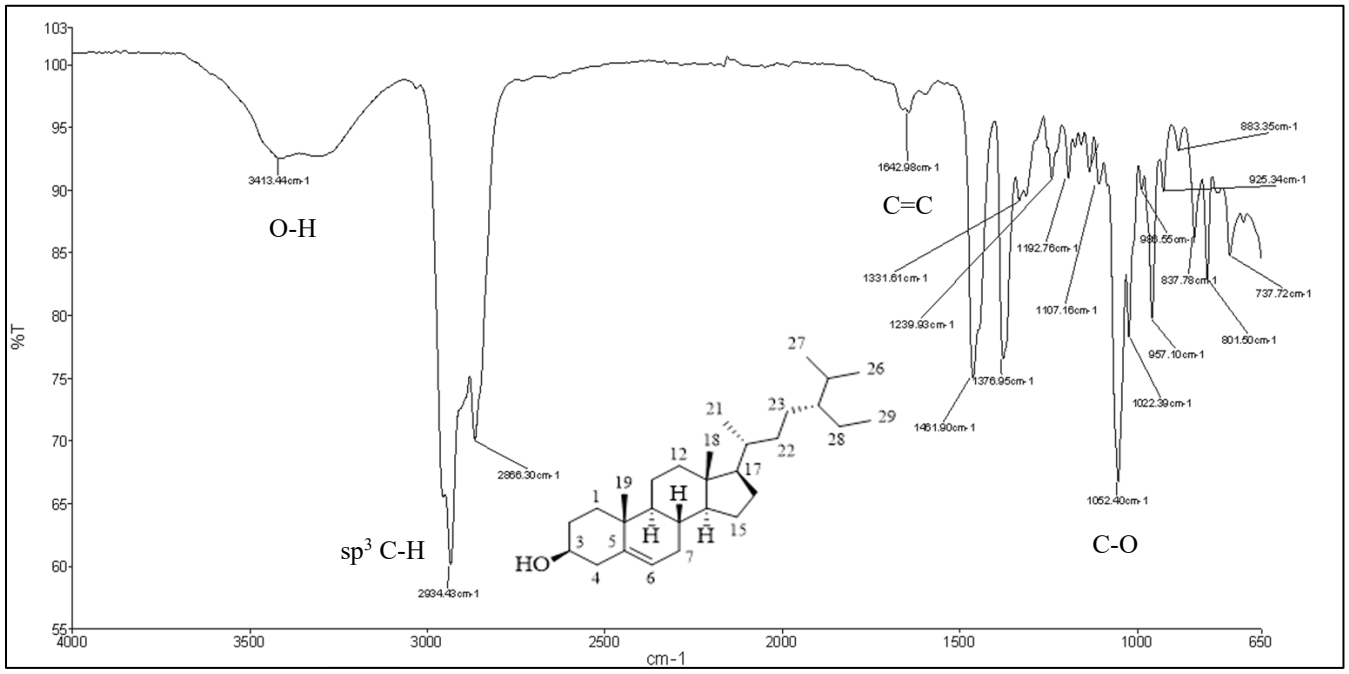

Figure 4. IR spectrum of compound **(2)**

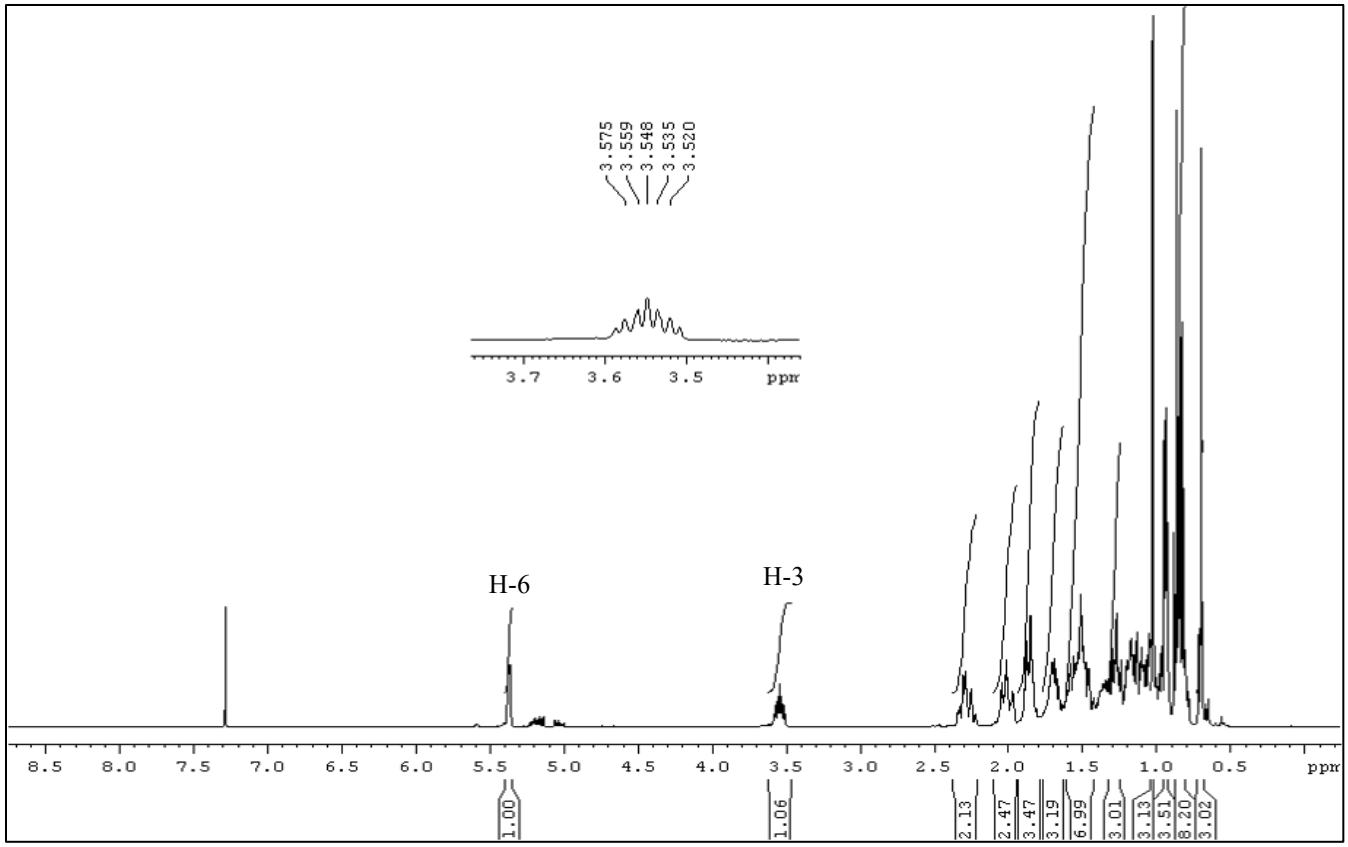

Figure 5. ^{1}H NMR spectrum of compound **(2)**

3.3 Antioxidant Activity

The crude extracts of *A. occidenatle* was analyzed for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay as described by Johari and Khong [7] with some modifications. Vitamin C was used as the positive control and the half-maximal inhibitory concentration (IC_{50}) of each crude extract was measured and expressed as µg/mL, which is defined as the concentration of substrate required to inhibit 50% of the radical.

The DPPH radical scavenging activity of *A. occidentale* stem bark extracts in comparing with commercial vitamin C as positive control is tabulated in **Table 1** along with the value of IC_{50}. Among the samples, methanol crude extract was most effective in its ability to scavenge free radical as it exhibited the highest radical scavenging activity towards DPPH from 38.39% at 62.50 µg/mL to 92.91% at 1000 µg/mL. The results revealed that methanolic extract of *A. occidentale* exhibited strong antioxidant activity in the DPPH assay with an IC_{50} value of 87.67 µg/mL.

Table 1. IC_{50} value of DPPH radical scavenging activity of *A. occidentale* extracts and commercial vitamin C

Sample	**Percentage of Inhibition (I%)**					**IC_{50} (µg/mL)**
	Concentration (ppm)					
	62.50	**125**	**250**	**500**	**1000**	
AOBH	3.32	9.42	16.86	23.32	36.05	1981
AOBC	4.30	7.80	15.43	20.45	45.29	1272
AOBM	38.39	60.00	87.89	92.20	92.91	87.67
Vit C	90.87	94.66	96.73	98.28	99.83	4.52

3.4 Correlation between Total Phenolic Content, Total Flavonoid Content and Antioxidant Activity

The total phenolic contents of *A. occidentale* stem bark extracts were measured using the Folin-Ciocalteau method with gallic acid as a reference standard. The value of total phenolic content of each extract was determined from the calibration curve of gallic acid ($y = 0.0056x + 0.0353$) with R^2 value of 0.9964. Based on the results tabulated in **Table 2**, methanol extract displayed the highest total phenolic content which was 81.91 mg GAE/g followed by chloroform and hexane extracts.

