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Phytochemical Investigation and Assessment of Antioxidant and Antimicrobial Activities of *Phagnalon barbeyanum* Aerial Parts

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ABSTRACT

Objectives: The present study aimed to investigate and isolate different phytochemical constituents and assess the antioxidant and antimicrobial activities of the total methanol extract and different fractions of *Phagnalon barbeyanum* aerial parts. **Methods:** Phytochemical screening of *Phagnalon barbeyanum* aerial parts was performed using specific test for each class of compounds. Different chromatographic techniques were used to isolate and purify compounds and their structures were elucidated by using different spectral techniques (^{1}H NMR and ^{13}C NMR). The antioxidant activity was evaluated by DPPH radical scavenging assay and antimicrobial activity was done by standard agar well diffusion assay. **Results:** Phytochemical investigation of *Phagnalon barbeyanum* aerial parts was done for the presence of carbohydrates and or glycosides, sterols and or triterpene, flavonoids, tannins and saponins and revealed the absence of alkaloids and anthraquinones. Compounds; *β*-sitosterol **(1)**, apigenin **(2)** and *β*-sitosterol-3-O-*β*-D- glucopyranoside **(3)** were isolated from methylene chloride and ethyl acetate fractions. The ethyl acetate fraction showed highest antioxidant activity followed by *n*-butanol and methylene chloride fractions. The total methanol extract and *n*-hexane fractions showed lowest antioxidant activity. The ethyl acetate fraction and total methanol extract showed moderate activity against *Staphylococcus aureus* and Vancomycin resistant *Staphylococcus aureus*. **Conclusion:** The aerial parts of *Phagnalon barbeyanum* is a rich source of different classes of active constituents as phenolics. The total methanol extract of *Phagnalon barbeyanum* aerial parts and some of its fractionated concentrates could be considered as antioxidant and antimicrobial agents.

Keywords: *Antimicrobial; Antioxidant; DPPH assay; Phagnalon barbeyanum; Phytochemical screening*

INTRODUCTION

Phagnalon is a small genus belonging to family Asteraceae. It is represented in Egypt by only five species. Folk medicinal use of these plants in various diseases was reported by some population in north Africa[1]. *Phagnalon saxatile* was reported for its use as carminative, analgesic, hypocholestermic[2] and for having potential antioxidant activity[3]. Phytochemical investigations on *Phagnalon* species are mainly devoted to the study of *Phagnalon rupestre* species, where it was reported having terpenoids, flavonoids, hydroquinone glycosides, and caffeoylquinic acid derivatives[3]. *Phagnalon barbeyanum*, which is known locally as Sanuf or Taam Elgamal is an herb growing in Egypt and widely used

among Bedouins living in Sinai for the relief of renal colic. A benzofuran derivative, 2-[1'-carbomethoxyvinyl-(2')]-3-acetoxy-5-[(1" angeloyloxy ethyl)] 2,3- dihydrobenzofuran, alongside with friedelinol and damaradienyl acetate have been previously isolated from the root of these species, in addition to apigenin and luteolin were isolated from the aerial parts of the same plant[1].

Polyphenols are widespread constituents in the majority of the plants and are the most abundant secondary metabolites of plant foods, with more than 8,000 phenolic structures currently known[4]. Generally polyphenolic compounds, including phenolic acids, flavonoids and tannins have shown potent antioxidant activity for their radical scavenging activity and their ability to form complex with heavy metal ions[5, 6]. The Phytochemical investigation of genus *Phagnalon* is very limited, thus it was interested for the authors to investigate its secondary metabolites as well as assess its antioxidant and antimicrobial activities.

MATERIAL AND METHODS

Plant materials

The aerial parts of *Phagnalon barbeyanum* were collected in April 2017 from Saint Catherin, South Siniai, Egypt. The plant was identified and authenticated by. Dr. Ahmed Fareed, Lecturer of Botany and Plant Taxonomy, Faculty of Science, Assuit University, Egypt. A voucher specimen (PBA-1) was kept in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assuit, Egypt.

