Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(2): 39-46
Received: 17-01-2016
Accepted: 18-02-2016

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Phytochemical investigation and antioxidant activity of *Hyophorbe verschaffeltii* (Arecaceae)

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Abstract
The investigation was carried out for isolation and characterization of the possible phytochemical compounds of leaves of *Hyophorbe verschaffeltii* and determination of its antioxidant activity. The air dried leaves of *Hyophorbe verschaffeltii* were extracted with 70% methanol. The chromatographic investigation for aqueous fraction lead to isolation of five compounds by Column chromatography, thin layer chromatography (TLC), Preparative thin layer chromatography (PTLC) and paper chromatography. The isolated compounds were identified by spectroscopic techniques as ^{1}H-NMR and ^{13}C-NMR. The 70% methanolic extract was assayed for its antioxidant activity in *vivo* by CCl_4-induced hepatic injury technique and levels of serum liver enzymes Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were determined, also Oxidative Damage Markers as superoxide dismutase (SOD) and malondialdehyde (MDA) in liver tissue were studied. *Hyophorbe verschaffeltii* (Arecaceae) afforded aqueous fraction from which five compounds Quercetin (compound H-1), Quercetin 7, 3', 4' trimethoxy (compound H-4), Luteolin (compound H-5), Cannigenin (compound H-2) and Brisbagenin (compound H-3) were identified for the first time. The *H. verschaffeltii* extract (200mg/kg) showed a remarkable hepatoprotective and antioxidant activity against CCl_4-induced hepatotoxicity as judged from the serum marker enzymes and antioxidant levels in liver tissues.CCl_4-induced a significant rise in ALT, AST, MDA and reduction in SOD level. Treatment of rats with *H. verschaffeltii* extract (200mg/kg) significantly (P<0.001) decrease serum liver enzymes ALT and AST against CCl_4-treated rats. Also the *H. verschaffeltii* extract showed significant (P<0.001) elevation of SOD level by 124.7 % as compared to control group and the CCl_4 intoxicated group treated with *H.verschaffeltii* showed significant and efficient decline in the level of MDA (P<0.001) compared to CCl_4 group by 40.25%. *Hyophorbe verschaffeltii* have the free radicle scavenging activity that controls the CC_4-induced oxidative stress in liver tissue and capable of boosting the intracellular antioxidant capabilities.

Keywords: *Hyophorbe verschaffeltii*, Arecaceae, antioxidant, spirostane, SOD, ALT, Quercetin.

Introduction
Arecaceae or Palm family (palmeae), in the order Arecales, it is among the famous plant families which include genera that embrace phenolic-rich species, it is a monophyletic group including 183 genera and 2364 species [1, 2]. The palm family (Arecaceae) has a long history of providing man with useful materials for his daily life [3]. Chemically; the family has been neglected despite of its economic importance, probably because of difficulty of collecting fresh material and getting it authenticated. Most work has been carried out on economically important plants such as *Phoenix dactylifera*, *Cocosnucifera* and other palms cultivated for their oils [4]. *Hyophorbe* is a genus of about five species of flowering plants in the Arecaceae family, it contains the following species: *Hyophorbe amaricaulis*, *Hyophorbe indica*, *Hyophorbe lagenicaulis*, *Hyophorbe vaughanii* and *Hyophorbe verschaffeltii* [2]. *Hyophorbe verschaffeltii* is a member of the palm family (Arecaceae, sub-family Arecoideae). This palm is endemic to the Mascarene Islands, which are located to the east of Madagascar in the Indian Ocean [5].
From literature reviews, there is no data reported about neither the phytochemical constituents nor antioxidant activity of *Hyophorbe verschaffeltti* H. Wendl. The aim of this study is investigation for the chemical constituents and *in vivo* investigation of antioxidant activity of the palm leaves extract.

Materials and method
Plant material
Fresh plant material leaves of *Hyophorbe verschaffeltti* H. Wendl. (Arecaceae) were collected from El-Zohreya Botanical garden, Cairo, Egypt. The plant was kindly identified by

Agricultural Engineer Teresa Labib, El Orman Botanical Garden. The fresh plant leaves were completely dried in shade place at room temperature and then powdered by electric mill. The dried powders were kept in a dark place until subjected to the extraction process.

