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Antioxidant activity of *Phyllanthus watsonii* extract and its major isolated constituents

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ABSTRACT. *Phyllanthus watsonii* is one of Malaysia's traditional medicinal herbs and is endemic to the Endau-Rompin area. Although several studies on the bioactivity and phytochemistry of *P. watsonii* have been reported, information on its antioxidant potential and major chemical constituents remains limited. Therefore, this study aimed to evaluate the *in vitro* antioxidant activity of the crude extract and its major isolated constituents. The antioxidant activity was assessed using the ferric thiocyanate (FTC) method, thiobarbituric acid (TBA) method and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. Isolation and purification of the major constituents were carried out using open column chromatography. The purified constituents were further identified and elucidated using spectroscopic techniques, including nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC-MS) and Fourier-transform infrared spectroscopy (FTIR). The crude extract of *P. watsonii* showed more than 90% inhibition in all three antioxidant assays. Among the isolated constituents, glochidonol (PW2) exhibited more than 90% inhibition in the FTC and TBA assays. Based on these findings, *P. watsonii* possesses strong antioxidant activity, with glochidonol (PW2) identified as one of the major contributors to this activity. These results suggest that *P. watsonii* is a promising source of natural antioxidants and has potential for further development in food and nutraceutical applications.

Key words: Phyllanthus watsonii, isolation, purification, identification, antioxidant activity

1. INTRODUCTION

Phyllanthus comprises over 600 species of shrubs, trees, and annual or biennial herbs (Bajwa et al. 2013). It is well distributed throughout the tropical and subtropical regions of both hemispheres. Most of the plants in this genus are usually in the form of small, erect, annual herb that grows up to 30 to 40 cm in height. It is indigenous to the rainforests of the Amazon and other tropical areas throughout the world, including the Bahamas, southern India, and China. There are 20 species of *Phyllanthus* commonly found throughout Malaysia. These are *Phyllanthus albidiscus, P. amarus, P. chamaepeuce, P. columnaris, P. debilis, P. elegans, P. emblica, P. filicifolius, P. gomphocarpus, P. gracilipes, P. oxyphyllus, P. pachyphyllus, P. pulcher, P. reticulates, P. ridleyanus, P. roseus, P. sikkimensis, P. urinaria, P. virgatus and P. watsonii* (Dasiman, and Bahari, 2021). Traditionally, *Phyllanthus* species are well known used as remedy for liver issues such as jaundice and hepatitis, kidney stones, diabetes, digestive problems including diarrhea and dysentery, fevers, and as diuretics (Husnunnisa et al., 2022; Joseph & Raj, 2011).

Previous study on Phyllanthus genus reported to possess class of secondary metabolite constituents such as alkaloids, terpenoids, and phenolic compounds which include flavonoids, phenolic acids, anthocyanins, coumarins and lignins

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(Husnunnisa et al., 2022; Nawfetrias et al., 2024). Alkaloids of the securine- and norsecurinine-type has been reported to be the characteristic of compounds found in *Phyllanthus* species. For example *P. simplex* have yielded simplexine and phyllanthine (Negi & Fakhir, 1988), while *P. amarus* (Houghton et al., 1996) and *P. niruroides* (Bila et al., 1995) have yielded the isomeric isobubialine and epibubialine, and niruroidine, respectively. Besides alkaloids, various triterpenes have also been reported from several species such as olean-12-en-3*β*,15α-diol, olean-12-en-3*β*,24-diol, olean-12-en-3*β*,15α,24-triol, oleana-11:13(18)-dien-3*β*,24-diol, lup-20(29)-en-3*β*,24-diol, *betulin* and ocotillol-II, from *P. flexuosus* (Tanaka et al., 1988), and 21α-hydroxyfriedelan-3-one and 21α-hydroxyfriedel-4(23)-en-3-one from *P. reticulates* (Hui et al., 1975). Flavonol glycosides such as quercetin-3-*O*-(2-*α*-L-rhamnopyranosyl)-*β*-D-glucuronopyranosyl methyl ester and quercetin-3-*O*-*β*-D-glucuronide methyl ester were also reported from *P. acidus* (Xu et al., 2022). Some common phenolic acids especially gallic acid was also identified from Phyllanthus sp (Sulaiman and Ooi, 2014). Natural pigment type of anthocyanins group known as delphinidin was discovered during study benefit of *P. reticulatus* for oral health and hygiene (Gagare et al., 2023). As for coumarins, scopoletin was reported widely distributed also in *P. reticulatus* (Begum et al., 2006) and *P. acuminatus* (Benavides et al., 2024). Some of Phyllanthus species also rich in lignins or lignans such as phyllanthin, hypophyllanthin, phyltetralin, hypophyllanthin, virgatusin, nirtetralin, lintetralin, niranthin, 5-demethoxyniranthin and urinatetralin (Azman et al., 2019; Wang & Lee, 2005).

The bioactivity of Phyllantus species study was also reported in previous study in order to maximize the potential of its usefulness. Santos et al., (2000) investigated the antinociceptive properties of several species of the genus reported that the alcoholic water extract of *P. amarus, P. orbiculatus, P. fraternus* and *P. stipulatus*, when given intraperitoneally to the animal model, produced significant inhibition of acetic acid-induced abdominal constrictions. Several *Phyllanthus* species have also been reported to exhibit significant antibacterial properties, such as *P. acuminatus* (Goun et al., 2003) and *P. amarus* (Kloucek et al., 2005). Although some study on *P. watsonii* on anti-cancer including cancers of the cervix, liver, lungs, macrophages, uterus, stomach, breast, and colon, and antioxidant *in-vivo* have been reported (Dasiman, & Bahari, 2021; Rao et al., 2016), there is still lack of information on other bioassay study and identification of its constituents. Hence, this report will provide the in vitro antioxidant data together with additional chemical constituents isolated from *P. watsonii*.

2. METHODOLOGY

2.1. General

Melting points were determined on a Leica Galen III apparatus or on a Kofler hot-stage apparatus, and were uncorrected. Optical rotation was measured on JASCO (Tokyo, Japan) Polarimeter P1030. UV spectra were recorded on a Varian UV-Vis CARY 100. UV Spectra Max Plus 38 was used for reading absorbance values in the bioassays. IR spectra (in KBr disc) were recorded on a Perkin Elmer 1650 FT-IR spectrometer. All ^{1}H and ^{13}C NMR as well as the two dimensional experiments (gHSQC and gHMBC) were recorded on a Varian Unity Inova (500 MHz) equipped with pulsed field gradients (PFG), using and Indirect Detection Probe. Deuterated solvents were used and chemical shifts (δ and δ_C) were given in ppm. MS were recorded on a Polaris Q GCMS and LCMS on a Finnigan LCQ Deca.

2.2. Pre-extraction

Phyllanthus watsonii were collected from Ulu Sungai, Endau Rompin National Reserve Park, Johor. Their respective voucher specimens (SK 186/02 and SK 682/ 03) were deposited at the mini herbarium of the Laboratory of Natural Products, Institute Bioscience, UPM. All plant samples were cut into small pieces, air dried and ground into fine powder using a Wiley mill.

2.3. Extraction Process

The *Phyllanthus watsonii* (1.0 kg) was soaked with 100% methanol (MeOH) for 4 days at room temperature after which the extract was decanted. The plant material was replenished with fresh MeOH and the same extraction procedure repeated two more times. The extracts collected from each soaking were pooled and rotary evaporated to dryness, yielding 42.7 g crude extract. The crude extract was resuspended in distilled water and solvent partitioned into hexane and EtOAc yielding 10.0 g and 21.6 g of the respective fractions.

2.4. Determination of Antioxidant Activity

The antioxidant activity was evaluated using three different assays as follows:

2.4.1. Ferric Thiocyanate (FTC) Method

This assay was carried out as described according to the modified method of Kim (2018). A mixture of 4.0 mg of crude extract (final concentration 0.02% w/v) or 2.0 mg of pure compounds dissolved in 4.0 ml of 99.5 % ethanol, 4.1 ml of 2.51% linoleic acid (Sigma, USA) in 99.5% ethanol (Scharlau, Barcelona, Spain), 8.0 ml of 0.02M phosphate buffer (pH 7.0) and 3.9 ml of distilled water contained in screw-cap vial (13 x 75mm) was placed in an oven at 40 ^{O}C in the dark. To measure the extent of antioxidant activity, 0.1 ml of the reaction mixture was transferred to a test tube (13 x 150 mm) and to it, 9.7 ml of 75% (v/v) aqueous ethanol followed by 0.1 ml of 30% aqueous ammonium thiocyanate (Sigma, USA) and 0.1 ml of 0.02 M ferrous chloride (Merck, Germany) in 3.5% hydrochloric acid were added. Three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance was measured at 500nm. The measurement was taken every 24 hours until one day after absorbance (Abs) of the control reached its maximum value.

2.4.2. Thiobarbituric Acid Method (TBA)

The test was conducted according to the method of Kim (2018). The same test samples prepared for the FTC method were used. On the on the final day of the FTC assay, 1.0 ml of 20% aqueous trichloroacetic acid (Wako, Japan) and 2.0 ml 0.67% aqueous thiobarbituric acid (Wako, Japan) solution was added to 2.0 ml of the test sample solution. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was centrifuged at 300 rpm for 20 minutes. Antioxidant activity was recorded based on the absorbance (Abs) of the supernatant at 532 nm.
In both the FTC and TBA methods, the antioxidant activity is described by percent inhibition;

$$\text{Percent inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control})]\ 100$$

2.4.3. Free radical Scavenging Activity (DPPH)

Determination of antioxidant activity was carried-out via radical scavenging activity as describe by Daud et al. (2018). The antioxidant potential of the crude extract of AhJ33 fruit rind extract and the pure isolated compounds was assessed on the basis of their scavenging activity on the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. All test samples were prepared by dissolving 1.0 mg of samples into 1.0 ml 70% aqueous ethanol. A solution of DPPH was prepared by dissolving 5.0 mg DPPH in 2.0 ml of methanol and the solution was kept in the dark at room temperature (25 °C). The test samples were prepared in 96-well microtiter plates. Five µl of methanolic DPPH solution was added to each well. The plate was shaken to ensure thorough mixing before being placed in the dark and wrapped with aluminum foil. After 30 minutes, the optical density of the solution was analyzed using an ELISA reader Spectramax Plus (Molecular Devices) at a wavelength of 517 nm. Percentage inhibition by sample treatment was determined by comparison with methanol treated control group. The concentration of the sample required for 50% inhibitory (IC_{50}) was calculated based on radical scavenging graph at different concentrations. All test analyses were run in triplicates and the reading were averaged. Quercetin (Sigma, USA) was used as standard (positive control). In the DPPH method, the antioxidant activity is described by percent inhibition;

Percent inhibition = [(Abs control–Abs sample)/Abs control)] 100

*$Abs_{control}$ = absorbance of DPPH radical + methanol

*Abs_{sample} = absorbance of DPPH radical + sample extract/standard (positive control)

2.4.4. Chromatographic methods

Open column chromatography is carried out using glass chromatography columns, slurry-packed with normal phase Silica gel 60 (Merck 7734) and size exclusion Sephadex LH-20 eluted with grade gradient elution. The elutes component were monitored using TLC and visualized under UV absorbing and evaluated according to their TLC profiles.