Instruments

^{1}H NMR and ^{13}C NMR spectra were measured on Bruker AMX- 600 and 400 Spectrometers with standard pulse sequences operating at 600 and 400 MHz in ^{1}H NMR and 150 and 100 MHz in ^{13}C NMR respectively, (Germany). Chemical shifts are given in δ values (*ppm*) using tetramethylsilane as the internal standard. Column chromatography was carried out on glass columns of different sizes using silica gel (70-230 and 230-400 mesh, E-Merck, Germany). Spectroscopic data were acquired using the Schimadzu 1601, UV/Visible Spectrophotometer (USA). Disposable cuvettes (1cm × l cm x 4.5cm) were used for visible absorbance measurements. Electric balance (Sartorius cpa 3245, Germany), automatic pipettes (AXYPet, Poland), autoclave (ALP, Japan), incubator (Thermo, Germany), laminar air flow cabinet (Nuarine, France), Vortex (Maxi mix II, Canada) and Spectrophotometer (Jenway 6304, UK).

Chemicals

Molish's and Dragendorff's reagents were prepared fresh[7,8]. Ammonia, ferric chloride solution, conc. sulphuric acid, acetic anhydride, *n*-hexane, methylene chloride, ethyl acetate, methanol and *n*-butanol were obtained from El-Nasr Pharmaceutical and Chemical Co. (All solvent used are of analytical grade). DPPH (2, 2-Diphenyl-1-picryl hydrazyl), ascorbic acid (were obtained from Sigma-Aldrich Chemicals Co, Germany). Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany), McFarland standard (Sigma Aldrich, Germany), normal physiological saline (Haydlina, Egypt), Mueller-Hinton agar (Oxoid, UK), Amoxicillin (amx) as antibacterial standard (E.P.I.Co, Egypt) and Cyclohexamide (CHX) as anti yeast standard (Bio basic ink, Canada).

Solvent systems were used for TLC:

I. Methylene chloride-methanol (95: 5 *v/v*)
II. Methylene chloride-methanol (90:10 *v/v*)
III. Methylene chloride-methanol (85:15 *v/v*)

Microorganisms

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922) *Pseudomonase aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231) and clinical isolate of *VRSA* (Vancomycin resistant *Staphylococcus aureus*), which were used for assessment of antimicrobial activity were obtained from Egyptian Company for Production of Vaccines, Sera and Drugs (VACSERA).

Methods

Extraction and fractionation

The air-dried aerial parts (360 g) of *Phagnalon barbeyanum* was extracted by maceration in aqueous methanol (70%) till complete exhaustion (three times each 3 L, overnight). The collected methanol extracts were concentrated under reduced pressure to give a dark brown syrupy residue (40 g). A part of the methanol concentrate (38 g) was subjected to successive liquid-liquid fractionation with *n*-hexane, methylene chloride, ethyl acetate and finally with *n*-butanol till complete exhaustion for each fraction to give *n*-hexane (2 g), methylene chloride (1.5 g), ethyl acetate (8 g) and *n*-butanol. (21 g) concentrates.

Phytochemical screening

1. Carbohydrates and / or glycoside: The alcoholic solution of total extract of *Phagnalon barbeyanum* aerial parts was combined with a small amount of Molisch's reagent (α-naphthol dissolved in ethanol) in test tube. After mixing, 1ml of concentrated sulfuric acid was slowly added down the sides of the sloping test-tube without mixing to form a layer, a purple ring at the interface between the aqueous and organic layer indicating the presence of carbohydrates and / or glycoside[7].

