Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2015; 3(6): 67-73
Received: 17-01-2015
Accepted: 01-03-2015

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Biological studies of isolated triterpenoids and phenolic compounds identified from *Wodyetia bifurcata* family Arecaceae

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Abstract
The chromatographic investigation of the aerial parts of *Wodyetia bifurcata* (family Arecaceae) revealed the presence of β-Amyrin, lupeol, apigenin, kaempferol, p- hydroxyl benzoic acid and gallic acid that isolated for first time from *Wodyetia bifurcata* and their structures are elucidated by different spectroscopic techniques as UV, ^{1}H-NMR, ^{13} C-NMR and EI/MS spectra. The biological activities of different plant extracts are tested for anti-inflammatory, antioxidant and cytotoxicity.

Keywords: *Wodyetia bifurcata,* Arecaceae, Aerial part, Triterpenoids, phenolic compounds, Anti-inflammatory, Antioxidant, Cytotoxicity.

1. Introduction
Wodeytia is a genus of trees belonging to family Arecaceae. *Wodyetia bifurcata* L (also called foxtail) is fast growing, large sized evergreen tree, up to 10 m tall, grows in El-Orman garden, Giza, Egypt. *Wodyetia bifurcata* L is endemic to Australia and it is found on north Queensland [18]. Triterpenoids are prevalent in the plant kingdom. Recent evidences support the beneficial effects of naturally occurring triterpenoids against several types of human diseases [27], anticancer [19], antioxidant [2], hepatoprotective [22], antifungal [28], antibacterial [16] and antileishmanial activity [9]. Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics, found ubiquitously in plants. Until now, more than 9000 different flavonoid compounds were described in plants, where they play important biological roles by affecting several developmental processes [37]. There has been increasing interest in the research of flavonoids from dietary sources, due to growing evidence of the versatile health benefits of flavonoids including antioxidant activity [10], coronary heart disease [35], hepatoprotective [34], anti-inflammatory, [23], anticancer activities [38], antibacterial [21] while some flavonoids exhibit potential antiviral activities [5].

2. Results and discussions
The MS of the amyrins is characterized by an M+ of *m/e* 426 and a major fragmentation yielding a component of *m/e* 218 [15]. Data of ^{1}H NMR for compound I showed eight methyl group corresponding to terpenoid of *olean* skeleton. The ^{13}C NMR and EI-MS of compound I were compared with that reported in that literature review [7, 8, 25]. The compound I is identified as β-Amyrin.
The ^{1}H NMR data of compound II showed seven tertiary methyl singlets and one secondary hydroxyl group. It also showed olefinic protons at δ 4.41 and 4.67. The compound showed 30 signals for the terpenoid of lupine skeleton which is represented by seven methyl groups. The carbon bonded to the hydroxyl group C-3 appeared at δ 79.1, while the alkenic carbons appeared at δ 151.1 and 109.5. EI-MS spectrum of compound II showed the molecular ion at m/z 426 (10%) [M^+] corresponding to the formula $C_{30}H_{50}O$, together with fragments at m/z 411 (6%) [M^+-15] and 408 (5%) [M^+_18] which were due to the loss of methyl group and a molecule of water from the molecular ion peak other fragments occurring at m/z 385 (4%) (M^+-41), m/z 220 (8%) (M^+-$C_{15}H_{26}$), m/z 218 (46%) (M^+-$C_{14}H_{20}O$), m/z 207(51%) (M^+-$C_{16}H_{27}$) and m/z 189 (70%) (M^+-$C_{14}H_{21}$) were characteristic for lupane series [15]. Data of ^{1}H NMR, ^{13}C NMR and EI-MS were compared with that reported in that literature review [1, 3, 13, 36]. The compound II is identified as lupeol.