Extraction and isolation

The air dried powdered leaves of *Hyophorbe Verschaffeltii* (3 kg) were extracted by maceration with 70% methanol (methanol: H_2O, 70:30) with occasional stirring at room temperature for 3 days. The process was repeated two times till exhaustion. The combined methanol extracts were concentrated under reduced pressure at 40º to yield (420g). The residue was suspended in water and diluted with acetone. The aqueous fraction after filtration was evaporated until dryness to give (41g). About 25 g of the aqueous fraction residue was chromatographed using silica gel column chromatography (100cm x 2.5cm) using gradient elution system of increasing polarity using chloroform and methanol to yield six main fractions (I-VI). Fraction II was eluted with chloroform-methanol (90:10) revealed the presence of yellow fluorescent spot with R_f = 0.95 using silica gel GF_{254} plates and BAW then subjected to Co-chromatograms using Whatmann No.1 MM paper chromatography using 2 solvent systems: BAW and 15% AcOH, these were examined under UV light before and after exposure to ammonia vapors then purified over preparative TLC and concentrated under reduced pressure to yield compound H-1 (40 mg). Fraction IV eluted with chloroform-methanol (70:30) revealed the presence of major green color spot with R_f = 0.737 after spraying with 10% v/v sulphuric acid followed by heating at 100º for 10 minutes, using silica gel GF_{254} plates and BAW. These fractions were combined together and concentrated under reduced pressure to afford a residue of group IV. This residue was subjected for further purification by another silica gel sub-column chromatography (66g, 75x2 cm) using (75:25 $CHCl_3$: MeOH) solvent system then gradient elution till 100% MeOH, Sub-fraction IV-I was purified over preparative TLC and concentrated to yield compound H-2(25mg), and sub-fraction IV-II was purified over preparative TLC and concentrated to yield compound H-3(30mg). Fraction V eluted with chloroform-methanol (50:50) revealed the presence of one bluish violet fluorescent major spot with R_f= 0.61 using silica gel GF_{254} plates and BAW, these fractions were combined together and concentrated under reduced pressure to afford a residue of fraction V. This residue was subjected for further purification by another Polyamide sub-column chromatography (25g, 50x1 cm) using H_2O only as solvent system then gradient elution till 100% MeOH and fractions of 100 ml each were collected, concentrated and monitored by silica gel TLC using BAW, Sub-fraction V-I was purified over preparative TLC and concentrated to yield compound H-4(35mg). Fraction VI eluted with methanol revealed the presence of one violet fluorescent major spot with R_f = 0.833 using silica gel GF_{254} plates and BAW, Co-chromatograms were made using Whatmann No.1 MM paper chromatography using 2 solvent systems BAW and 15% AcOH these were examined under UV light before and after exposure to ammonia vapors. The fractions were purified over preparative TLC and concentrated under reduced pressure to yield compound H-5 (49 mg).

Compound (H-1)

Yellow amorphous powder, R_f value of 0.75 & 0.3 upon using system BAW and 15% AcOH, respectively. It gave yellow fluorescence under UV-light changed to orange when sprayed with Naturstroff reagent using precoated silica gel TLC. ^{1}H NMR (CD_3OD, 500MHz): δ ppm 7.8 (1H,d, *J* =2.1Hz,H-2'), 7.36 (1H,dd, *J* =2.1, 8.5Hz,H-6'), 6.8 (1H,d,*J* =8.2Hz, H-5'), 6.42 (1H,d,*J* =1.8 Hz, H-6), 6.18 (1H,d, *J* =1.8 Hz, H-8). ^{13}C NMR: δ ppm 178.0 (C-4), 165.2 (C-7), 162.5 (C-5), 158.7(C-9), 148.8 (C-4'), 147.5 (C-2), 145.0 (C-3'), 137.2 (C-3), 124.5 (C-1'), 120.8 (C-6'), 116.0 (C-5'), 115.8 (C-2'), 104.4 (C-10), 99.1 (C-6), 94.5 (C-8).

Compound (H-2)

Crystallized from methanol, TLC investigation using precoated silica gel G TLC plates and solvent BAW a reddish brown color with spraying reagent 10% H_2SO_4 with R_f=0.40. ^{1}H NMR spectral data (500MHz, CD_3OD): δ ppm 4.35 (1H,m,H-16), 4.01 (1H,m,H-3), 3.78 (1H,m,H-1), 3.48 (2H,m,H-26), 0.92 (3H,d,*J* =7 Hz, H-21), 0.87 (3H,*d*,*J* =7 Hz, H-27), 0.85 (3H,s,H-19), 0.75(3H,s,H-18). ^{13}C NMR: δ ppm 109.8 (C-22), 79.9 (C-16), 72.5 (C-1), 65.7 (C-26), 64.6 (C-3), 62.2 (C-17), 55.9 (C-14), 54.7 (C-9), 41 (C-13), 39.5 (C-2), 37.3 (C-5), 36.2 (C-4), 35.3 (C-8), 31.8 (C-15 & C-23), 30.8 (C-25), 29.5 (C-7), 28.5 (C-24), 27 (C-6), 23.5 (C-11), 14.1 (C-21), 16.6 (C-27).

Compound (H-3)

Crystallized from methanol, TLC investigation using precoated silica gel G TLC plates and solvent BAW a reddish brown color with spraying reagent 10% H_2SO_4 with R_f =0.415. ^{1}H NMR spectral data (500MHz, CD_3OD): δ ppm 4.18 (1H, m,H-16), 3.99 (1H,dd, *J* =11.3, 4.6 Hz,H-3), 3.73 (1H,dd, *J* =11.3, 4.4 Hz, H-1), 3.63 (1H, dd, *J* =10.5, 3.5Hz, H-26ax.), 3.36 (1H, dd, *J* =10.5, 10.5Hz, H-26eq.), 0.96 (3H,d, *J* =7.0 Hz, H-21), 0.88 (3H,s, H-19), 0.85 (3H,d, *J* =5.6 Hz, H-27). ^{13}C NMR: δ ppm 108.9 (C-22), 81.8 (C-16), 77 (C-1), 67.8 (C-3), 66.2 (C-26), 62.9 (C-17), 59.15 (C-14), 54.8 (C-9), 44.1 (C-2), 42.9 (C-5), 42.5 (C-20), 42.1 (C-10), 41.1 (C-12), 40.5 (C-13), 39(C-4), 36.3 (C-25), 36 (C-8), 33.3 (C-15), 32.3 (C-23), 32.2 (C-7), 29.9 (C-24), 29.7 (C-6), 24.5 (C-11), 18 (C-27), 16.8 (C-18), 13.1 (C-21), 6.8 (C-19).