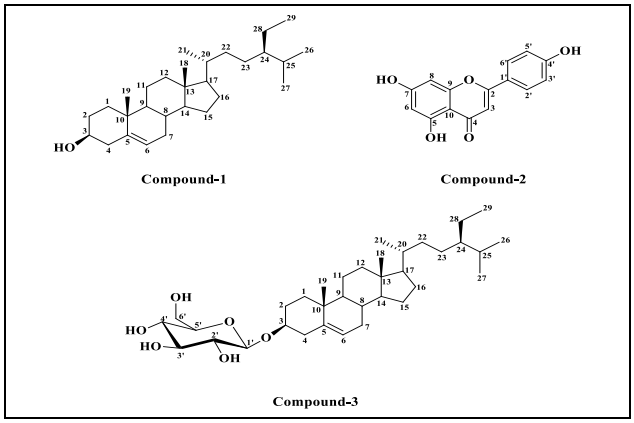

Figure 1. Structures of the isolated compounds from *Phagnalon barbeyanum* aerial parts

2. Sterols and/or triterpenes: The chloroformic solution of total extract of *Phagnalon barbeyanum* aerial parts was treated with 2 ml of acetic anhydride in presence of concentrated sulfuric acid; a violet ring is formed indicating the presence of sterols and or triterpens[7].

3. Flavonoids: The total extract of *Phagnalon barbeyanum* aerial parts was mixed with 2 ml of 2% NaOH solution, intensive yellow color is formed indicating the presence of flavonoids[7].

4. Tannins: The total extract of *Phagnalon barbeyanum* aerial parts aerial parts was mixed with 2 ml of 2% solution of $FeCl_3$, black or blue-green color is formed indicating the presence of tannins[7].

5. Alkaloids: The chloroformic solution of total extract of *Phagnalon barbeyanum* aerial parts was evaporated till dryness and the extract was dissolved in 2 ml of HCl, the formation of very faint brown precipitate with Wagner's reagent indicating the presence of alkaloids and / or nitrogen bases[8].

6. Anthraquinone: The alcoholic solution of the total extract of *Phagnalon barbeyanum* aerial parts was boiled with 1ml dilute solution of H_2 So_4 then shacked with chloroform and finally the chloroformic layer was mixed with dilute solution of ammonia, a rose red color indicating the presence of anthraquinone[8, 9].

7. Saponins: The alcoholic solution of total extract of *Phagnalon barbeyanum* aerial parts was treated with equal volume of suspension of RBCs in normal saline and shacked gently, a clear red solution indicating the presence of saponins[10].

Isolation of compounds

A part of the methylene chloride soluble fraction (1.2 g) was chromatographed on silica gel column chromatography (45 g), eluted with *n*-hexane (100%), *n*-hexane-methylen chloride gradient elution, methylene chloride (100%), methylene chloride-methanol gradient elution and finally eluted with MeOH (100%). Fractions of 100 ml, each were collected. Similar fractions were grouped and pooled together, concentrated under reduced pressure to give six subfractions labeled (MC-Pb1) to (MC-Pb6). Subfraction (MC-Pb2) was purified on silica gel column chromatography and eluted with *n*-hexane followed by *n*-hexane- ethyl acetate gradient elution from (99:1) to (80:20), the fractions eluted with *n*-hexane- ethyl acetate (85:15) afforded compound **1** (20 mg). Subfraction (MC-Pb5) was purified on silica gel column chromatography and eluted with methylene chloride- methanol gradient elution from (98:2) to (85:15), the fractions eluted with methylene chloride-methanol (90:10) afforded compound **2** (8 mg).

A part of the ethyl acetate-soluble fraction (7 g) was chromatographed over silica gel column chromatography (210 g), eluted with *n*-hexane (100%), *n*-hexane-ethyl acetate gradient elution from (99:1) to (75:25), ethyl acetate (100%) and finally eluted with methanol (100%). Fractions 100 ml, each were collected. Similar fractions were grouped and pooled together, concentrated under reduced pressure to give 10 subfractions labeled (EA-Pb1) to (EA-Pb10). Subfraction (EA-Pb9) was purified on silica gel column chromatography and eluted with methylene chloride-methanol gradient elution from (99:1) to (80:20), the fractions eluted with methylene chloride- methanol (85:15) afforded compound **3** (8 mg).