UV spectral data of compound III showed two major absorption bands; band (I) at 331 nm and band II at 272 nm, which is typical of a flavone nucleus with no hydroxyl group at position 3 [20]. The addition of sodium methoxide resulted in a bathochromic shift (+Δ 69 nm) in band I with an increase in intensity, which proved that position 4` has a free OH group [20]. The bathochromic shift in band I (+ Δ 56 nm) on addition of $AlCl_3$; compared with the same band in MeOH, which is still stable even after the addition of HCl indicated the presence of free 5-OH group. The bathochromic shift in band II on addition of NaOAc (+ Δ 9 nm) compared with the same band in MeOH suggested the presence of a free hydroxyl group at carbon number 7. On addition of H_3BO_3 to NaOAc the hypochromic shift in band I (- Δ 45 nm) suggested the absence of any *ortho*-dihydroxyl groups. The ^{1}H-NMR spectrum of compound III revealed the presence of two aromatic protons of the A-ring revealed as two doublet at δ 6.2 and δ 6.0 each proton has J=1.5 Hz due to *meta* coupling assigned to H-8 and H-6, respectively. H-3 appeared at δ 6.4 as a single signal as no neighbor proton appears .The aromatic protons of the B-ring as two doublets at δ 7.6 and δ 7.0 each doublet with J= 5.7 Hz due to *ortho* coupling assigned to H-2`, 6` (H-2`, 6`are superimposed) and H-3`, 5`,(H-3`, 5`are superimposed) respectively .Note that the H-3`,5` doublet occurs upfield from H-2`, 6`due to shielding effect of the hydroxyl group and the deshielding influence of C-ring on H-2`, 6`. The EI-MS spectrum of compound III showed the molecular ion peak as the base peak (M^+) at m/z 270 (95%) corresponding to molecular formula $C_{15}H_{10}O_5$. Peaks at m/z 152 (43%) and m/z 118 (48%) it suggests the fragments of ring (A) and ring (B). Others peaks suggests another pathway of decomposition of compound and splitting of ring (A) and ring (B) at m/z 121 (20%) and m/z 149 (26%). Through the UV, EI-MS and H^1-NMR the compound was identified as apigenin (isolated for the first time from *Wodeytia bifurcata*). Data of ^{1}H NMR, UV spectra and EI-MS were compared with that reported in that literature review (Guang-ying Chen, *et al*., 2010; Fairouz Moussaoui *et al.,* 2010; Venkata Sai Prakash Chaturvedula and Indra Prakash, 2013; Jie Zhanga, *et al.,* 2005). The compound III is identified as.

UV spectral data of compound IV in methanol showed two major absorption bands; band (I) at 367 nm and band II at 265 nm, which is typical of a flavanol [20]. The addition of sodium methoxide resulted in a bathochromic shift (+Δ 53 nm) in band I with an no decrease in intensity, which proved that position 4` has a free OH group [20]. The bathochromic shift in band I (+ Δ 47 nm) on addition of $AlCl_3$; compared with the same band in MeOH, which is still stable even after the addition of HCl indicated the presence of free 5-OH group. The bathochromic shift in band II on addition of NaOAc (+ Δ 11 nm) compared with the same band in MeOH suggested the presence of a free hydroxyl group at carbon number 7 and in band I there is lesser than NaOMe indicate that there is no substitution of hydroxyl group number 7. On addition of H_3BO_3 to NaOAc the hypochromic shift in band I (- Δ 15 nm) suggested the absence of any ortho-dihydroxyl groups. ^{1}H-NMR spectrum of compound IV showed the two aromatic protons of the A-ring revealed as two doublet at δ 6.5 and δ 6.2 each proton has J=1.2 Hz due to *meta* coupling assigned to H-8 and H-6, respectively. The aromatic protons of the B-ring as two doublets at δ 7.94 and δ 6.9 each doublet with J=5.4 Hz due to *ortho* coupling assigned to H-2`, 6` (H-2`, 6`are superimposed) and H-3`, 5`, (H-3`, 5`are superimposed) respectively .Note that the H-3`,5` doublet occurs upfield from H-2`, 6`due to shielding effect of the hydroxyl group and the deshielding influence of C-ring on H-2`, 6`. The EI-MS spectrum of compound IV showed the molecular ion peak as the base peak (M^+) at m/z 287 (84%) corresponding to molecular formula $C_{15}H_{11}O_6$. Spectra showed peak at 269 m/z indicate that molecular formula $C_{15}H_9O_5$ (M-18) and removing of H_2O due to flavanol. It showed peak at m/z 259 (12%) suggest of (M– CO) and m/z 258 (15%) of (M– CHO). Peaks at m/z 153 (42%) and m/z 133 (48%) it suggests separation of ring (A) and ring (B). Others peaks suggests another pathway of decomposition of compound and splitting of ring (A) and ring (B) at m/z 121 (8%) and m/z 165 (%14). Through the UV, EI-MS and ^{1}H-NMR compound (F2) was identified as kaempferol (was isolated for the first time from *Wodeytia bifurcata*) data of ^{1}H NMR, UV spectra and EI-MS were compared with that reported in that literature review [6, 24, 31]. The compound IV is identified as kaempferol.