Compound (H-4)

Yellow powder, It has R_f value of 0.88 upon using BAW. It gave yellow fluorescence under UV-light showed no change on exposure to ammonia vapors and changed to orange when sprayed with Naturstroff reagent using precoated silica gel TLC. The EI/MS *m/z*(%): 344(17), 329(5), 315(8), 301(4), 207(13), 165(40), 167(13), 150(38), 147(40), 57(100).^{1}H NMR (DMSO-*d6*, 500MHZ):δ ppm 7.50 (1H, d, *J* =2.0 Hz, H-2'), 7.22 (1H,dd, *J* =2.0, 8.4 Hz, H-6'), 7.08 (1H, d,*J*=8.4 Hz, H-5'), 6.80 (1H,d,*J* =2.0 Hz, H-8), 6.53 (1H,d, *J* =2.0 Hz, H-6), 3.83, 3.86, 3.90 (each 3H, br s, OMe).

Compound (H-5)

Yellow amorphous powder, R_f value of 0.83 upon using BAW. It gave dark purple fluorescence under UV-light changed to orange when sprayed with Naturstroff reagent using pre-coated silica gel TLC and changed to yellow fluorescence on exposure to ammonia vapors and gave green color with $FeCl_3$ spray reagent.. ^{1}H NMR (CD_3OD, 500MHz): δ ppm 7.36 (2H, m, H-2' and H-6'), 6.9 (1H, d, *J* =8.5 Hz, H-5'), 6.53 (1H,s,H-3), 6.42 (1H,d,*J* =2 Hz, H-8), 6.2 (1H,d,*J* =2 Hz, H-6).^{13}C NMR: δ ppm 181.7 (C-4), 164.3 (C-7), 163.8 (C-2),161.7 (C-9), 157.6 (C-5), 149.7 (C-4'), 146.5 (C-3'), 121 (C-6'), 119.0

(C-1'), 115.8 (C-5'), 113.2 (C-2'), 103.8 (C-10), 103 (C-3), 100 (C-6), 99.1 (C-6), 94.8 (C-8).

Biological assay

Acute toxicity

The 70% total extract of *Hyophorbe verschaffeltii* was dissolved in distilled water then given orally to mice in graded doses up to 2 g/kg. The control group received the same volumes of distilled water. The percentage mortality for extract was recorded 24 hours later. The mice were divided into two groups of six mice each as follows: First control group: mice were given distilled water. Second group: mice were given a single oral dose of 2 g /kg. Observation of rats 14 days, for any changes in the skin, fur, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity and behaviour pattern. Particular observation for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma were done.

Antioxidant activity

Experimental groups

36 rats were randomly divided into six groups (6rats per group). Group I: Received Saline and served as control, rats were given a daily oral dose of 1ml saline alone for successive 7 days. Group II: (CCl_4 group) Received CCl_4 only, rats were given 0.5 ml of CCl_4 (10%CCl_4 in olive oil) intraperitoneally. Group III: (Reference hepatoprotective group) Received Silymarin, rats were given a daily oral dose of silymarin 25 mg/kg (Bhandari *et al.,* 2003). Group IV: Rats received a daily oral dose of aqueous suspension of MeOH extract of leaves of *Hyophorbe verschaffeltii* (200 mg/kg) alone for successive 7 days. Group V: Pre-treated group, rats were given a daily oral dose of Silymarin alone for successive 7 days followed by a single dose of CCl_4 administration (0.5 ml of 10%CCl_4 in olive oil).Group VI: Pre-treated group, rats were given a daily oral dose of aqueous suspension of MeOH extract of leaves of *Hyophorbe verschaffeltii* (200 mg/kg) alone for successive 7 days followed by a single dose of CCl_4 administration. (0.5 ml of 10%CCl_4 in olive oil). Rats had free access to food and drinking water during the study a single oral dose of $CCl_4$was given 6 h after the last dose of administration of extract and saline for groups I, V, VI on the 7th day.

Preparation of serum and tissue homogenate

After 24 h, blood samples were obtained from all groups of rats after being lightly anesthetized with ether by puncturing the retro-orbital vein plexuses [6], the blood was allowed to flow into a clean dry centrifuge tube and left to stand 30 min. before centrifugation to avoid haemolysis. Then blood samples were centrifuged for 15 min. at 2500 rpm the clear supernatant serum was separated and collected by Pasteur pipette into a dry clean tube and stored for determination of serum ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase) according to (IFCC) the international Federation of clinical chemistry. Then animals were sacrificed by cervical dislocation and the liver was rapidly isolated and washed with ice-cold isotonic saline (0.9%). Then, they were stored at –80 °C till they were homogenized in ice-cold 0.15 M KCl (w/v) using Sonicator homogenizer (4710 Ultrasonic homogenizer, Cole-Parmer instrument Company, USA). The homogenate was then made into aliquots by centrifuging for 10 min in a refrigerated centrifuge at 4 °C at 3000 rpm and stored at – 20 ºC prior to use for the determination of liver contents of Malondialdehyde (MDA), and enzymatic activities of Superoxide dismutase (SOD).