2.5. Isolation and purification of most active antioxidant compound

The hexane fraction contained substantial amounts of green pigments and thus was subjected to gel filtration over Sephadex LH-20 to remove these chlorophyllic constituents. Fractions devoid of the green pigments were recombined and subjected to open column chromatography over silica gel, eluted with varying gradients of hexane : $CHCl_3$ solvent system, starting from a less polar to a more polar gradient. Fractions 3.4 and 5 exhibited the same pattern on TLC profile were combined and yielded 50 mg of white crystalline powder (PW1). The EtOAc fraction was subjected to open column chromatography over silica gel, eluted with varying gradients of EtOAc:MeOH solvent system, starting from a less polar to a more polar gradient. Based on similar TLC profile, fractions 14 to 17 were recombined and rechromatographed over silica gel, eluted with varying gradients of $CHCl_3$:EtOAc solvent system, starting from a less polar to a more polar gradient. Fractions 8 and 9 exhibited the same pattern on TLC profile were combined and yielded 6.0 mg of white crystalline powder (PW2). The structure of the isolated and purified compounds was further elucidated using spectroscopic methods together with its antioxidant activity.

3. RESULTS AND DISCUSSION

3.1 Isolation and purification of major active antioxidant compound

PW1

Compound PW1 was obtained as white crystalline needles with melting point 190-191 ^{O}C. The EI-MS (Figure 1) exhibited a molecular ion peak (M^+) at m/z 412.8 corresponding to the molecular formula $C_{29}H_{48}O$. Other fragments ion at m/z 397.7, 379.7, 189.6 could also be observed which usually exhibited the characteristic of lupane-type triterpenoid constituent (Suttiarporn et al., 2015). The IR spectrum (Figure 2) showed major peaks exhibited values of 3401.8, 1647 and 813 cm^{-1}, and 1717 cm^{-1} which usually represent the presence of a hydroxyl group, double bond group and saturated ring ketone, respectively (Shi et al., 2019).

The ^{1}H NMR spectrum (Figure 3a and b), which exhibited six methyl peaks, one of which appearing as a doublet, in the region δ 0.88 to 1.04, was typical of a pentacyclic triterpene. This was supported by the ^{13}C NMR spectrum (Figure 4), which exhibited a total of 29 carbon resonances, seven of which were ^{1}J correlated to the methyl peaks as seen in the HSQC spectrum (Figure 5a and b).

The ^{13}C NMR spectrum also showed the presence of a carbinol carbon (C-OH) at δ_c 72.9 and two highly deshielded carbons at δ_c 115.7, for an sp^2 methine carbon, and 143.28, for a quaternary sp^2 carbon. The protonated carbons were ^{1}J correlated to the proton signals at δ 3.78 (1H, broad) and 5.17 (1H, doublet J = 6 Hz), respectively. The former was assignable to H-3 of a triterpene carrying a hydroxyl group on C-3 and the later was characteristic to an olefinic proton on C-15 of a 14-en friedoolean-type triterpene as seen also in some previous report (Boonya-Udtayan et al., 2019).

The HMBC spectrum (Figure 6a, b and c), showed some diagnostic correlations in which H-1 correlation with C-14 quaternary carbon. In addition, H-1 also exhibited clear other correlation signal with C-18 (δ_c 46.4), C-22 (δ_c 38.1), C-16 (δ_c 31.1), besides overlapping signal with C-13 (δ_c 39.2) and C-17 (δ_c 34.0) due to small difference in chemical shift range. The correlation between the hydroxyl group carbon with nearest proton could also be observed. Based on all these data, the structure for PW1 was proposed to be that of 26-nor-D: A-friedoolean-14-en-3β-ol which had been isolated from the same species before by Matsunaga et al., (1993). Comparison of the NMR chemical shift data on Table 1 obtained from PW1 with the reported values confirmed the assignment.