Compound V was isolated from soluble part of successive methanol extract of *Wodeytia bifurcata* as Colorless crystals; it gave violet color with ferric chloride solution indicating the presence of phenolic group with melting point 215-216 º. The ^{1}H-NMR of compound V showed signals at δ; 7.58 (2H, d, J = 2.1 Hz, H-2, 6), 6.92 (2H, d, J = 1.2 Hz, H-3, 5). Data of ^{1}H NMR, was compared with that reported in that literature review [29]. The compound V is identified as para hydroxyl benzoic acid.

The compound VI is identified by co-chromatography with authentic using paper chromatography with solvent system butanol : acetic acid : water (5:4:1) and acetic acid 6%. Spots visualized under UV light show violet color and gave blue color with $Fecl_3$ with R_f = 0.53 and 0.56 respectively. The compound VI is identified as Gallic acid.

The biological experiments showed that methanol extract have potent inhibitory activity (82.1%) against LPS (Lipo-Poly-Saccharide) - induced nitric oxide to extent compared with that of dexamethasone. Butanol showed the least by (27.9%) and chloroform and n-hexane (42.6%) and (65.8%) respectively.

3. Experimental

3.1 Plant Material

The aerial parts of *Wodeytia bifurcata* were collected from the Egyptian Orman garden, Giza, Egypt at June 2011. The plant was identified by Agricultural Engineer Terese Labib, El Orman Botanical Garden.

3.2 Extraction and isolation

The air dried powdered leaves and stems of *Wodeytia bifurcata* (1 kg) were macerated in 3 L 80% aqueous methanol with occasional stirring at room temperature for three days. The methanolic extract was concentrated and dried under vacuum. The dried residue (85 g) was suspended with H_2O and extracted with butanol. The butanol extract was separated and evaporated to dryness under reduced pressure to yield a semisolid residue (Fraction I, 28 g). The aqueous liquor was evaporated till dryness under vacuum to yield a residue (Fraction II, 35 g).

The fraction I subjected to column silica gel achieved with gradient elution using chloroform: methanol (100% $CHCl_3$ to 100% MeOH) to yield (fraction I-a, 85:15 chloroform: methanol) and (fraction I-b, 80:20 chloroform: methanol) which are subjected to further purification by silica gel to yield compound I and compound II subjeted to more purification with preparative-TLC silica gel G F_{245} eluate with $CHCl_3$: MeOH: H_2O (13:7:2).

The fraction II subjected to column silica gel achieved with gradient elution using chloroform: methanol (100% $CHCl_3$ to 100% MeOH) to yield (fraction II-c, 70:30 chloroform: methanol) and (fraction II-d, 40:60 chloroform: methanol) which are major fraction which is subjected to further purification by silica gel column to yield fractions which were further purified by rechromatography on sephadex LH-20 column using MeOH, MeOH/H2O and H2O as eluents to yield compound III , compound IV, compound V and compound VI.