Method of Assessment of liver function parameters

Diagnostic kits: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed in serum samples obtained from all groups of rats by a colorimetric method [7]. The activities of ALT, AST were expressed as U/L. All the colorimetric kits were obtained from biodiagnostic Co., Cairo, Egypt.

Method of Assessment of Oxidative Damage Markers

Estimation of liver Super oxide dismutase (SOD) activity

The level of SOD activity was determined in liver homogenate according to the method described [8]. This assay relies on the inhibition of pyrogallol auto-oxidation by SOD. The inhibition is directly proportional to the activity of SOD in the tested sample. The level of SOD activity in liver homogenate was assayed spectrophotometrically by measuring the % inhibition of the auto-oxidation of pyrogallol in the presence of SOD enzyme [9] using Spectrophotometer. The inhibition is directly proportional to the activity of SOD in the tested sample. One unit of SOD represents the amount of enzymes required to inhibit the rate of pyrogallol oxidation by 50% at 25º. The activity was expressed as units/mg protein**.**

Estimation of liver malondialdehyde (MDA) content

Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA) according to method described [10]. Colorimetric determination of TBARS is based on the reaction of one molecule of MDA with two molecules of thiobarbituric acid at low pH (2-3) and a temperature of 95 °C for 45 min. The resultant pink colour was extracted with *n*-butanol and the absorbance was determined as 535 nm and 520 nm spectrophotometrically. The difference in optical density between both wavelengths was used in measurement of MDA content. The liver content of MDA was determined from the standard curve which was constructed using serial dilutions of 1, 1,3,3-tetraethoxypropane (0.125- 4 nmol / ml).

Statistical analysis

Values were expressed as mean ± SEM (n=6). Statistical significance was determined using one way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Values of $P<0.001$, 0.01, 0.05 were considered significant.

Results and Discussion

Phytochemical investigation

Phytochemical investigation of the aqueous fraction of *H. verschaffelttii* leaves led to the isolation of 5 compounds identified as three flavonoids (H-1) quercetin, (H-3) quercetin-7, 3', 4'-trimethoxy, and (H-4) luteolin and two spirostane (H-2) Cannigenin (1β, 3α diol, 5α, 25R spirostane) and (H-3) Brisbagenin (1β, 3β diol, 5α, 25R spirostane) (Figure 1).

The ^{1}H NMR spectral data of compound (H-1) showed that the aromatic region exhibited an ABX spin coupling system at δ 7.7 (1H, *d*, *J* = 2.1 Hz, H-2'), 7.4 (1H, *dd*, *J* = 2.1,8.5 Hz, H-6'), and 6.8 (1H, *d*, *J* = 8.2Hz, H-5′) due to a 3′, 4′ disubstitution of ring B and a typical *meta*-coupled pattern for H-8 and H-6 protons (δ 6.18 and 6.42).The ^{1}H NMR spectral data were in agreement with those reported in the literature [11-15]. The ^{13}C NMR spectra compound (H-1), showed signals for

a carbonyl group at δ178 (C-4), seven oxygenated quaternary carbons (δ147.5, 137.2, 162.5, 165.2, 158.7, 145, and 148.8), two sp^2 quaternary carbons (δ104.4 and 124.5) and five sp^2 tertiary carbons (δ 99.1,94.5, 115.8, 116.0 and 120.8) The ^{13}C NMR spectral data were in agreement with those reported in the literature [12, 14, 15]. The paper chromatography was used to confirm compound (H-1) by CoPC with authentic sample with two different solvent system (BAW and 15%AC.acid) the R_f values was 0.75 in BAW and 0.3 in 15% Ac. Acid on PC. The R_f values were within the range of a flavonoid aglycone [16]. From the ^{1}H-NMR, ^{13}C-NMR and CoPC against authentic sample on PC the compound (H-1) is Quercetin.

The ^{1}H-NMR spectrum of compound (H-4), showed that the aromatic region exhibited an ABX spin coupling system at δ 7.50 and δ 7.22 assigned to H-2' and H-6', respectively and δ 7.08 assigned to H-5' due to a 3′, 4′ disubstitution of ring B and a typical *meta*-coupled pattern for H-8 and H-6 protons (δ 6.8 and 6.53).Three aromatic methoxy groups appeared at δ 3.83, 3.86 and 3.90. The ^{1}H NMR spectral data were in agreement with those reported in the literature [17]. The EI/MS of compound (H-4) revealed a molecular ion peak at *m/z* 344 corresponding to $[C_{18}H_{16}O_7]^+$. Also, Retro-Diels-Alder fragmentation resulted in ions at *m/z* 165 ($C_9H_9O_3$), 150 ($C_8H_6O_3$), 147 ($C_9H_7O_2$). In addition to diagnostic peaks at *m/z* 57 (O=C=C•-OH) 329 ($C_{18}H_{14}O_6$), 315 ($C_{17}H_{13}O_7$), and 301($C_{16}H_{13}O_6$), 207($C_{10}H_7O_5$) [18, 19]. The B_2^+ ion (165) along with the $[A_1 + H]^+$ (167) and $[M\text{-}CH3\ \text{-}CO]^+$ (301) fragments defined much of the substitution patterns for a series of naturally occurring quercetin methyl ethers [18]. Compound (H-4) identified as Quercetin 7, 3', 4'-trimethoxy.