The total flavonoid contents of *A. occidentale* extracts were determined using aluminium chloride colorimetric method with quercetin as a reference standard. The values of total flavonoid contents of the extracts were measured from the quercetin calibration curve equation ($y = 0.0012x + 0.0224$) with R^2 value of 0.9863. The highest value of total flavonoid content was observed in methanolic stem bark extract of *A. occidentale* with the value of 56.33 mg QE/g as shown in **Table 2**.

Both total phenolic content and total flavonoid content showed significant correlations with antioxidant activity of *A. occidentale* extracts. The total phenolic content and total flavonoid content were significantly correlated with antioxidant activity with the correlation coefficients of $R^2 = 0.8668$ and $R^2 = 0.9188$, respectively. These results indicated that flavonoids and phenolic compounds were the major factors contributing to the antioxidant capacity of *A. occidentale* bark extracts.

Table 2. Total phenolic content and total flavonoid content of *A. occidentale*

Sample	TPC (mg GAE/g)	TFC (mg QE/g)
AOBH	6.20	14.67
AOBC	6.73	18.83
AOBM	81.91	56.33

4. CONCLUSION

The study on the phytochemicals and biological activities of *A. occidentale* was successfully carried out. Cold maceration of the stem barks of *A. occidentale* for 72 hours at room temperature using *n*-hexane, chloroform, and methanol followed by the evaporation of each extract using a rotary evaporator had yielded a green solid of *n*-hexane crude extract (0.92 g, 0.10%), brown gummy of chloroform (1.29 g, 0.14%), and methanol (3.30 g, 0.37%) crude extracts. Purification of *n*-hexane crude extract had successfully yielded two compounds, identified as stearic acid **(1)** (0.01 g, 1.09%) and β-sitosterol **(2)** (0.02 g, 2.17%) as white solid. The antioxidant activity of *A. occidentale* was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and vitamin C as the positive control. The crude extract of methanol of *A. occidentale* exhibited strong antioxidant activity in the DPPH assay with an IC_{50} value of 87.67 μg/mL. The total phenolic and flavonoid contents showed significant correlations with the antioxidant activity suggested that phenolic and flavonoid groups contributed greatly to the antioxidant capacity of the extracts. The stem barks of *A. occidentale* can be considered as an effective source of natural antioxidant.

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