Compound (I)

^{1}H NMR ($CDCl_3$, 300 MHZ) at δ; 0.68, 0.78, 0.80, 086, 0.90, 0.93, 1.05, 1.18 (each 3H, s, C-25, C-23, C-30, C-29, C-24, C-26, C-27, C-28), 3.1(dd, *J=7.2, 3.1* Hz, H-3) 5.7(d, *J=7.2,* Hz, H-12).

^{13}C NMR ($CDCl_3$, 300 MHZ): at δ ; 15.12 (C-24), 15.12 (C-25),16.82 (C-26), 18.77 (C-6), 20.53 (C-11), 23.15 (C-30), 23.44 (C-27), 24.06 (C-16), 25.69 (C-15), 26.54 (C-2), 27.48 (C-23), 28.43 (C-28), 30.12 (C-20), 31.42 (C-7), 33.21 (C-17), 33.78 (C-29), 34.42 (C-21), 35.25 (C-10), 35.94 (C-22), 37.25 (C-4), 37.40 (C-1), 39.32 (C-8), 41.42 (C-14), 46.12 (C-9), 48.42 (C-19), 48.70 (C-18),55.12 (C-5), 79.32 (C-3), 122.91 (C-12), 145.66 (C-13). EI-MS spectrum: showed the molecular ion at *m/z* 426 (14%) [M+] corresponding to the formula $C_{30}H_{50}O$, together with fragments *m/z* 411(5%), 275 (3%), *m/z* 257 *(2%),* 218 (95%), 203 *(46%),* 189 (33%), 175 (*17%),* 161 (3%).

Compound (II)

^{1}H NMR (CDCl3, 300 MHZ) at δ; 0.77, 086, 0.88, 0.90, 0.92, 0.96, 1.11 (each 3H,s), 3.64 (1H, dd, *J=5.2* Hz,H-3) 4.41 (1H,s,H-29a), 4.67 (1H,s,H-29b) showed seven tertiary methyl singlets and one secondary hydroxyl group. It also showed olefinic protons at δ 4.41 and 4.67. ^{13}C NMR (CDCl3,300MHZ): at δ; 14.36 (C-27), 15.73 (C-24), 16.77 (C-26) , 17.62 (C-25), 18.42 (C-28), 19.12 (C-6), 20.53 (C-30), 21.33 (C-11), 25.55 (C-2), 26.63 (C-12), 27.21 (C-15), 28.43 (C-23), 30.48 (C-21), 34.33 (C-7), 35.32 (C-16), 37.66 (C-10), 38.42 (C-1), 39.88 (C-4), 40.22 (C-13), 41.82 (C-22), 42.12 (C-8), 43.42 (C-14), 44.22 (C-17), 49.56 (C-19), 49.84 (C-18), 50.12 (C-9), 54.29 (C-5), 77.40 (C-3), 110.89 (C-29), 150.63 (C-20).

Compound (III)

UV spectral data at λ_{max} (nm): MeOH; 272, 331, NaOMe; 268, 332, 400, $AlCl_3$; 279, 306, 351, 387, $AlCl_3$/HCl; 279, 304, 346, 386, NaOAc; 281, 306, 387, NaOAc/H_3BO_3; 268, 342. ^{1}H NMR (DMSO-d_6, 270 MHZ) at δ; 7.6 (2H, d, *J*= 5.7 Hz, H-2′, H-6′), 7.0 (2H, d, *J*= 5.7 Hz, H-3′, H-5′), 6.4 (1H, s, H-3), 6.2 (1H, d, *J*= 1.5 Hz, H-8), 6.0 (1H, d, *J*= 1.5 Hz, H-6). The EI-MS spectrum showed the molecular ion peak as the base peak (M^+) at m/z 270 (95%) corresponding to molecular formula $C_{15}H_{10}O_5$ together with fragments at m/z 152 (43%), 118 (48%), 121 (20%) and 149 (26%).