The ^{1}H-NMR spectrum of compound (H-5) exhibited the characteristic pattern of a flavone structure [20, 21] represented by ABX spin coupling system at δ 7.36 (m) and δ 6.9(d) assigned to H-2'/H-6' and H-5', respectively, characteristic for a 3', 4' di-substituted B-ring. Also, an aromatic singlet at δ 6.53 assigned to H-3 together with a pair of *meta* coupled aromatic protons of H-6 and H-8 at δ 6.2 and δ 6.42, respectively. The ^{1}H-NMR spectral data were in agreement with those reported in the literature [13, 14, 22, 23]. The ^{13}C NMR spectra showed signals for a carbonyl group at atδ181.7 (C-4), six oxygenated quaternary carbons (δ163.8, 157.6, 164.3, 161.7, 146.5 and 149.7), two sp^2 quaternary carbons (δ103.8 and 119) and six sp^2 tertiary carbons (δ 103, 100, 94.8, 113.2, 115.8 and 121). The ^{13}C NMR spectral data were in agreement with those reported in the literature [14, 22, 24, 25]. The paper chromatography was used to confirm compound (H-5) by Co PC with authentic sample with two different solvent system (BAW and 15%AC.acid) the R_f values was 0.73 in BAW and 0.07 in 15% Ac. Acid on PC. The R_f values were within the range of a flavonoid aglycone [16]. From the ^{1}H-NMR,^{13}C-NMR and Co PC against authentic sample on PC the compound (H-5) is Luteolin.

The ^{1} H-NMR spectrum of (H-2) showed resonances for two angular tertiary methyl signals at δ0.75(3H,s,H-18), δ0.85(3H,s,H-19),two secondary methyl signals at δ0.92(3H,d,*J* = 7Hz,H-21),0.87(3H,d,*J*=7Hz, H-27) which were recognized as typical steroid methyls [26], also two protons on carbon atoms bearing hydroxyl groups (lH, m, 3.78 and 4.01) that assigned for H-1 & H-3,respectively. Finally, three protons on carbon atoms bearing ether oxygen atoms (lH, m, 4.35 and 2H, m, 3.48) assigned for H-16 & H-26 that are generally considered suggestive that the compound might be a steroidal sapogenin [27]. The ^{1}H NMR spectral data were in agreement with those reported in the literature [27-29]. The three protons at position 27 appeared at δ0.87 upper field of the corresponding resonance of (25S) spirostane [30]. The ^{13}C NMR spectrum of H-2contained 27 signals including four methyl groups, nine non-oxygenated and one oxygenated methylene, seven non oxygenated and three oxygenated methine and two non-oxygenated and one oxygenated quaternary carbon signals. A quaternary carbon (C-22) signal appeared at 109.8ppm. These data indicated a dihydroxy spirostane skeleton with an exocyclic axial secondary methyl group (25*R*) in ring F. According to the ^{13}C NMR spectral data, the multiplicity and chemical shift of C-22 are of prime importance in establishing the parent skeleton. It is of the quaternary type for skeleton of spoirostane [30]. The (25 R) configuration of basic spirostane skeleton was confirmed by the downfield shift of^{13}C resonances of the ring F atoms C-23, C-24, C-25, C-26 and C-27 at δ 31.8, 28.5, 30.8, 65.7 and 16.6, respectively. Comparable to those of (25S) configuration [30, 31]. The A/B Ring junction was established as *trans* by the examination of ^{13}C NMR chemical shifts of C-5, C-7and C-9 (37.3, 29.5, 54.7) which were consistent with the presence of 5α-spirostnae steroidal skeleton rather than a 5β-spirostane skeleton [30, 31]. A comparison of the chemical shifts and the shapes of the signals of protons at C- 1 and C-3 of a series of derivatives of the sapogenins with published chemical shifts and shapes [27] and 1H-NMR spectrum clearly shows signal of 3β-H at (δ 4.01,1H, m) so that the orientation of C-1 hydroxyl group was established to be equatorial (β-) and the orientation of 3-OH group was shown to be axial (α-). The^{13}C NMR spectral data were in agreement with those reported in the literature [27, 30]. Consequently compound (H-2) was confirmed to be Cannigenin (1β, 3α diol, 5α, 25R spirostane).