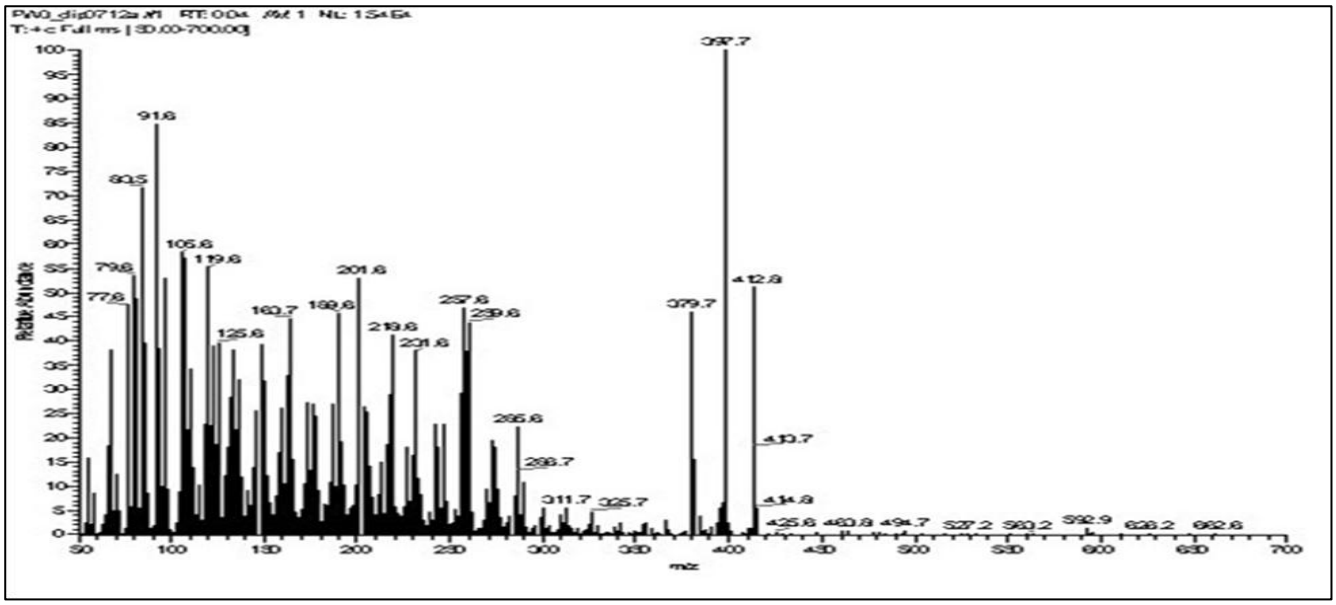


Figure 1. EI-MS spectra of PW1

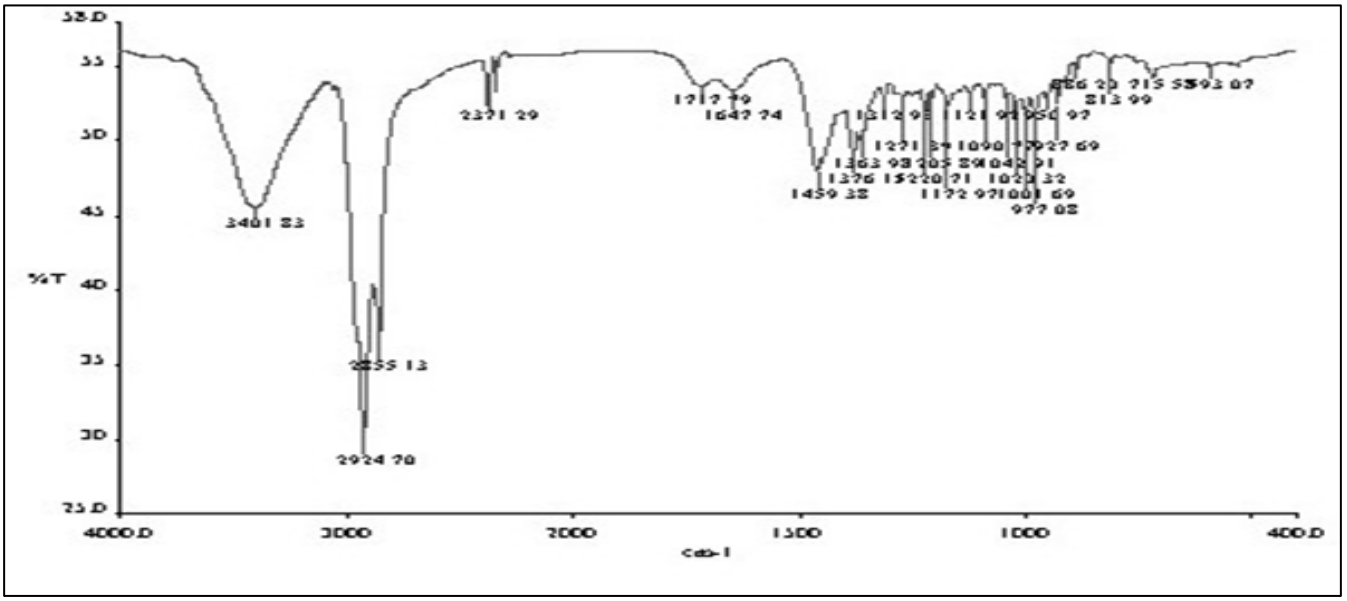


Figure 2. IR spectrum of compound PW1

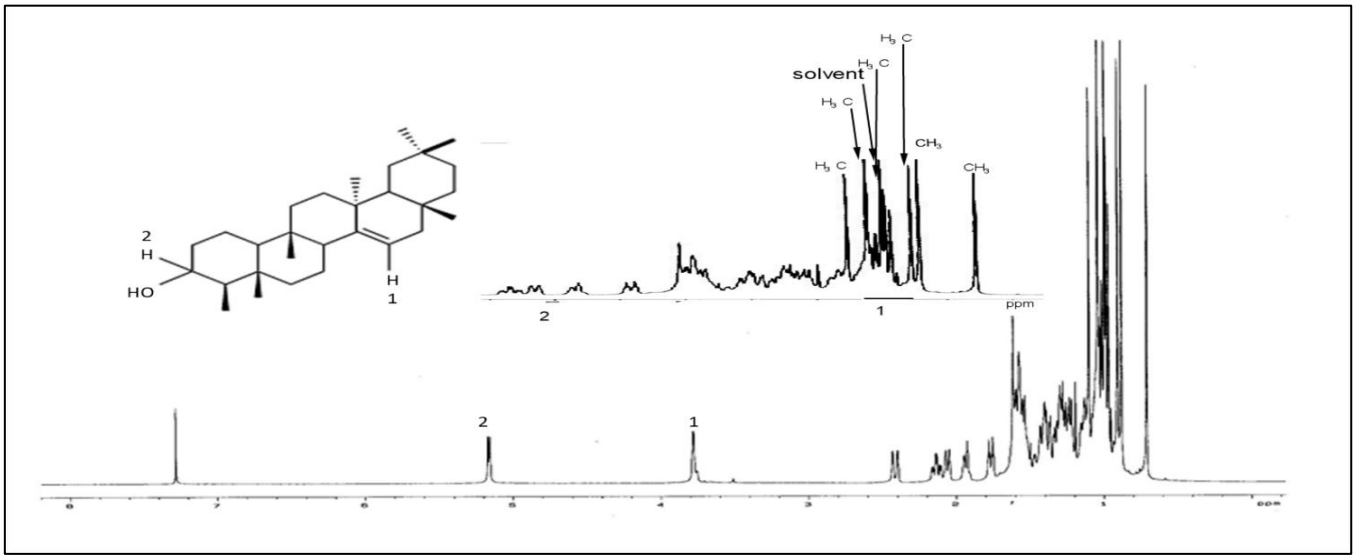


Figure 3a. ^{1}H NMR spectra of compound PW1 in $CDCl_3$

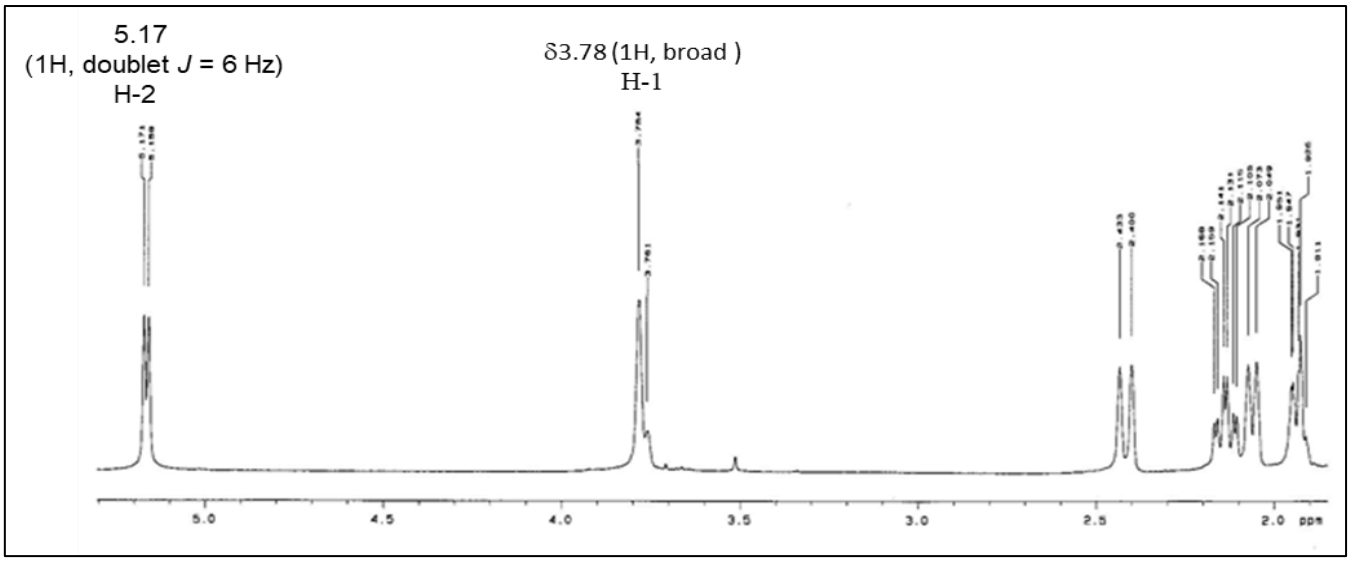


Figure 3b. ^{1}H NMR spectra of compound PW1 in $CDCl_3$

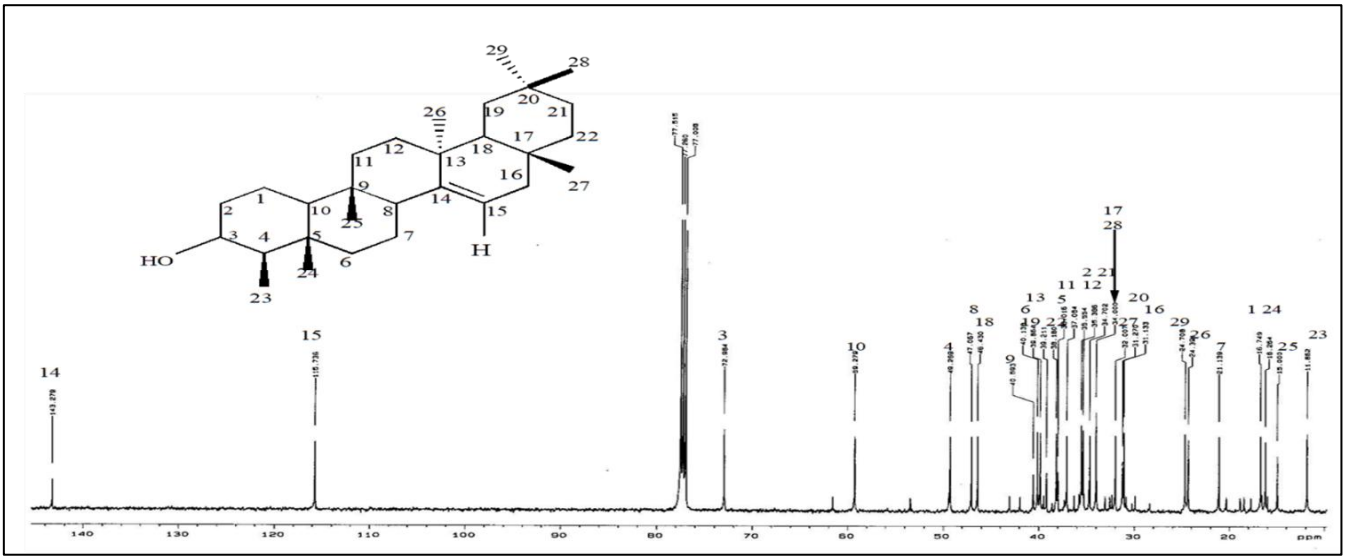


Figure 4. ^{13}CNMR spectrum of compound PW1 in $CDCl_3$

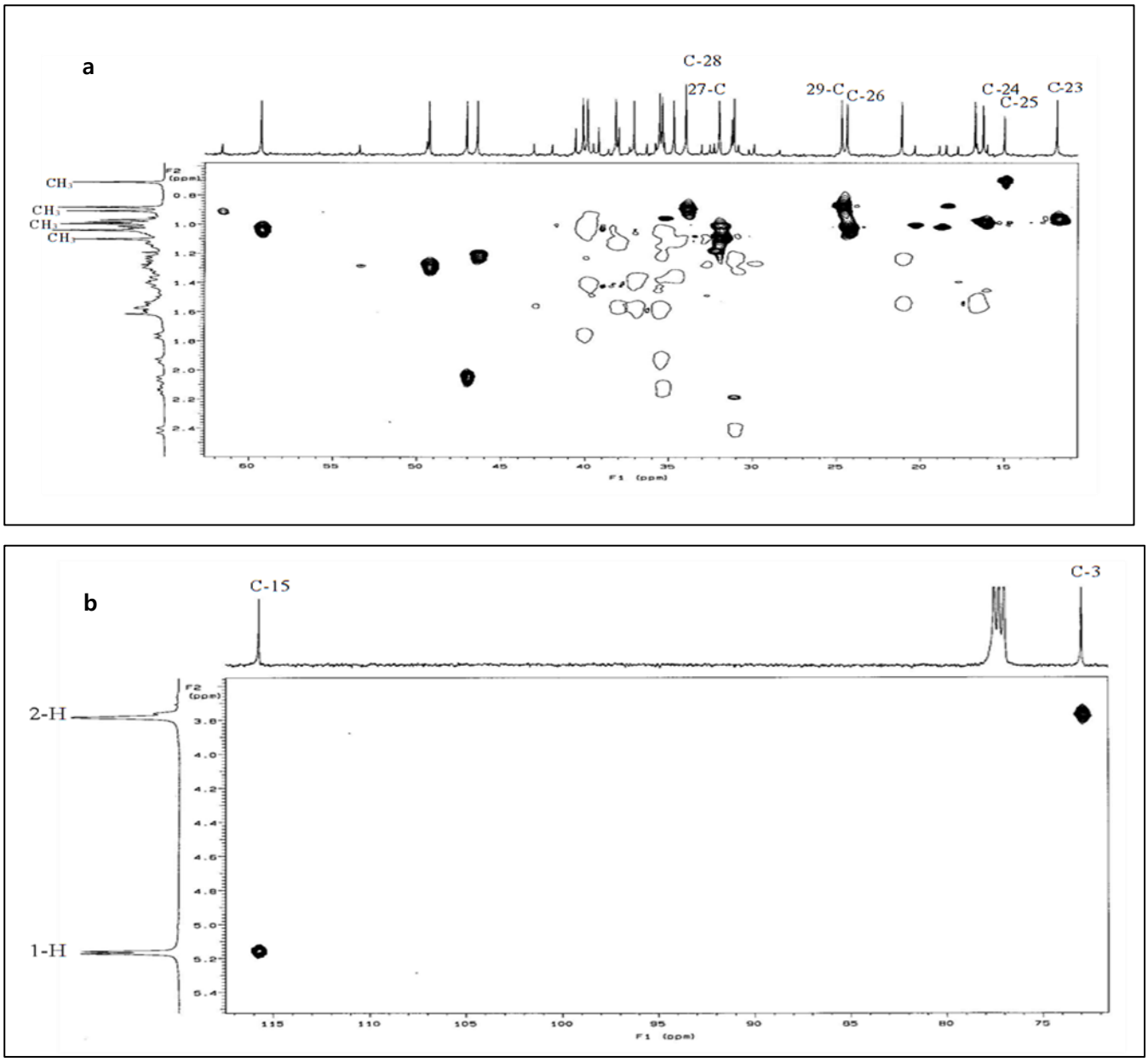


Figure 5. HSQC spectrum of compound PW1 (a) ^{1}J correlation carbon to proton (b) correlation of hydroxyl carbon to proton (C-3 with H-2) and double bond carbon to proton (C-15 with H-1)