Compound (IV)

UV spectral data at λ_{max} (nm): MeOH; 265, 294, 322, 367, NaOMe; 277, 294, 315, 420, 420, $AlCl_3$; 268, 303, 350, 424, $AlCl_3$/HCl; 269, 303, 348, 424, NaOAc; 276, 302, 387, NaOAc/H_3BO_3; 266, 248, 320, 372. ^{1}H-NMR (DMSO-d_6, 270 MHZ) at δ; 7.94 (2H, d, *J*= 5.4 Hz, H-2′, H-6′), 6.9 (2H, d, *J*= 5.4 Hz, H-3′, H-5′), 6.5 (1H, d, *J*= 1.2 Hz, H-8), 6.2 H, d, *J*= 1.2 Hz, H-6). The EI-MS spectrum showed the molecular ion peak as the base peak (M^+) at m/z 287 (84%) corresponding to molecular formula $C_{15}H_{11}O_6$ together with fragments at m/z 269, 259 (12%), 258 (15%), 153 (42%) and 133 (48%)

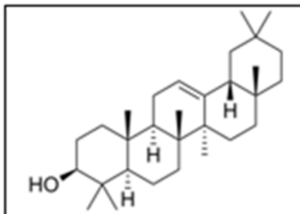
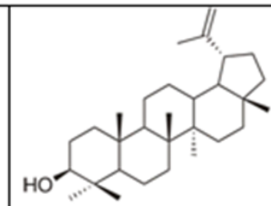
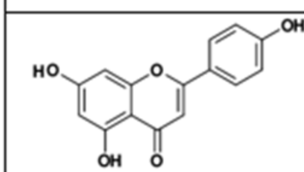
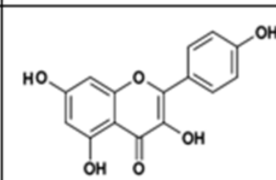
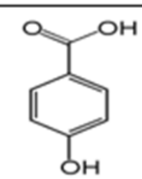
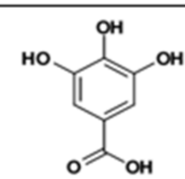
Compound (V)

Compound is colorless crystals that gave violet color with ferric chloride solution with melting point 215-216 º. The ^{1}H-NMR (CD3OD, 300 MHz) showed signsals at δ; 7.58 (2H, d, *J* = 2.1 Hz, H-2, 6), 6.92 (2H, d, *J* = 1.2 Hz, H-3, 5).

Compound (VI)

Compound VI is identified by co-chromatography with authentic using paper chromatography with solvent system butanol: acetic acid: water (5:4:1) and acetic acid 6%. Spots visualized under UV light show violet color and gave blue color with $Fecl_3$ with R_f = 0.53 and 0.56 respectively.

Table 1: of isolated compounds

 **β-Amyrin**	 **Lupeol**
Compound (I)	Compound (II)
 **Apigenin**	 **Kaempferol**
Compound (III)	Compound (IV)
 **4-Hydroxybenzoic acid**	 **Gallic acid**
Compound (V)	Compound (VI)

4. Biological activities

4.1 Evaluation of anti-inflammatory activity

The accumulation of nitrite, an indicator of NO synthesis was measured by Griess reagent (Gerhauser, *et al.,* 2003). A standard curve was plotted using serial concentration of sodium nitrite (Fig. 1). The nitrite content was normalized to cellular protein content as measured by bicinchoninic acid assay [12, 32]. The NO inhibition percentage was calculated by submitting the nitrite content of the cell supernatant of cultures treated with DMSO (control) LPS or LPS/treated compounds (fig. 2).