The ^{1} H-NMR spectrum of (H-3) showed signals for four typical steroid methyls; two appeared as singlets at δ 0.86 and δ0.88 and other two as doublets at δ0.85(*J*=6Hz)and δ0.96(*J*=7Hz) (26), also two protons on carbon atoms bearing hydroxyl groups (δ3.73 and δ 3.99) that assigned for H-1 & H-3, respectively. Finally, three protons on carbon atoms bearing ether oxygen atoms (4.18, 3.63 and 3.36) assigned for H-16 & H-26axial and equatorial protons that are generally considered suggestive of a spiroketal BAW indicated that the compound might be a steroidal sapogenin [27]. The^{1}H NMR spectral data were in agreement with those reported in the literature [27, 29, 32, 33]. The signal due to the 3β-H (δ 4.01) found in cannigenin was absent in the spectrum of compound H-3 and shifted to (δ3.99) that is assigned for 3α-H, so that the orientation of C-1and C-3 hydroxyl group were established to be equatorial (β-).The three protons at position 27 appeared at δ0.85 upper field of the corresponding resonance of (25S) spirostane [30]. The ^{13}C NMR spectrum of H-3 contained 27 signals including four methyl groups, nine non-oxygenated and one oxygenated methylene, seven non-oxygenated and three oxygenated methine and two non-oxygenated and oneoxygenated quaternary carbon signals. A quaternary carbon (C-22) signal appeared at 110 ppm. These data indicated a dihydroxy spirostane skeleton with an exocyclic axial secondary methyl group (25*R*) in ring F.According to the ^{13}C NMR spectral data, the fundamental structure of (H-3) based upon a steroid of (25R)-spirostanol was suggested by the ^{13}C-NMR (δ108.9) quaternary carbon signal assignable to C-22 of spirostanol, also The (25 R) configuration of basic spirostane skeleton was confirmed by the downfield shift of C-23, C-24, C-25, C-26 and C-27 at 32.3, 29.9, 36.3, 66.2 and 18, respectively Comparable to those of (25S) configuration [31]. The A/B Ring junction was established as *trans* by the examination of ^{13}C

NMR chemical shifts of C-5, C-7and C-9 (42.9, 32.2, 54.8) which were consistent with the presence of 5α-spirostnae steroidal skeleton rather than a 5β-spirostane skeleton [30, 31]. The ^{13}C NMR spectral data were in agreement with those reported in the literature [27, 30, 33]. Consequently compound (H-3) is confirmed to be Brisbagenin (1β, 3β diol, 5α, 25R spirostane).

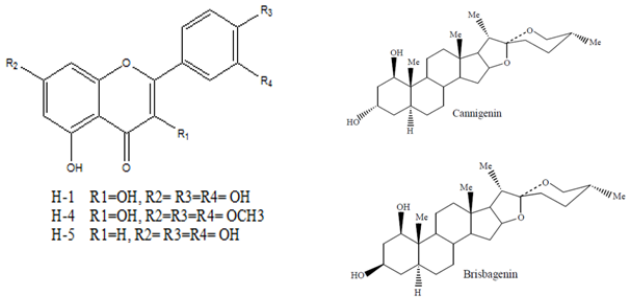


Figure 1. Compounds isolated from leaves of *H.verschaffeltii*

Biological assay

Acute toxicity

The results of this study showed no mortality after 24 hours of oral administration of 70% aqueous extract of *Hyophorbe verschaffeltii* at graded dose up to a 2 g/kg b.wt. After 15 days of single oral administration of *Hyophorbe verschaffeltii* extract, the mice did not show any signs of toxicity or changes in general behavior or other physiological activities.

Antioxidant activity

Assessment of liver function parameters

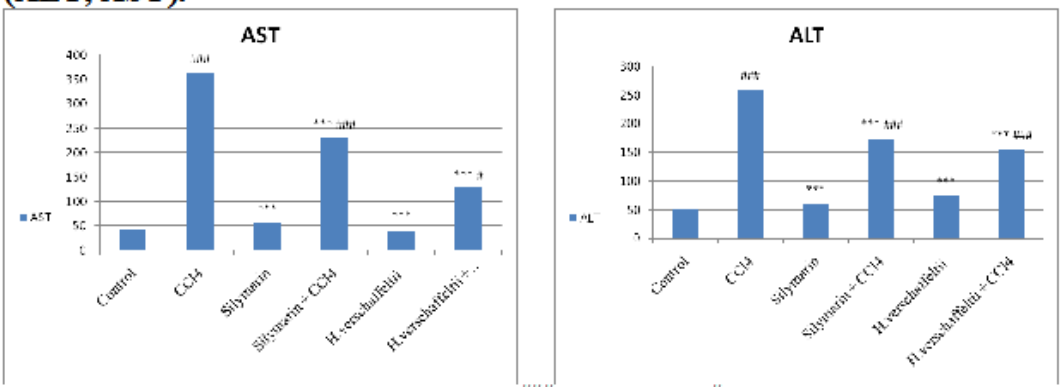
Exposing rats to CCl_4 induced severe hepatic injury and abnormal liver functions parameters represented by elevation of serum levels of hepatic enzymes ALT and AST. It showed a significant elevation in the serum enzymes levels of AST & ALT levels by 363.2 and 259.3 Iu/ Liter, respectively after 24 hours as compared with control group (P<0.001) that are indicators of hepatic damage. The treatment by extract of *Hyophorbe verschaffeltii* leaves showed a significant reduction in elevated serum AST and ALT levels by 64.15% and 40.53% at dose level of 200 mg/kg b.w.t respectively as compared with CCl_4 treated group(*P*<0.001). Compared to group treated with silymarin (25mg/kg) exhibited reduction in serum AST & ALT levels by 36.48% & 32.89% respectively as compared with CCl_4 treated group. (Table1 & Figure 2) Our data revealed that treatment with *H. verschaffeltii* leaves extract significantly diminished the CCl_4-induced elevations of liver function parameters (ALT and AST) in serum and restored their normal levels (P<0.001&P<0.05, respectively). The reduction of ALT and AST enzymes levels in rats treated with *H. verschaffeltii* leaves extract clearly establishes the hepatoprotective effect that might be able to induce accelerated regeneration of liver cells, reducing the leakage of the above enzymes into the blood.