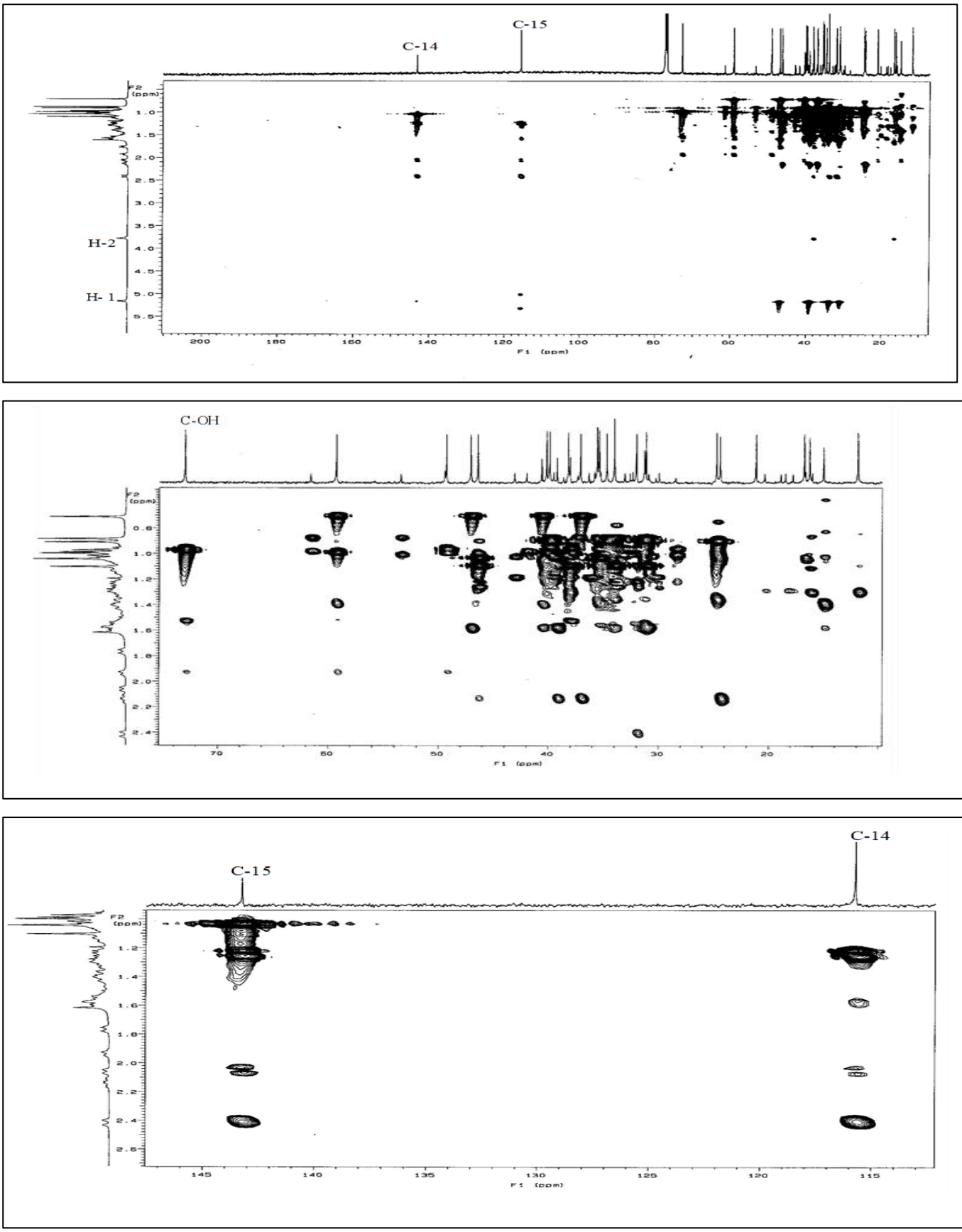


Figure 6. HMBC spectrum of compound PW1 (a) H-1 correlation signal with carbon 18, 22, 16, 13 and 17, (b) correlation between the hydroxyl group (OH) carbon with proton, (c) correlations H-1 correlation with C-14 quaternary carbon.

Table 1. Comparison of ^{13}C values for PW1 with 26-nor-D: A-friedoolean-14-en-3*β*-ol **(**Matsunaga et al., 1993)

Carbon No.	^{13}C PW1	^{13}C 26-nor-D: A-friedoolean-14-en-3*β*-ol
1	16.7	16.5
2	35.3	35.3
3	72.9	72.7
4	49.2	49.0
5	38.0	37.8
6	40.1	39.9
7	21.1	20.9
8	47.0	46.8
9	40.5	40.4
10	59.2	59.0
11	37.0	36.8
12	35.6	35.1
13	39.2	39.0
14	143.3	143.0
15	115.7	115.5
16	31.1	30.9
17	34.0	33.8
18	46.4	46.2
19	39.8	39.6
20	31.2	31.0
21	34.7	34.4
22	38.1	37.9
23	11.9	11.6
24	16.2	16.0
25	15.0	14.8
26	24.3	24.1
27	32.0	31.8
28	34.0	33.8
29	24.7	24.5

PW2

Compound PW2 was obtained as white crystalline needles with melting point 228-230^0. The IR spectrum (Figure 7) showed the presence of a hydroxyl group at 3423.1 cm^{-1} and 1638.1 cm^{-1} (carbonyl, C=O). The ^1H NMR spectrum (Figure 8) was characteristic of a triterpene, exhibiting signals for seven methyl groups. All the methyl singlets were observable in the region of δ 0.82 to 1.23, except for a deshielded methyl singlet which appeared at δ 1.70. A pair of singlet at δ 4.70 (singlet) and 4.59 (singlet) was typical of a pair exomethylene protons H-29a and H-29b of a lup-20(29)ene-type triterpene, as seen in lupeol and related lupanes. The ^1H NMR spectrum also showed a broad 1H singlet at δ 3.92 indicating the presence of an oxygenated or hydroxylated CH also known as oxymethine in the molecule.

The ^{13}C NMR spectrum (Figure 9a and b) exhibited signals for thirty carbons, consistent with the molecular formula indicated from the mass spectrum. Altogether, it exhibited signals for the seven methyls, ten methylenes (including the exomethylene), six methines (including the oxymethine), and seven quaternary carbons. A carbonyl signal was observed at δ_c 216.1 for a ketone and two highly deshielded carbons at δ_c151.0 (quaternary sp^2 C) and 109.7 (sp^2 CH_2) could be assigned to the exomethylene group of the lup-20(29)ene skeleton, C-20 and C-29 respectively. The other

deshielded carbon at δ_c 79.9 belonged to the oxymethine carbon. The direct H-C correlation (^1J) for both the exomethylene and oxymethine groups were supported by an HSQC experiment (Figure10b).

Analysis of the HMBC spectra (Figure 11a, b and c) allowed the assignment of the chemical shifts of the compound PW2 and their assignment are tabulated in Table 2. Based on the above data, PW2 is assigned as glochidonol**.** Confirmation of the compound as glochidonol was established by comparison with literature data (Puapairoj et al., 2005) as shown in Table 3. The EI-MS (Figure 12) did not exhibit a molecular ion peak (M^+) at m/z 440 corresponding to the molecular formula $C_{30}H_{48}O_2$. The presence of oxymethine could strongly influences the fragmentation patterns in mass analysis as reported in many studies (Cheng et al., 2021; El-Bondkly et al 2008) which has affected the accuracy for the mass data of PW2. A fragment ion at m/z 205 which usually represent the presence of triterpenoid class of compound could be observed as reported in many studies (Cordova-Gonzalez et al., 2021; Vilegas et al., 1997).