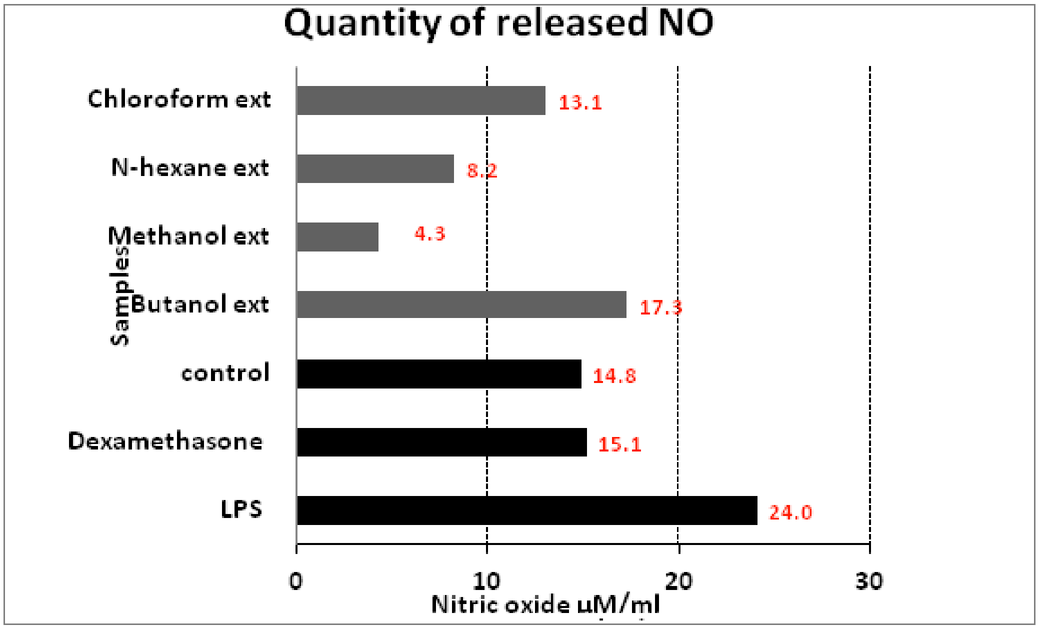


Fig 1: The level of Nitric oxide in RAW 264.7 cells supermatant after the treatment with the samples (50 µg/ml) compared with LPS-treated cell (200 µg/ml), as measured by Griess assay.

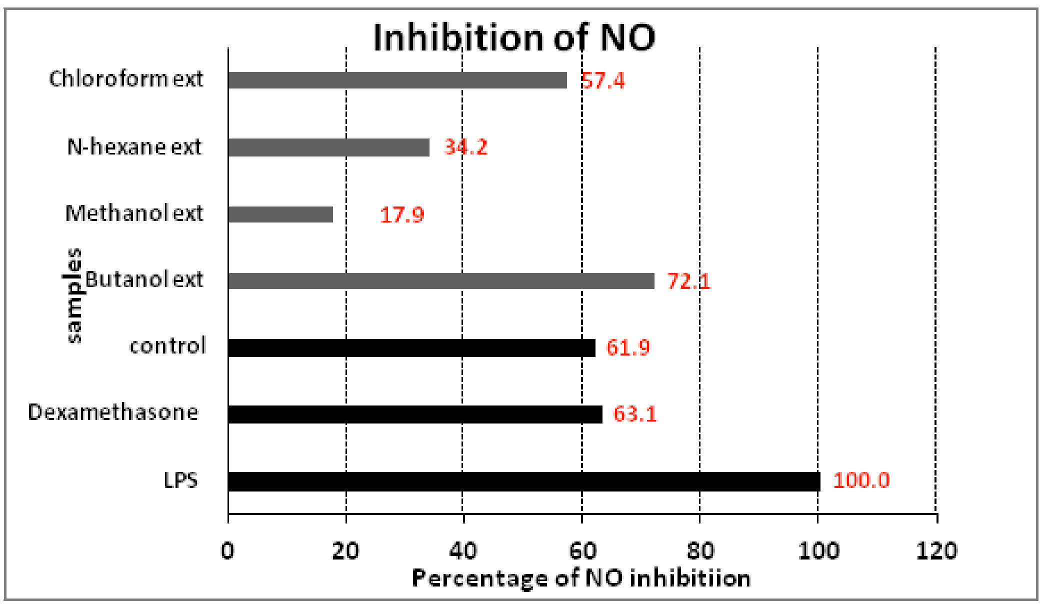


Fig 2: The percentage of inhibition of Nitric oxide in LPS0stimulated RAW 264.7 cells supernatant after the treatment with the samples (50 µg/ml) compared to LPS treated cells, as measured by Griess assay.