Compound 1: Obtained as transparent crystalline needles (methanol), m.p. 134-136°C, R_f = 0.56 (I). It is soluble in chloroform and methylene chloride but insoluble in methanol and water. It gave a red color with Salkowiski test and violet ring with Libermann-Burchard's test[12]. ^{1}H NMR ($CDCl_3$, 600 MHz): δ_H 3.52 (1H, *m*, H-3), 2.24 (2H, *m*, H-4), 5.34 (1H, *dd*, *J*= 1.8 & 4.8 Hz, H-6), 0.65 (3H, *s*, H-18), 1.00 (3H, *s*, H-19), 0.85 (3H, *d*, *J*= 6 Hz, H-21), 0.91 (3H, *d*, *J*= 8.4 Hz, H-26), 0.93 (3H, *d*, *J*= 7.2 Hz, H-27) and 0.78 (3H, *dd*, *J*= 4.2 & 6.6 Hz, H-29). ^{13}C-NMR ($CDCl_3$, 150 MHz): δc 37.35 (C-1), 31.99 (C-2), 71.88 (C-3), 39.70 (C-4), 140.84 (C-5), 121.80 (C-6), 31.99 (C-7), 31.55 (C-8), 50.22 (C-9), 34.81 (C-10), 21.39 (C-11), 42.38 (C-12), 42.86 (C-13), 56.72 (C-14), 22.28 (C-15), 28.33 (C-16), 56.09 (C-17), 11.95 (C-18), 19.50 (C-19), 36.28 (C-20), 18.99 (C-21), 33.81 (C-22), 25.89 (C-23), 50.22 (C-24), 28.33 (C-25), 18.99 (C-26), 19.50 (C-27), 24.39 (C-28) and 12.00 (C-29).

Compound 2: Obtained as yellow amorphous powder (methanol), m.p. 346-348°C. R_f = 0.50 (II). It is insoluble in *n*-hexane and ether, but soluble in methanol and ethanol. It gave yellow color with dilute solution of sodium hydroxide[13]. ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 6.77 (1H, *s*, H-3), 6.17 (1H, *d*, *J*= 1.7, H-6), 6.46

(1H, *d*, *J*= 1.7, H-8), 7.91 (1H, *d*, *J*= 8.7 Hz, H-2' & H-6') and 6.91 (1H, *d*, *J*= 8.7 Hz, H-3' & H-5'). ^{13}C-NMR (DMSO-d_6, 100 MHz): *δc* 164.10 (C-2), 103.25 (C-3), 182.11 (C-4), 161.90 (C-5), 99.47 (C-6), 164.48 (C-7), 94.51 (C-8), 157.83 (C-9), 103.99 (C-10), 121.60 (C-1'), 128.90 (C-2' & C-6'), 116.44 (C-3' & C-5') and 161.70 (C-4').