Table (1):The effect of oral administration of methanol extract of *Hyophorbe verschaffeltii* leaves (200 mg/Kg) on ALT and AST serum activity in CCl_4 induced hepatotoxicity in rats, Data are presented as mean±SE (n=6).

Groups	AST (Iu / Liter)	ALT (Iu / Liter)
Control	44.33±3.159	52±3.055
CCl_4	$363.2\pm33.17^{\#\#\#}$	$259.3\pm17.96^{\#\#\#}$
Silymarin(25 mg/kg)	$55.5\pm4.815^{***}$	$60.33\pm8.015^{***}$
Silymarin + CCl_4	$230.7\pm14.81^{***\#\#\#}$	$174\pm5.398^{***\#\#\#}$
H.verschaffeltii (200 mg /Kg)	$41.5\pm2.907^{***}$	$72.83\pm10.76^{***}$
H.verschaffeltii + CCl_4	$130.2\pm17.51^{***\#}$	$154.2\pm14.27^{***\#\#}$

$^{\#\#\#}$= P<0.001, $^{\#}$= P<0.05: Statistically significant from control group.
***= P<0.001: Statistically significant from CCl_4 group by using one way ANOVA followed by Tukey's Multiple Comparison Test.

Fig (2): Effects of *H. verschaffeltii* leaves extract and silymarin onCCl4-induced alterations in serum liver function parameters (ALT, AST).



Data are presented as mean±SE, n=6, $^{\#\#\#}$= P<0.001, $^{\#}$= P< 0.05 as compared with the control group. ***= P<0.001 as compared withCCl_4-treatedgroup.

Assessment of Oxidative Damage Markers

Estimation of liver Super oxide dismutase (SOD) activity

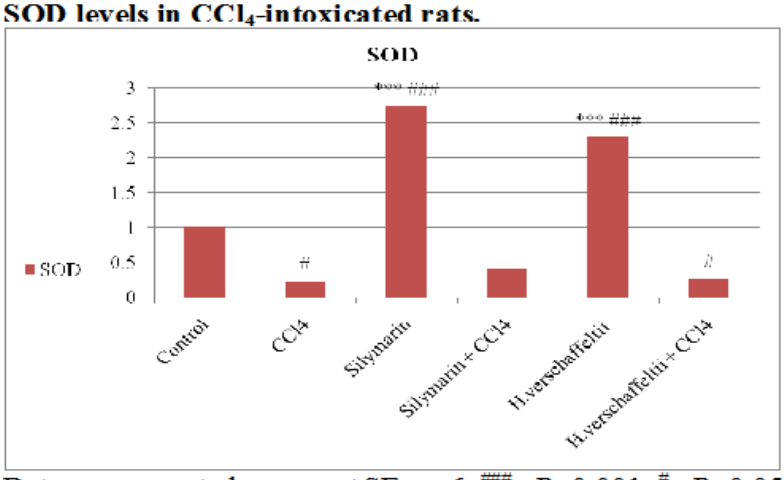
SOD is a metallo protein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O_2. The activity of SOD is expressed as units/mg protein. To investigate the ability of *H. verschaffeltii* leaves to enhance the antioxidant capacity of liver, we assessed superoxide dismutase (SOD) in liver tissue homogenate (Table 2 & Figure 3).

Table (2): The effect of oral administration of methanol extract of *Hyophorbe verschaffeltii* leaves (200 mg/Kg) on Liver SOD activity in CCl_4 induced hepatotoxicity in rats, Data are presented as mean±SE (n=6).

Groups	SOD activity (units/mg protein)
Control	1.02±0.179
CCl_4	0.24±0.038$^{\#}$
Silymarin (25 mg/kg)	2.73±0.29$^{***\#\#\#}$
Silymarin + CCl_4	0.435±0.046
***H.verschaffeltii* (200 mg /Kg)**	2.292±0.1279$^{***\#\#\#}$
***H.verschaffeltii* + CCl_4**	0.286±0.066$^{\#}$

$^{\#\#\#}$= P<0.001, $^{\#}$= P<0.05: Statistically significant from saline control group.
***= P<0.001: Statistically significant from CCl_4 group by using one way ANOVA followed by Tukey's Multiple Comparison Test.