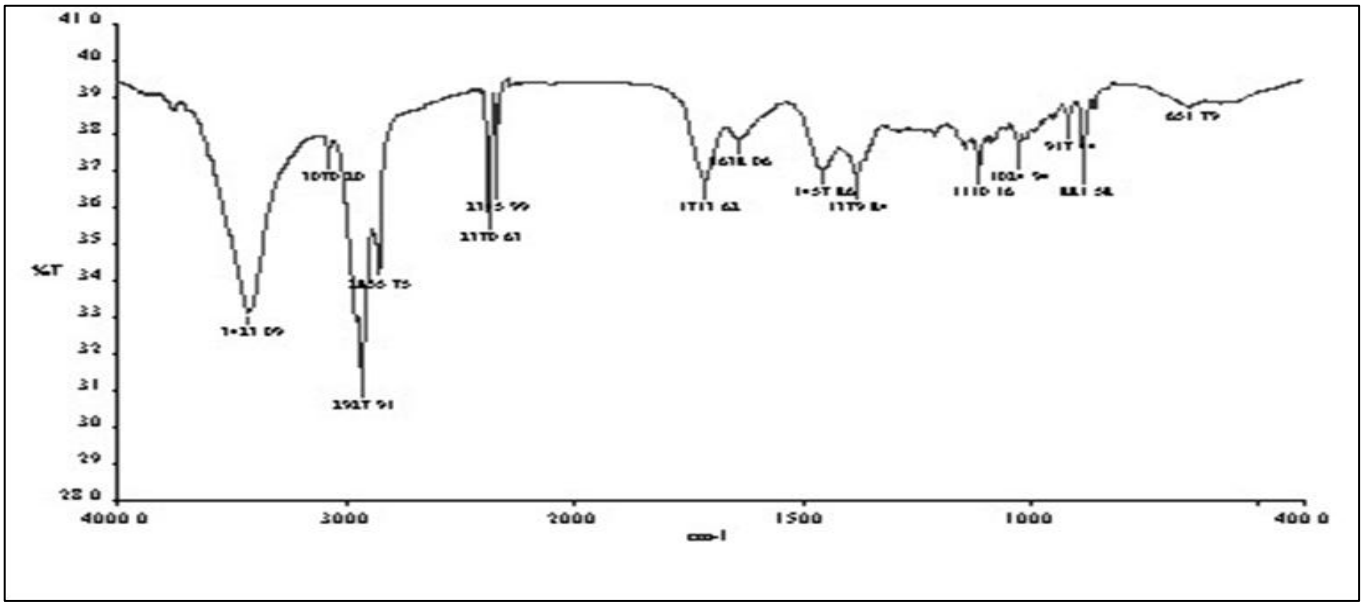


Figure 7. IR spectrum of compound PW2

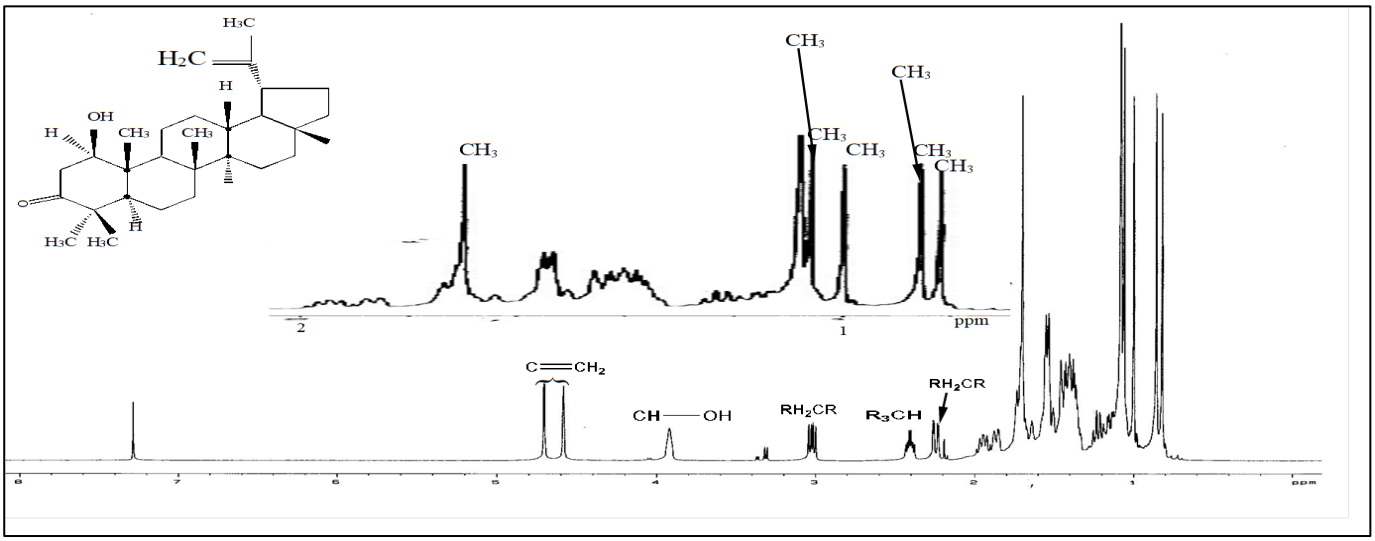


Figure 8. ^{1}H NMR spectra of compound PW2 in $CDCl_3$

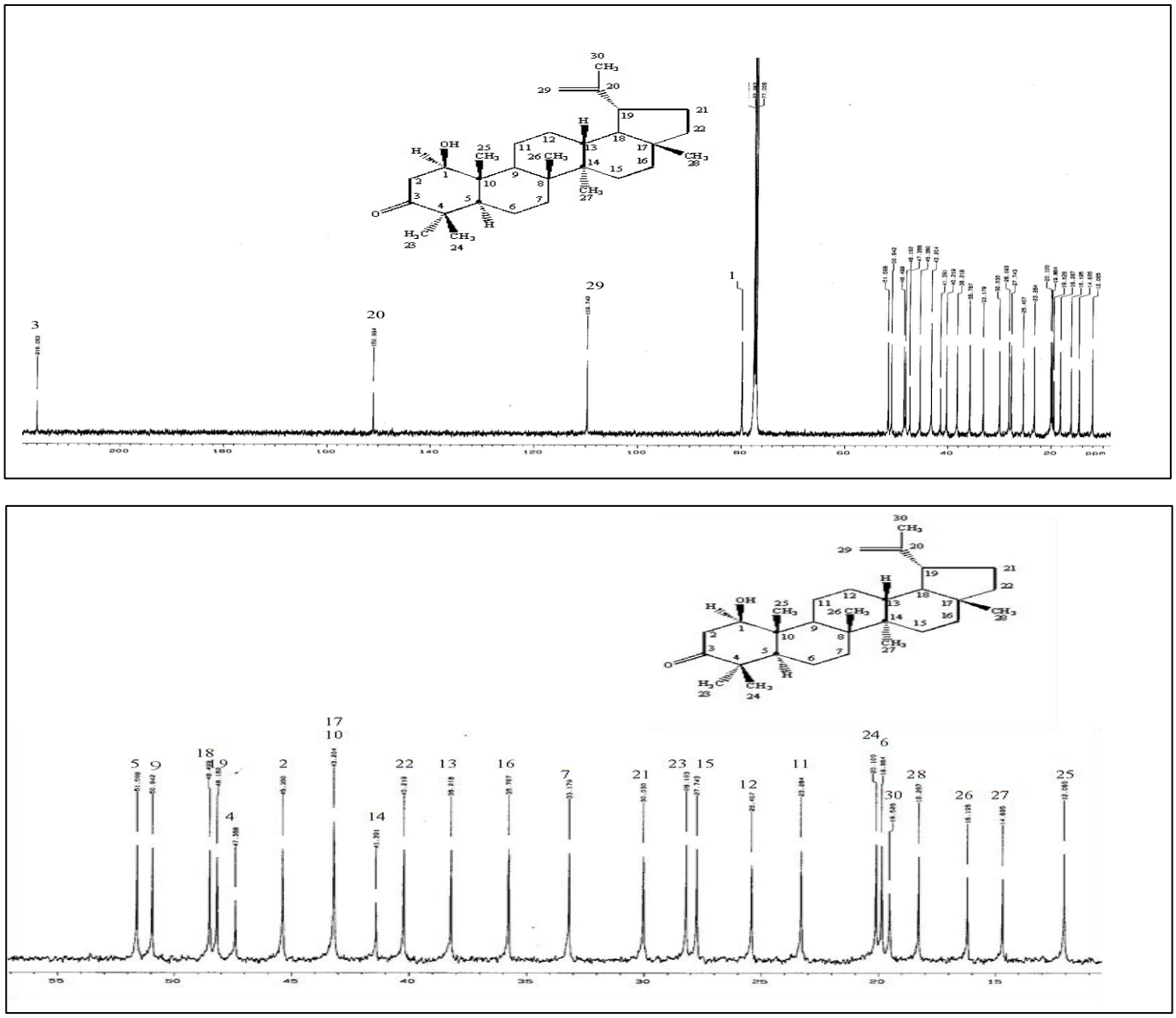
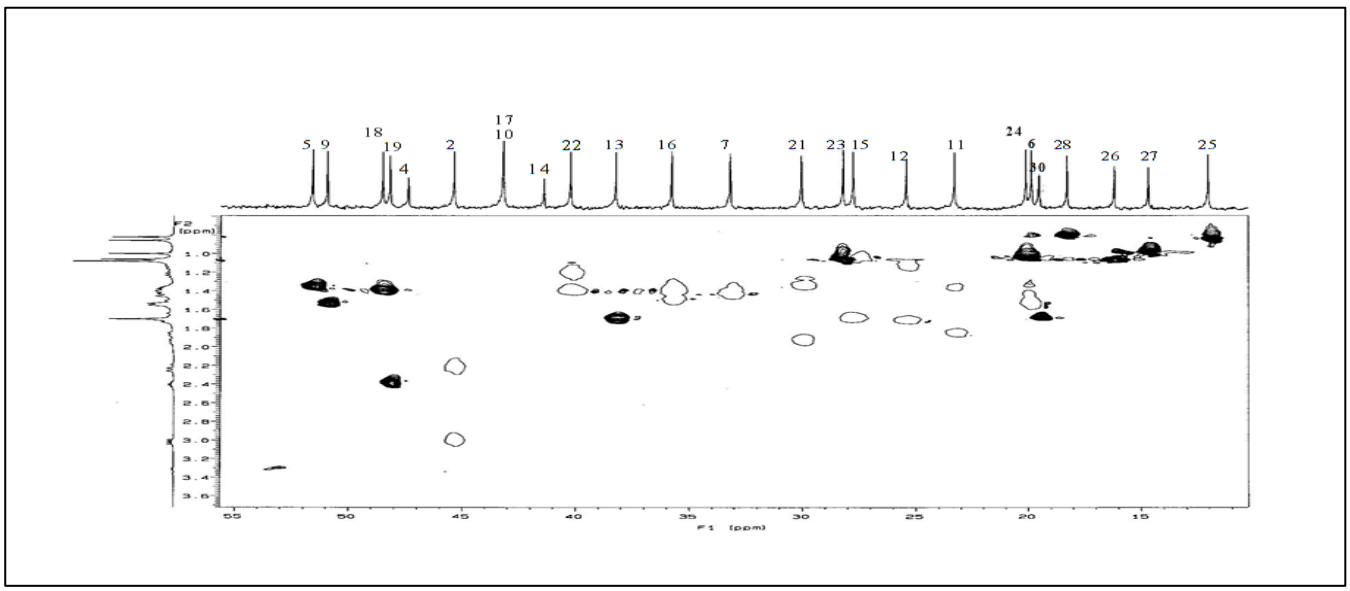


Figure 9. ^{13}C NMR spectra of compound PW2 in $CDCl_3$ (a) carbon 1, 29, 20 and 5, (b) other carbons



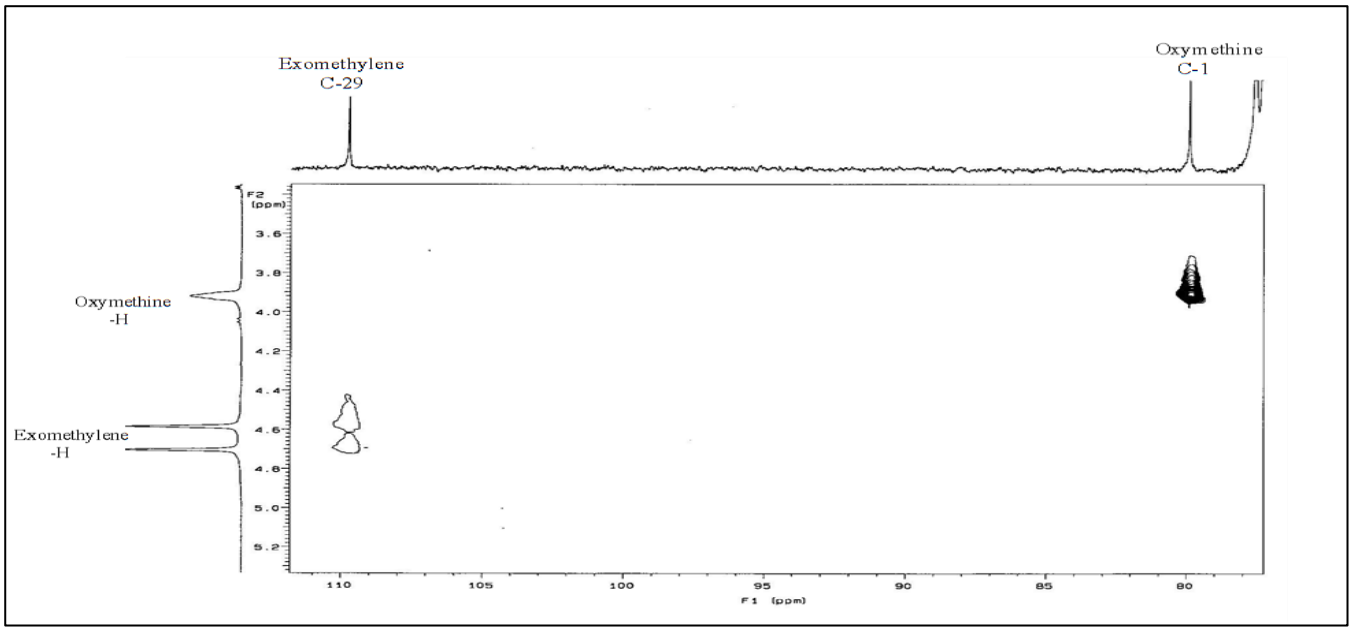
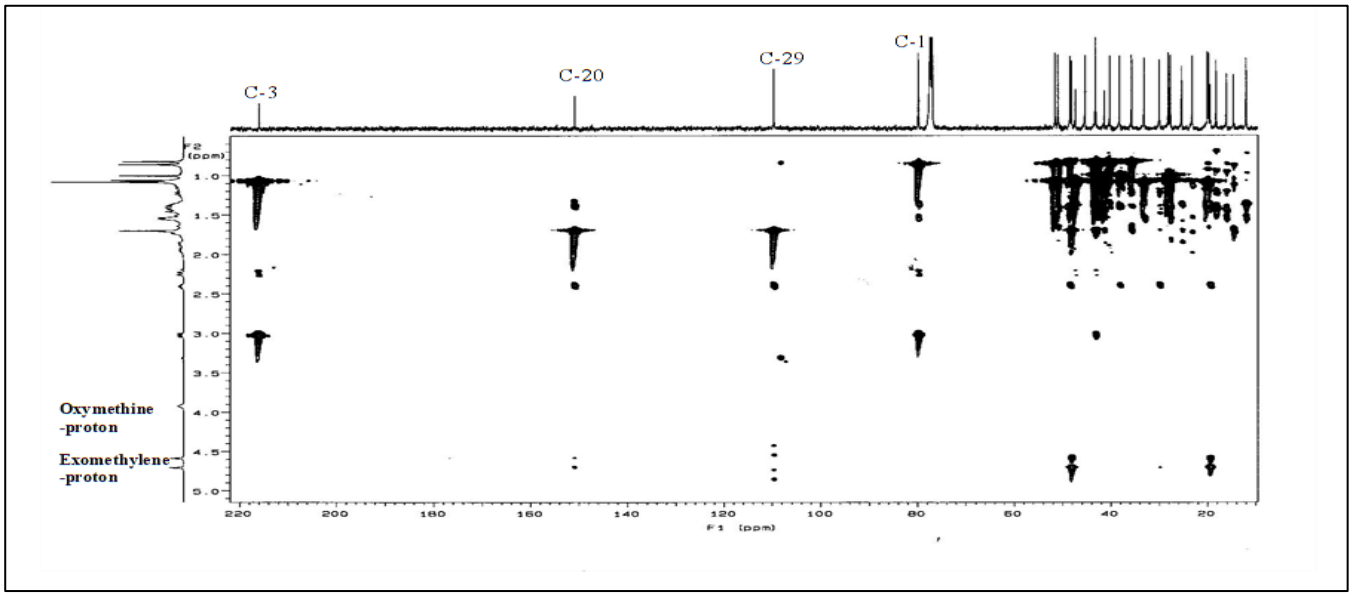