4.2 Evaluation of antioxidant activity

DPPH is a deep violet radical due to its unpaired electron, which in the presence of antioxidant radical scavenger (donate an electron to DPPH) decolorizes to the pale yellow non-radical form [30]. The change in the colorization and subsequent fall in absorbance were monitored spectrophotometrically at 520 nm. A standard calibration curve was plotted using serial dilutions of ascorbic acid in concentrations ranging from 0 to 100 µg/ml in distilled water (fig. 3). The half maximal scavenging capacity (SC_{50}) values for each tested sample and ascorbic acid were estimated via dose curve and calculated from curve equation (fig. 4).

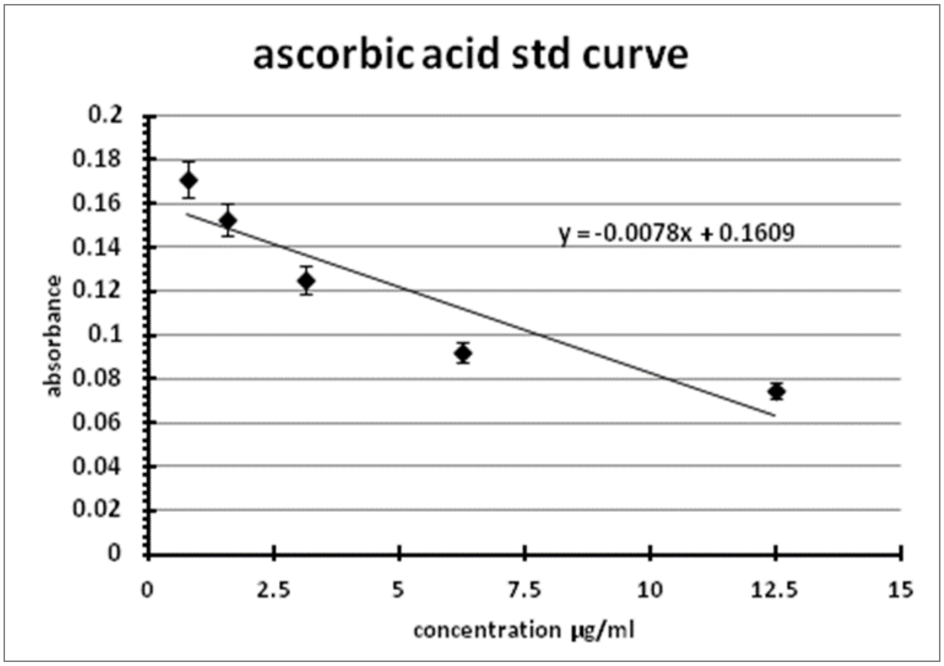


Fig 3: Antioxidant activity against DPPH radicals

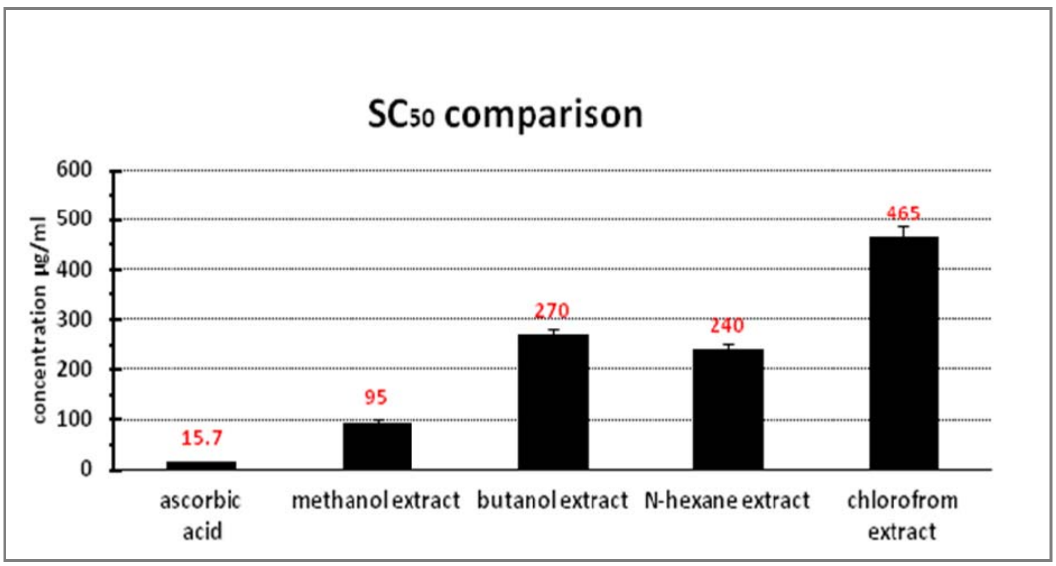


Fig 4: Antioxidant activity against DPPH radicals

4.3 Evaluation of cytotoxic activity

Cytotoxicity of the tested extract was measured against Hep-G_2 cells using MTT cell viability assay, which is based on ability of active mitochondrial dehydrogenase enzymes of living cells to cleave the