Compound 3: Obtained as white granular powder (methanol), m.p. 276 – 278ºC. R_f = 0.54 (III). It is insoluble in *n*-hexane and chloroform, sparingly soluble in cold ethanol and methanol, soluble in hot methanol and ethanol. It gave red color with Salkowski`s test and violet ring with Liebermann-Burchard`s test[12]. ^{1}H NMR (DMSO-d_6, 600 MHz): δ_H 0.99, 1.78 (1H, *m* & 1H, *m*, H-1), 1.47 (2H, *m*, H-2), 3.45 (1H, *m*, H-3), 2.12, 2.36 (1H, *m* & 1H, *m*, H-4), 5.32 (1H, *m*, H-6), 1.91 (2H, *m*, H-7), 1.38 (1H, *m*, H-8), 0.87 (1H, *m*, H-9), 1.38, 1.47 (1H, *m* & 1H, *m*, H-11), 1.13, 1.94 (1H, *m* & 1H, *m*, H-12), 1.08 (1H, *m*, H-14), 1.18, 1.24 (1H, *m* & 1H, *m*, H-15), 1.14 (2H, *m*, H-16), 0.98 (1H, *m*, H-17), 0.64 (3H, *s*, H-18), 0.95 (3H, *s*, H-19), 1.31 (1H, *m*, H-20), 0.89 (3H, *d*, *J*= 6.6 Hz, H-21), 1.30, 1.00 (1H, *m* & 1H, *m*, H-22), 1.53 (2H, *m*, H-23), 0.90 (1H, *m*, H-24), 1.62 (1H, *m*, H-25), 0.81 (3H, *d*, *J*= 6.6 Hz, H-26), 0.87 (3H, *d*, *J*= 7.2 Hz, H-27), 1.80, 1.47 (1H, *m* & 1H,*m*, H-28), 0.80 (3H, *t*, *J*= 7.2 Hz, H-29), 4.2 (1H, *d*, *J*= 7.8 Hz, H-1'), 2.88 (1H, *m*, H-2'), 3.11 (1H, *m*, H-3'), 3.00 (1H, *m*, H-4'), 3.05 (1H, *m*, H-5') and 3.40, 3.64 (1H, *dd* & 1H, *dd*, *J*= 11.4, 1.2 & 11.4, 3.0 Hz, H-6'). ^{13}C-NMR (DMSO-d_6, 150 MHz): *δc* 36.81 (C-1), 28.76 (C-2), 76.86 (C-3), 38.28 (C-4), 140.41 (C-5), 121.20 (C-6), 31.40 (C-7), 31.35 (C-8), 49.58 (C-9), 36.20 (C-10), 20.58 (C-11), 40.03 (C-12), 41.84 (C-13), 55.40 (C-14), 22.58 (C-15), 25.39 (C-16), 56.15 (C-17), 11.77 (C-18), 19.09 (C-19), 35.47 (C-20), 18.60 (C-21), 33.32 (C-22), 23.85 (C-23), 45.11 (C-24), 27.78 (C-25), 11.66 (C-26), 18.92 (C-27), 29.25 (C-28), 19.71 (C-29), 100.75 (C-1'), 73.44 (C-2'), 76.86 (C-3'), 70.06 (C-4'), 76.74 (C-5') and 61.06 (C-6').

Antioxidant activity

Antioxidant activity was determined by DPPH radical scavenging method [13].DPPH 10 × 10^{-5} M solution was prepared by dissolving 40 mg of DPPH in 1000 ml ethanol. Ethanolic solution (0.2 ml) of each different fractions of *Phagnalon barbeyanum* aerial parts of different concentrations (0.0625, 0.125, 0.25, 0.5, 1mg/ml) were mixed with 2 ml of ethanolic solution of DPPH (0.1mM). Similarly; 0.2 ml ethanolic solution of ascorbic acid of different concentrations (0.625, 0.125, 0.25, 0.5, 1mg/ml) were mixed with 2 ml of DPPH solution. A mixture of 0.2 ml of ethanol and 2 ml of ethanolic solution of DPPH (0.1 mM) served as control. Solutions were shaked and left for 30 min at room temperature. Absorbance was measured at λ_{max} 517 nm, using UV-Visible Spectrophotometer.

The experiments were carried out in triplicate manner using ascorbic acid as a reference standard and antioxidant activity was expressed as percentage of DPPH radical scavenging relative to control using the following equation[14].

$$\% \text{ DPPH radical scavinging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \text{X } 100$$

The scavenging effect (antioxidant activity) of each tested sample was expressed as SC_{50}, which is the concentration of the extract required for 50% scavenging of DPPH radicals compared with that of standard ascorbic acid. The decrease in the absorbance indicates an increase in DPPH radical scavenging activity.

Antimicrobial activity

Total methanol extract, methylene chloride, ethyl acetate and *n*-butanol fractions dissolved in DMSO and adjusted at concentration 50 mg/ml[15]. Overnight cultures of standard pathogenic strains were prepared in nutrient agar at 37ºC. Pure colonies of overnight plates were picked up and inoculated in 10 ml of 0.9% normal saline, and the turbidity adjusted to be equivalent to 0.5 McFarland standards, then the optical densities (ODs) of cell suspensions were adjusted to obtain 0.45 absorbance units at λ_{max} 650 nm. The adjusted suspensions were used within 15 minutes. Using the standard well diffusion assay, 500 µl from each cell suspension was placed in sterile petri dish and 20- 25 ml of Mueller-Hinton medium were poured into the plates for preparation of seeded media. After solidifying, two wells were made in agar using 0.7 cm cork porer, and each one was inoculated with 100 µl of each tested extract. The effect of DMSO against all used pathogenic strains was tested as a negative control and the effect of standard antibiotics (amoxicillin and cyclohexamide, at concentrations 50 mg/ml), also tested as a positive control. All plates were incubated at 37ºC for 24 hours. After incubation period, the diameters of inhibition zones around the wells were measured to assess the antimicrobial activity quantitatively[16].