Figure 10. HSQC spectrum of compound PW2 (a) H-C correlation (^1J) carbon to proton (b) H-C correlation (^1J) of exomethylene and oxymethine groups



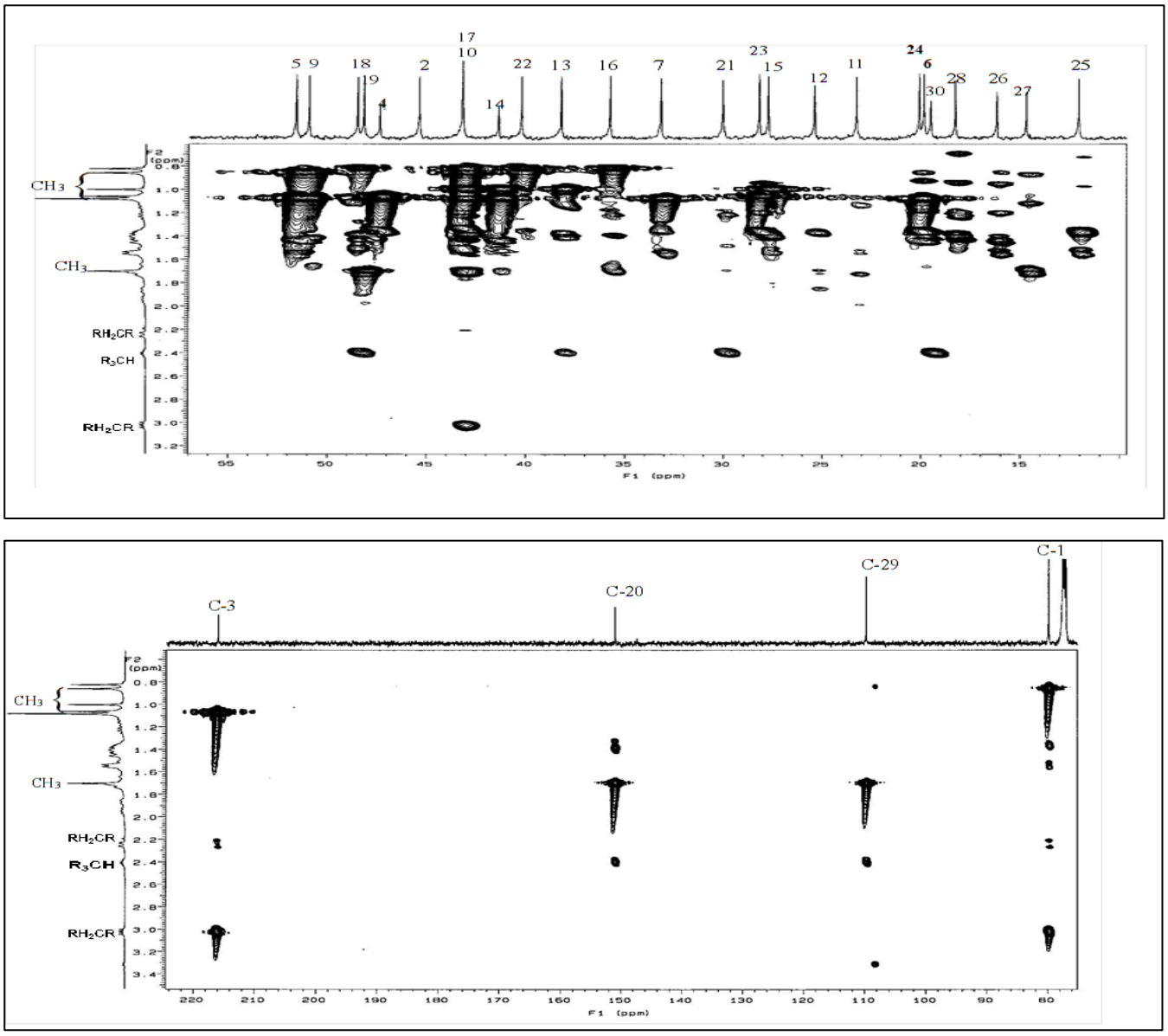


Figure 11. HMBC spectrum of compound PW2 (a) exomethylene correlation, (b) other carbon with proton correlation, (c) carbonyl (C=O) correlation

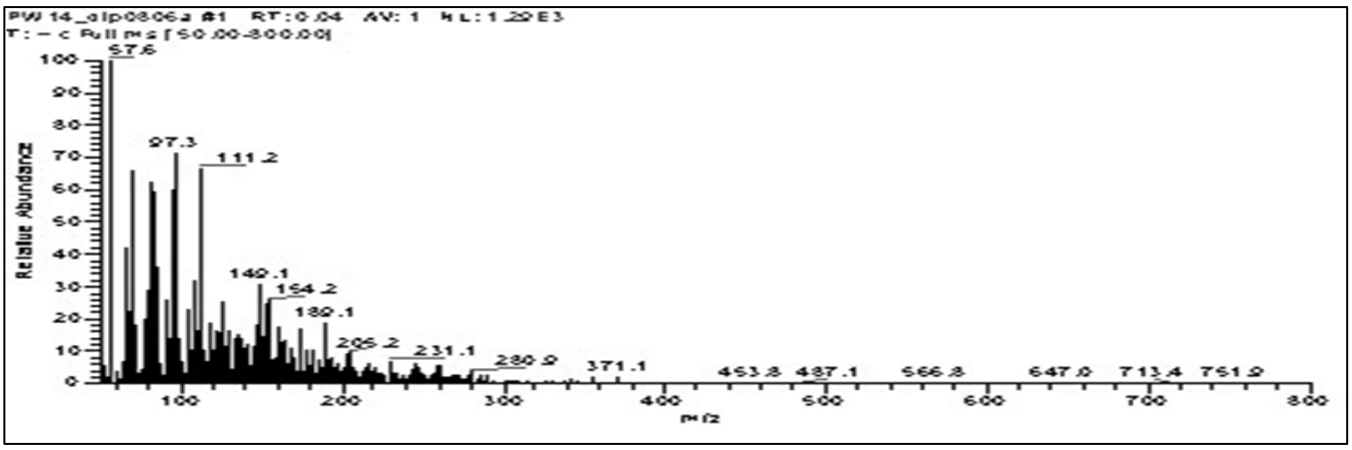


Figure 12. EI-MS spectra of PW2

Table 2. ^{1}H-^{13}C correlations based on HMBC experiment on PW2 and C, H assignments for PW2

Carbon Assignment	δ_C	Attached proton	^{2}J	^{3}J
1	79.87 (OCH)	3.93	-	-
2	45.38(RCH_2R)	2.25,3.02	216.06,79.87	43.20
3	216.06(C=O)	-	-	-
4	47.39(R_4C)	-	-	-
5	51.59(R_3CH)	1.35	47.39,43.20,19.53	33.18,216.06,12.07,79.87
6	19.86(RCH_2R)	1.35,1.43	33.18,51.59	43.20,47.39
7	33.18(RCH_2R)	1.35,1.43	43.20,19.86	41.39,51.59, 16.20
8	43.20(R_4C)	-	-	-
9	50.94(R_3CH)	1.54	43.20,23.28	12.7,16.20
10	43.20(R_4C)	-	-	-
11	23.28(RCH_2R)	1.35, 1.85	50.54,25.41	38.21,43.20
12	25.41(RCH_2R)	1.06,1.70	38.21,23.28	41.39,50.94,4850
13	38.21(R_3CH)	1.70	25.41,48.50,41.39	43.20,48.18,23.28,14.70,43.20
14	41.39(R_4C)	-	-	-
15	27.74(RCH_2R)	1.06,1.70	41.39,35.77	14.70,43.20,38.21
16	35.77(RCH_2R)	1.35,1.55	43.20,27.74	18.29,40.22,48.50,41.39
17	43.20(R_4C)	-	-	-
18	48.50(R_3CH)	1.35	38.21,48.18,43.20	150.99,16.20,25.41,41.39
19	48.18(R_3CH)	2.41	150.99,30.03,48.50	19.53,109.74,38.21
20	150.99(R_2C=R)	-	-	-
21	30.03(RCH_2R)	1.33,1.94	40.22, 48.18	150.99,43.20
22	40.22(RCH_2R)	1.20,1.40	30.03,43.20	18.29,48.18,35.77,48.50
23	28.19(RCH_3)	1.00	47.39	51.59
24	20.10(RCH_3)	1.06	47.39	216.99,51.59,28.19
25	12.07(RCH_3)	0.86	43.20	50.94,79.87,51.59
26	16.20(RCH_3)	1.06	43.20	41.39,50.94,33.18
27	14.70(RCH_3)	1.00	41.39	43.20,27.74,38.21
28	18.29(RCH_3)	0.82	43.20	40.22,35.77,48.50
29	109.74(H_2C=)	4.56,4.70	150.99	19.53,48.18
30	19.53(RCH_3)	1.70	150.99	109.74,48.18

Table 3. Comparison of ^{13}C chemical shifts for PW2 with glochidonol (Puapairoj et al., 2005)

Carbon No.	**^{13}C Pw2**	**^{13}C Glochidonol**
1	79.87	79.58
2	45.38	45.07
3	216.06	215.99
4	47.39	47.11
5	51.59	51.26
6	19.86	19.58
7	33.18	32.85
8	43.20	42.86
9	50.94	50.63
10	43.20	42.90
11	23.28	22.96
12	25.41	25.08
13	38.21	37.90
14	41.39	41.08
15	27.74	27.44
16	35.77	35.46
17	43.20	42.86
18	48.50	48.18
19	48.18	47.89
20	150.99	150.73
21	30.03	29.72
22	40.22	39.93

23	28.19	27.94
24	20.10	19.58
25	12.07	11.81
26	16.20	15.90
27	14.70	14.00
28	18.29	18.00
29	109.74	109.47
30	19.53	19.24

3.2. Evaluation of antioxidant activity

The methanolic crude extract and purified compounds of *P. watsonii* were tested for their antioxidant activity via three antioxidant assays which include Ferric thiocyanate assay (FTC), Thiobarbituric Acid Method (TBA) and Free radical Scavenging Activity (DPPH).