tetrazolium rings of the yellow MTT and form dark blue insoluble formazan crystals which are largely impermeable to cell membranes, results in its accumulation within healthy cells (result in the liberation of crystals, which are then solubilized). The number of viable cells is directly proportional to the level formazan dark blue color. The extent of reduction of MTT was quantified by measuring the absorbance at 570 nm [14]. The percentage of relative viability was calculated using the following equation (absorbance of treated cells / absorbance of control cell)*100. Then the half maximal inhibitory concentration (IC_{50}) was calculated from the equation of dose response curve. Results showed that butanol extracts had weak cytotoxicity activity against Hep-G_2 cells, IC50 = 568.5 µg/ml (fig. 5), (Olajire and azeez, 2011).

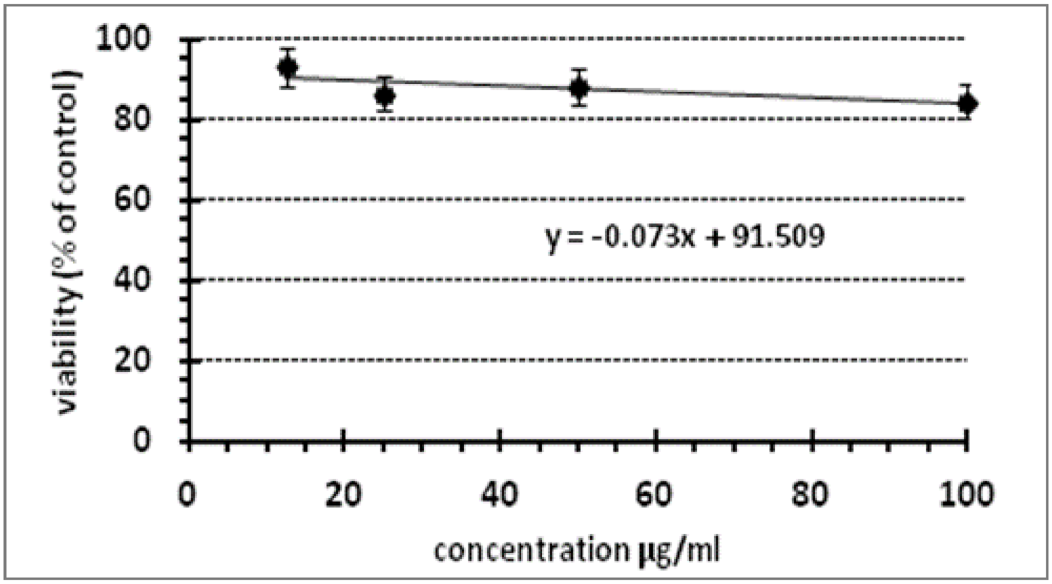


Fig 5: Cytotoxic effect of different samples against Hep-G2 cells using MTT assay (n=4), data expressed as the mean value of cell viability (% of control) ± S.E.

5. References

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