Fig (3): Effects of *H. verschaffeltii* leaves extract and silymarin on SOD levels in CCl_4-intoxicated rats.



Data are presented as mean±SE, n=6, $^{\#\#\#}$= P<0.001, $^{\#}$= P<0.05as compared with the control group. ***= P<0.001 as compared withCCl_4-treatedgroup.

We found that CCl_4 treatment induced significant decline in SOD enzyme activity in the liver homogenate by 76.47% as compared to the control group (P<0.05).The liver homogenate of groups treated with silymarin and *H. verschaffeltii* leaves only showed significant elevation of SOD level by167.64% and 186.27 %, respectively as compared to control group(P<0.001).By comparing the statistical results of the two groups treated with silymarin and *H. verschaffeltii* followed by CCl_4 we noticed the non-significant relationship that means the equivalence of *H. verschaffeltii* effect on CCl_4 intoxication as compared to group treated with silymarin. This indicates that *H. verschaffeltii* capable of boosting the intracellular antioxidant capabilities.

Estimation of liver malondialdehyde (MDA) content

The liver peroxidation levels were measured according to the concentration of thiobarbituric acid reactive species (TBARs) [34] and the amount of produced malondialdehyde (MDA) was used as an index of lipid peroxidation. Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process [35]. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals which is accepted as an indicator of lipid peroxidation [36].

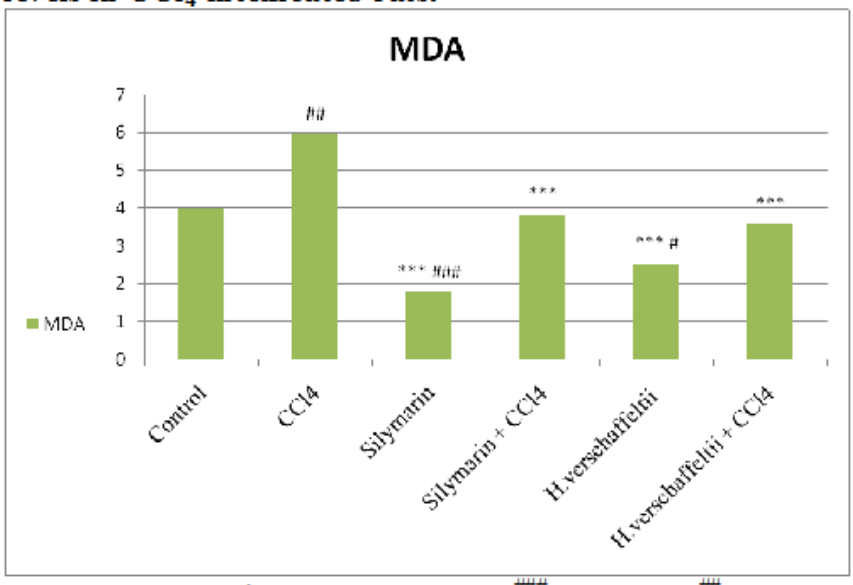
By evaluation of MDA level (marker of lipid peroxidation) in liver homogenate, we noticed the significant elevation of MDA in CCl_4 treated group as compared to control group by 49.25% (P<0.01). In contrast the level of MDA attenuated significantly by administration of Silymarin and *H. verschaffeltii* as compared to control group by 56% and 37.075 %, respectively (P<0.001). CCl_4 intoxicated group treated with Silymarin and *H. verschaffeltii* showed significant and efficient decline in the level of MDA (P<0.001) compared to CCl_4 group by 36.06% and 40.25%, respectively (Table 4, Figure 4). The equivalence of treatment by *H. verschaffeltii* and silymarin is noticed by the statistical non-significant relationship of MDA level that means *H. verschaffeltii* have the free radicle scavenging activity that controls the CCl_4-induced oxidative stress in liver tissue (Table 3 & Fig. 4).

Table (3): The effect of oral administration of methanol extract of *Hyophorbe verschaffeltii* leaves (200 mg/Kg) on Liver MDA content in CCl_4 induced hepatotoxicity in rats,Data are presented as mean±SE (n=6).

Groups	MDA level nmol/ml
Control	4±0.327
CCl_4	5.97±0.382$^{\#\#}$
Silymarin (25 mg/kg)	1.76±0.1685***$^{\#\#\#}$
Silymarin + CCl_4	3.817±0.44***
***H.verschaffeltii* (200 mg /Kg)**	2.517±0.2509***$^{\#}$
***H.verschaffeltii* + CCl_4**	3.567±0.1406***

$^{\#\#\#}$= P<0.001, $^{\#\#}$= P<0.01, $^{\#}$= P<0.05: Statistically significant from saline control group.
***= P<0.001: Statistically significant from CCl_4 group by using one way ANOVA followed by Tukey's Multiple Comparison Test.

Fig (4): Effects of *H. verschaffeltii* leaves extract and silymarin on MDA levels in CCl_4-intoxicated rats.



Data are presented as mean±SE, n=6, $^{\#\#\#}$= P<0.001, $^{\#\#}$= P<0.01, $^{\#}$= P<0.05 as compared with the control group. ***= P<0.001 as compared withCCl_4-treated group.

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