3.2.1. Ferric thiocyanate assay (FTC) Method

The FTC method measures the amount of peroxide produced during the initial stages of oxidation. Higher level of activity is denoted by lower absorbance values. As shown in Figure 13, the crude extract of *P. watsonii* showed higher antioxidant activity compared to the control and vitamin E. The percent inhibition value on the last day ranged from 13.3-95.5 of peroxide production. The extract may thus contain active constituents capable of scavenging the hydroxyl radical or transition metal ion such as Fe^{2+} and Cu^{2+} (Noguchi and Niki, 1998) during lipid peroxidation. Glochidonol, one of the purified compounds from the extract is probably contributing to the observed bioactivity of *P. watsonii* extract since it gave very strong antioxidant activity, almost comparable to the standard drug butylated hydroxy toluene (BHT) and quercetin. The inhibition value for glochidonol on the last day was 95.5% of peroxide production (Table 4). As reported from previous antioxidant study on *Glochidion* species, glochidonol has demonstrated as one of the antioxidant constituents (Linh et al., 2024).

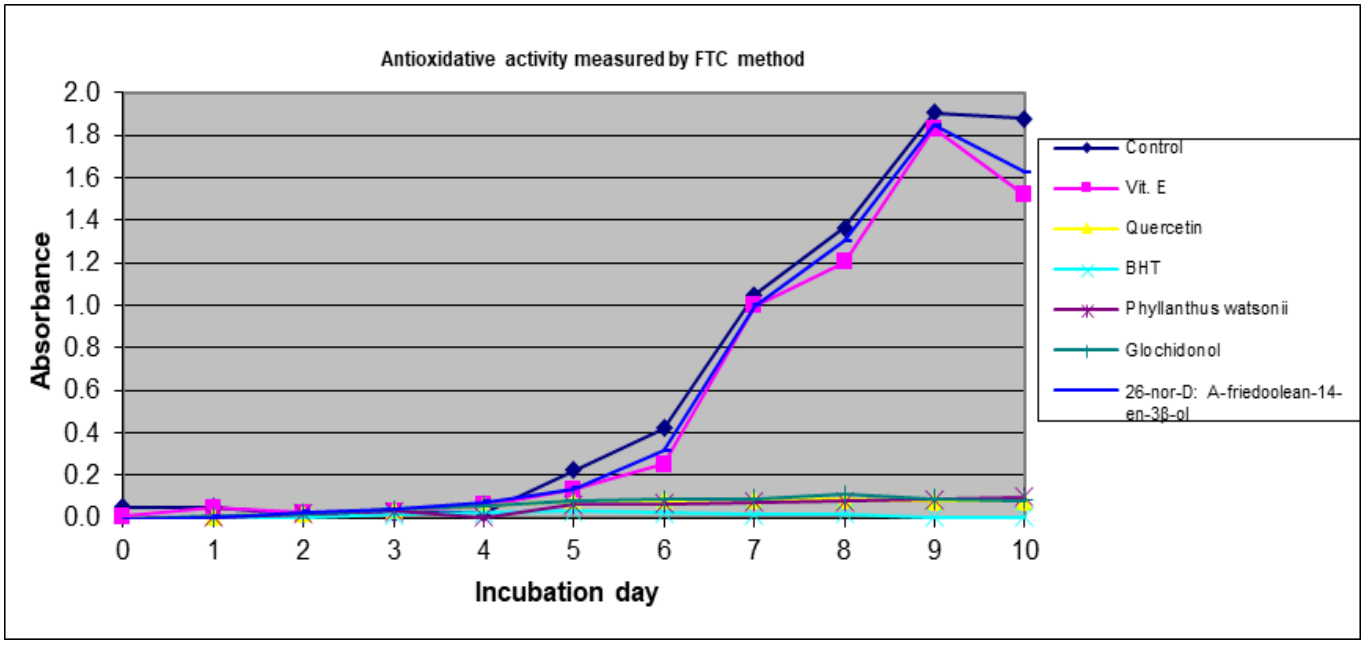


Figure 13. Antioxidative activity of the samples measured by FTC method

3.2.2. Thiobarbituric Acid Method (TBA)

The TBA assay involved a measurement of total peroxide content at a later stage of lipid oxidation, involving the quantitation of the secondary products formed form oxidation. Higher level of activity is denoted by lower absorbance values. The results from TBA assay on Figure 14, the crude extract of *P. watsonii* and glochidonol consistently showed high level of antioxidant activity similar to the FTC result. The comparison of TBA assay with FTC assay percent inhibition of linoleic acid peroxidation is show in Table 4. From the results, glochidonol and crude extract of *P. watsonii* show more than 90% level of antioxidant compared to control.

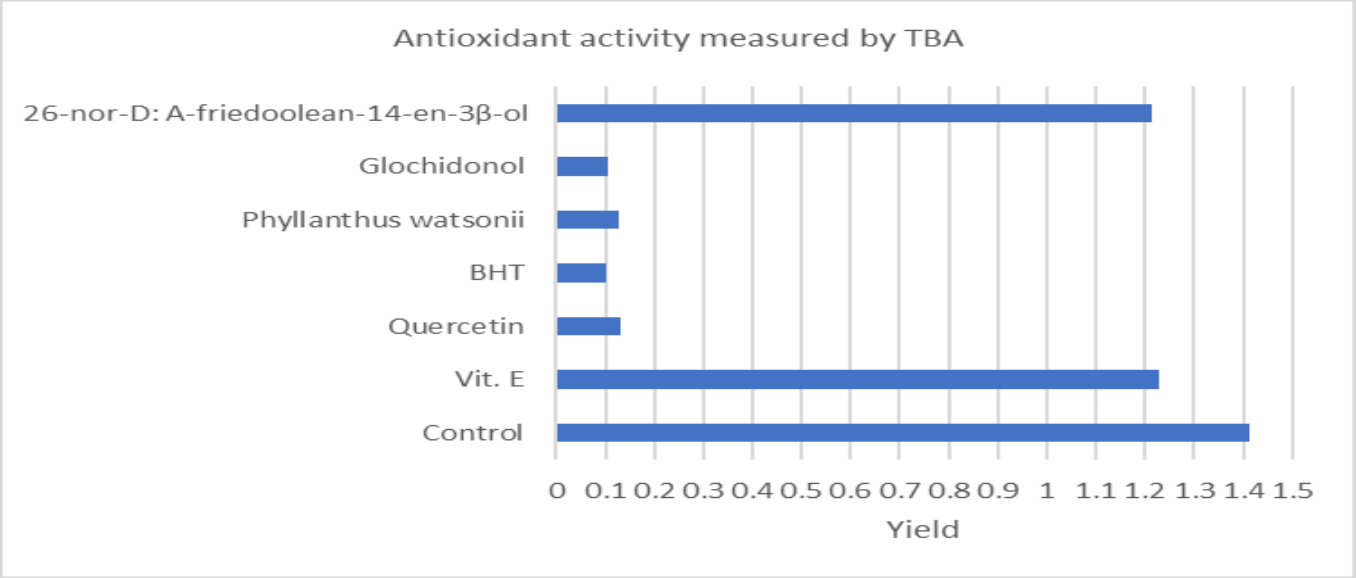


Figure 14. Antioxidative activity of the samples measured by TBA method

Table 4. Comparison of absorbance values and percent inhibition of linoleic acid peroxidation as measured by the FTC and TBA antioxidant assays

Sample	Absorbance* (FTC)	Percent Inhibition (%) (FTC)	Absorbance* (TBA)	Percent Inhibition (%) (TBA)
Control	1.8770	0	1.4144	0
Vit. E	1.5204	11.9	1.2291	13.1
Quercetin	0.0788	95.8	0.1315	90.7
BHT	0.0027	99.9	0.1001	92.9
Phyllanthus watsonii (crude)	0.0965	94.9	0.1259	91.1
Glochidonol	0.0841	95.5	0.1066	92.5
26-nor-D:A-friedoolean-14-en-3*β*-ol	1.6271	13.3	1.2150	14.1

* Reading on day 10 (one day after control reached maximum)

3.2.3. Free radical Scavenging Activity (DPPH)

The antioxidant property of an extract or a compound may also be measured by their ability to inhibit or scavenge stable free radicals such the stable 1,1-dipheyl-2-picrylhydrazyl (DPPH) free radicals. 1,1-dipheyl-2-picrylhydrazyl (DPPH) radicals give a deep violet colour and absorbs strongly at 517 nm. As its odd electron becomes paired off in

the presence of a free radical scavenger, the absorption decreases (colour fades and disappears). The decrease in absorbance is proportional to the number of DPPH molecules being scavenged. The change in absorbance is measured to evaluate the antioxidant potential of the test samples. As shown in table 5, *P. watsonii* gave the highest percent inhibition of 97.05 % inhibition with IC_{50} value of 15.6 µg/ml. The triterpene isolated from *P. watsonii*, 26-nor-D: A-friedoolean-14-en-3*β*-ol showed moderate inhibition of 50.9% at a concentration of 100 µM. Although glochidonol showed positive results on FTC and TBA method but it could not be analysed further using DPPH method due to insufficient amount.

Table 5. Percent inhibition of the test samples measured by DPPH method

Sample	Concentration (µg/ml)	% Inhibition
Phyllanthus watsonii	1000.0	97.05
(crude extract)	500.0	94.16
	250.0	91.00
	125.0	90.83
	62.5	86.92
	31.3	62.77
	15.6	50.00
Samples	**Concentration (µM)**	**% Inhibition**
26-nor-D: A-friedoolean-14-en-3*β*-ol	100.00	50.90
	50.00	20.46
	25.00	22.86
	12.50	38.33
	6.25	22.20
	3.13	23.22
Glochidonol	-	-

CONCLUSION

The methanolic crude extract of *P. watsonii* exhibited the highest antioxidant activity via DPPH assay with value of 97.1% followed by FTC and TBA with value of 94.9 and 91.1% respectively. The methanolic crude extract was further undergone isolation and purification of its major chemical constituents which obtained two major compounds. Both compound were identified as 26-nor-D:A-friedoolean-14-en-3*β*-ol (PW1) and glochidonol (PW2) using spectroscopic method. Based on three assays data for 26-nor-D:A-friedoolean-14-en-3*β*-ol (PW1), the highest value of antioxidant activity of it was 50.9%. The glochidonol antioxidant activity could not be conducted via DPPH assay due to insufficient amount. As for FTC and TBA assays, glochidonol possess the highest antioxidant activity with value of 95.5% on FTC assay followed by TBA assay with value of 92.5%. The data obtained revealed the antioxidant potential of *P. watsonii* with glochidonol could be the major contributor to its antioxidant activity.

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AUTHOR CONTRIBUTIONS

Mohd Nazrul Hisham Bin Daud responsible for data collection, NMR, LCMS, FTIR and antioxidant analysis, and preparation of the original draft.

Agustono Wibowo is responsible for the NMR and FTIR data analysis and preparation of the original draft.

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DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

The author(s) declared that no AI tool was used during the writing process

DATA AVAILABILITY

Not applicable.