The experiments were carried out in triplicate manner using amoxicillin as a reference antibacterial standard and cyclohexamide as a reference anti yeast standard.

Statistical Analysis

Experimental results are expressed as mean ± standard error. Results were statistically analyzed using analysis of variance (one-way ANOVA) followed by

Tukey's t test for comparison between different groups. SPSS 20 version was used for the statistical analysis.

RESULTS AND DISCUSSION

Phytochemical screening

The total methanol extract of *Phagnalon barbeyanum* aerial parts were subjected to preliminary phytochemical screening for the following constituents: carbohydrates and/or glycosides, sterols and/or triterpenes, flavonoids, tannins, alkaloids and/or nitrogenous bases, saponins, and anthraquinone. The results of phytochemical screening are compiled in the **Table 1**.

Identification of isolated compounds

Compound 1: Chromatographic and chemical properties of compound **1** suggested its steroidal or triterpenoidal nature[11]. ^{1}H NMR data (**Table 2**) showed six methyl group signals, two of them are tertiary at δ_H 0.65 and 1.00 assigned to H-18 &H-19 respectively, three secondary ones at δ_H 0.85, 0.91 and 0.93, which assigned to H-21, H-26 and H-27 respectively and primary methyl group at δ_H 0.78 which assigned to H-29. In addition to presence of olefinic proton at δ_H 5.34 which assigned to H-6. Furthermore multiplet signal at δ_H 3.52 which assigned to H-3. The data obtained from ^{1}H NMR suggested the compound **1** is *β*-sitosterol. These findings were confirmed by ^{13}C-NMR data (**Table 2**), which revealed the presence of two olefinic carbons signals at δ_C 140.84 assigned to C-5 and δ_C 121.80 assigned to C-6, an oxygenated carbon at δ_C 71.88 assigned to C-3, as well as a cluster of resonances at upfeild shift δ_C 11.95–56.72 assignable to –CH– and –CH2– groups of *β*–sitosterol. From previous mentioned physical, chemical, chromatographic and spectral data (^{1}H-NMR and ^{13}C-NMR) and by comparing with published data[17], in addition to co-chromatography with an authentic sample, it was concluded that the compound **1** is *β*-sitosterol and this is the first report for its isolation from *Phagnalon barbeyanum* aerial parts.

Table 1. Results of phytochemical screening of the aerial parts of *Phagnalon barbeyanum*

Constituents	Results
Carbohydrates and/or glycosides	+
Sterols and/or triterpenes	+
Flavonoids	+
Tannins	+
Alkaloids	-
Saponins	+
Anthraquinone	-

(+) = present, (-) = absent.

Compound 2: Chromatographic and chemical properties of compound **2** indicated its flavonoidal aglycone nature[8]. ^{1}H-NMR data (Table 3) showed a characteristic pattern of flavones[12], represented by two doublets at δ_H 6.46 and 6.17, each (1H, *d*, *J*=1.7 Hz) assigned for meta coupled aromatic protons (H-8 and H-6, respectively). In addition to presence of two sets of *ortho*-coupled aromatic protons at δ_H 7.81 and 6.87, each (2H, *d*, *J*=8.7 Hz) assigned to H-2′, 6′ and H-3′, 5′ respectively. Furthermore, a singlet proton at δ_H 6.77 assigned to H-3. ^{13}C-NMR data (**Table 3**) showed the presence of aromatic signals representing 15 carbons. The spectrum also showed the presence of two oxygenated carbons of ring A at δ_C 163.6 and 161.2, which were assigned to C-7 and C-5 respectively. In addition to another oxygenated carbon of ring B at δ_C 161.0 assigned to C-4′. ^{13}C-NMR spectral data of compound **2** were in good agreement with those reported for apigenin[18]. From the previously mentioned physical, chemical, chromatographic and spectral data (^{1}H NMR and ^{13}C NMR) and reviewing literatures for apigenin in addition to co-chromatography with an authentic sample, compound **2** was concluded to be apigenin[18], and this is the second report for its isolation from *Phagnalon barbeyanum* aerial parts.[1]