COMPETING INTEREST

The authors declare that there are no competing interests.

COMPLIANCE OF ETHICAL STANDARDS

The authors declare that this research did not involve human or animal subjects and this research does not include any ethical issue.

SUPPLEMENTARY MATERIAL

No supplementary material is associated with this article.

REFERENCES

Azman, N.A.Z., Raman, I.A., Jantan, I., & Derawi, D. (2019). Formulation screening of palm-based nanoemulsion for an oral drug vehicle of phyllanthin. *Malaysian Journal of Chemistry, 21*(2), 43-49.

Bajwa, R.K., Kulkarni, S.S., Tekale, P.P., Shinde, N.M., & Bagwe, K.A. (2013). Proximate Analysis of *Phyllanthus Amarus*. *International Journal of Research in Pharmacy and Chemistry, 3(2)*, 221-227.

Begum, T., Rahman, M. S., & Rashid, M. A. (2006). Phytochemical and biological investigations of *Phyllanthus reticulatus*. *Dhaka University Journal of Pharmaceutical Sciences*, *5*(1), 21-23. https://doi.org/10.3329/dujps.v5i1.223

Benavides, K., Sánchez-Kopper, A., Jiménez-Quesada, K., Perez, R., & Garro-Monge, G. (2023). Evaluation of salicylic acid and methyl jasmonate as elicitors in *Phyllanthus acuminatus* hairy roots by non-targeted analysis using high-resolution mass spectrometry. *Molecules*, *29*(1), 80. https://doi.org/10.3390/molecules29010080

Bila, B., Gedris, T.E., & Herz, W. (1995). Niruroidine, a Norsecurinine-Type alkaloid from *Phyllanthus niruroides*. *Phytochemistry 41*(5), 1441-1443. https://doi.org/10.1016/0031-9422(95)00738-5

Boonya-Udtayan, S., Thasana, N., Jarussophon, N, & Ruchirawat S. (2019). *Serratene triterpenoids* and their biological activities from Lycopodiaceae plants. *Fitoterapia, 136*, 104181. https://doi.org/10.1016/j.fitote.2019.104181

Cheng, C. R., Yang, M., Wu, Z. Y., Wang, Y., Zeng, F., Wu, W. Y., Guan, S. H., & Guo, D. A. (2021). Fragmentation pathways of oxygenated tetracyclic triterpenoids and their application in the qualitative analysis of *Ganoderma lucidum* by multistage tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 25*(9), 1323-1335. https://doi.org/10.1002/rcm.4989

Cordova-Gonzalez, A., Birgel, D., Kappler, A., & Peckmann, J. (2021). Variation of salinity and nitrogen concentration affects the pentacyclic triterpenoid inventory of the haloalkaliphilic aerobic methanotrophic bacterium *Methylotuvimicrobium alcaliphilum*. *Extremophiles*, *25*(3), 285-299. https://doi.org/10.1007/s00792-021-01228-x

Dasiman, R., & Bahari, E. A. (2021). Malaysian Phyllanthus sp: An updated review of their impacts on the reproductive functions and fertility. *Malaysian Journal of Medicine & Health Sciences*, *17*(SUPP3), 343-350.

Daud, M. N. H., Wibowo, A., Abdullah, N., & Ahmad, R. (2018). Bioassay-guided fractionation of Artocarpus heterophyllus L. J33 variety fruit waste extract and identification of its antioxidant constituents by TOF-LCMS. *Food Chemistry*, *266*, 200-214. https://doi.org/10.1016/j.foodchem.2018.05.120

El-Bondkly, A. M., Abd-Alla, H. I., Shaaban, M., & Shaaban, K. A. (2008). The electrospray ionization-mass spectra of erythromycin a obtained from a marine Streptomyces sp. mutant. *Indian Journal of Pharmaceutical Sciences*, *70*(3), 310. https://doi.org/10.4103/0250-474X.42979

Gagare, S.S., Jain, A.S., Sankhe, S., Salunkhe, A., Sawant, A., Sagvekar, V., & Sawant, G. (2023). Modification And Evaluation of Modified Formulations of Oral Health and Hygiene by Utilization of The Parts of *Phyllanthus reticulates*. *International Journal of Pharmaceutical Sciences and Research, 14*(10), 4878-4886. https://doi.org/10.13040/IJPSR.0975-8232.14(10).4878-86

Goun, E., Cunningham, G., Chu, D., Nguyen, C., & Miles, D. (2003). Antibacterial and antifungal activity of Indonesian ethnomedical plants. *Fitoterapia*, *74*(6), 592-596. https://doi.org/10.1016/S0367-326X(03)00117-5

Houghton, P. J., Woldemariam, T. Z., O'Shea, S., & Thyagarajan, S. P. (1996). Two Securinega-Type Alkaloids from *Phyllanthus amarus*. *Phytochemistry 43*(3), 715-717.

Hui, W. H., Li, M. M., & Wong, K. M. (1976). A new compound, 21α-hydroxyfriedel-4 (23)-en-3-one and other triterpenoids from *Phyllanthus reticulatus*. *Phytochemistry*, *15*(5), 797-798. https://doi.org/10.1016/S0031-9422(00)94448-0

Husnunnisa, H., Hartati, R., Mauludin, R., & Insanu, M. (2022) A review of the Phyllanthus genus plants: Their phytochemistry, traditional uses, and potential inhibition of xanthine oxidase. *Pharmacia 69*(3), 681–687. https://doi.org/10.3897/pharmacia.69.e87013

Joseph, B., & Raj, S. J. (2011). An overview: Pharmacognostic properties of *Phyllanthus amarus* Linn. *International Journal of Pharmacology 7*(1), 40-45.

Kim, J. S. (2018). Evaluation of *in vitro* antioxidant activity of the water extract obtained from dried pine needle (*Pinus densiflora*). *Preventive Nutrition and Food Science*, *23*(2), 134. https://doi.org/10.3746/pnf.2018.23.2.134

Kloucek, P., Polesny, Z., Svobodova, B., Vlkova, E., & Kokoska, L. (2005). Antibacterial screening of some Peruvian medicinal plants used in Calleria District. *Journal of Ethnopharmacology*, 99(2), 309-312. https://doi.org/10.1016/j.jep.2005.01.062

Linh, N. N., Hop, N. Q., Nhung, P. T. T., Dao, P. T. B., Manh, V. Q., Pham, T. V., & Son, N. T. (2024). Glochidion species: A review on phytochemistry and pharmacology. *Natural Product Communications*, *19*(9), 1-30. https://doi.org/10.1177/1934578X241276962

Matsunaga, S., Tanaka, R., Takaoka, Y., In, Y., Ishida, T., Rahmani, M., & Ismail, H. B. M. (1993). 26-Nor-D: A-friedooleanane triterpenes from *Phyllanthus watsonii*. *Phytochemistry*, *32*(1), 165-170. https://doi.org/10.1016/0031-9422(92)80125-X

Noguchi, N., & Niki, E. (1998). Chemistry of active oxygen species and antioxidants. In: Papas, A. M. (Ed) Antioxidant status, diet, nutrition, and health. CRC Press, Boca Raton, pp. 1-20. https://doi.org/10.1201/9780367811099

Negi, R. S., & Fakhir, T. M. (1988). Simplexine (14-hydroxy-4-methoxy-13,14-dihydronorsecurinine): An alkaloid from *Phyllanthus simplex*. *Phytochemistry 27*(9), 3027-3028. https://doi.org/10.1016/0031-9422(88)80724-6

Nawfetrias, W., Devy, L., Esyanti, R. R., & Faizal, A. (2024). *Phyllanthus lignans*: A review of biological activity and elicitation. *Horticulturae*, *10*(2), 195. https://doi.org/10.3390/horticulturae10020195

Puapairoj. P., Naengchomnong, W., Kijjoa, A., Pinto, M.M., Pedro, M., Nascimento, M.S., Silva, A.M., & Herz, W. (2005). Cytotoxic activity of lupane-type triterpenes from *Glochidion sphaerogynum* and *Glochidion eriocarpum* two of which induce apoptosis. *Planta medica*, *71*(03), 208-213. https://doi.org/10.1055/s-2005-837818

Rao, N.K., Bethala, B.K., Sisinthy, C.S.P., & Manickam, D.S. (2016). *In vivo* antioxidant activity of *Phyllanthus watsonii* A. Shaw leaves in streptozotocin induced type 2 diabetic rats. *An International Journal of Advances in Pharmaceutical Sciences, 7(1)*, 3024-3026.

Santos, A. R., De Campos, R. O., Miguel, O. G., Cechinel Filho, V., Siani, A. C., Yunes, R. A., & Calixto, J. B. (2000). Antinociceptive properties of extracts of new species of plants of the genus Phyllanthus (Euphorbiaceae). *Journal of Ethnopharmacology*, *72*(1-2), 229-238. https://doi.org/10.1016/S0378-8741(00)00256-7

Shi, Z. N., Wang, Y. D., Gong, Y., Li, H. F., & Zhu, Y. (2019). New triterpenoid saponins with cytotoxic activities from *Ligularia przewalskii*. *Phytochemistry Letters*, 30, 215-219. https://doi.org/10.1016/j.phytol.2019.02.024

Sulaiman, S. F., & Ooi, K. L. (2014). Antioxidant and α-glucosidase inhibitory activities of 40 tropical juices from Malaysia and identification of phenolics from the bioactive fruit juices of *Barringtonia racemosa* and *Phyllanthus acidus*. *Journal of Agricultural and Food Chemistry*, *62*(39), 9576-9585. https://doi.org/10.1021/jf502912t

Suttiarporn, P., Chumpolsri, W., Mahatheeranont, S., Luangkamin, S., Teepsawang, S., & Leardkamolkarn, V. (2015). Structures of phytosterols and triterpenoids with potential anti-cancer activity in bran of black non-glutinous rice. *Nutrients*, *7*(3), 1672-1687. https://doi.org/10.3390/nu7031672

Tanaka, R., Tabuse, M., & Matsunaga, S. (1988). Triterpenes from the stem bark of *Phyllanthus flexuosus*. *Phytochemistry*, *27*(11), 3563-3567. https://doi.org/10.1016/0031-9422(88)80769-6

Vilegas, J. H., Lanças, F. M., Vilegas, W., & Pozetti, G. L. (1997). Further triterpenes, steroids and furocoumarins from Brazilian medicinal plants of Dorstenia genus (Moraceae). *Journal of the Brazilian Chemical Society*, *8*(5), 529-535. https://doi.org/10.1590/S0103-50531997000500016

Wang, C. Y. & Lee, S. S. (2005). Analysis and identification of lignans in *Phyllanthus urinaria* by HPLC-SPE-NMR. *Phytochemical Analysis*, *16*(2), 120-126. https://doi.org/10.1002/pca.830

Xu, J., Xin, Y., Zhu, H. T., Kong, Q. H., Yang, W. N., Wang, D., Yang, C. R. & Zhang, Y. J. (2022). Flavonoids from the fruits of *Phyllanthus ac*idus (L.) Skeels with anti-α-glucosidase activity. *Natural Product Research*, *31*(12), 1986-1992. https://doi.org/10.1080/14786419.2022.2116704