Table 2. ^{1}H-NMR and ^{13}C NMR spectral data of compound 1 ($CDCl_3$, 600 and 150 MHz, respectively)

No	δ_H	δ_C	No	δ_H	δ_C
1	-	37.35	16	-	28.33
2	-	31.99	17	-	56.09
3	3.52 (1H, *m*)	71.88	18	0.65 (3H, *s*)	11.95
4	2.24 (2H, *m*)	39.70	19	1.00 (3H, *s*)	19.50
5	-	140.84	20	-	36.28
6	5.34 (1H, *dd*, *J*=1.8, 4.8)	121.80	21	0.85 (3H, *d*, *J*= 6)	18.99
7	-	31.99	22	-	33.81
8	-	31.55	23	-	25.89
9	-	50.22	24	-	50.22
10	-	34.81	25	-	28.33
11	-	21.39	26	0.91 (3H, *d*, *J*= 8.4)	18.99
12	-	42.38	27	0.93 (3H, *d*, *J*= 7.2)	19.50
13	-	42.86	28	-	24.39
14	-	56.72	29	0.78 (3H, *dd*, *J*= 4.2, 6.6)	12.00
15	-	22.28			

Compound 3: Chromatographic and chemical properties of compound **3** suggested its steroidal or triterpenoidal nature[11]. ^{1}H NMR data (**Table 4**) showed the presence of six methyl groups at δ_H 0.64 (3H, *s*), 0.80 (3H, *t*), 0.81 (3H, *d*), 0.87 (3H, *d*), 0.95 (3H, *s*) and 0.89 (3H, *d*) could be assigned to H-18, 29, 26, 27, 19 and 21 respectively. A doublet proton signal at δ_H 4.2

(1H, *d*, *J*= 7.8) could be assigned to anomeric sugar proton, the large coupling constant indicates its *β* configuration. Moreover a multiplet proton signal at δ_H 3.45 (1H, *m*) could be assigned to H-3 and finally the olefenic proton at δ_H 5.32 (1H, *m*) could be assigned to H-6, suggesting that compound **3** is a *β*-sitosterolglucoside. ^{13}C-NMR data (**Table 4**) has confirmed the presence of *β*-sitosterol nucleus as it showed 29 carbons distributed as six methyls, eleven methylenes, nine methines and three quaternary carbons. The carbon resonances at *δc* 100.74 (C-1′), 73.43 (C-2′), 76. 86 (C-3′), 70.06 (C-4′), 76.73 (C-5′) and 61.06 (C-6′) confirmed the presence of glucose moiety. From the previous mentioned physical, chemical, chromatographic and spectral data, it could be concluded that compound **3** is identified as *β*-sitosterol-3-O-*β*-D-glucopyranoside; this was confirmed by authentication with authentic reference material.

Table 3. ^{1}H-NMR and ^{13}C NMR spectral data of compound 2 (DMSO, d_6, 400 and 100 MHz respectively)

No	δ_H	δ_C	No	δ_H	δ_C
2	-	164.10	9	-	157.83
3	6,77 (1H, *s*)	103.25	10	-	103.99
4	-	182.11	1″	-	121.60
5	-	161.90	2′, 6′	7.91 (1H, *d*, *J*= 8.7)	128.90
6	6.17 (1H, *d*, *J*= 1.7)	99.47	3′, 5′	6.91 (1H, *d*, *J*= 8.7)	116.44
7	-	164.48	4′	-	161.70
8	6.46 (1H, *d*, *J*= 1.7)	94.51			

Antioxidant activity

The obtained results (**Table 5**, **Figure 2**) indicated that, the ethyl acetate fraction showed the highest antioxidant activity followed by *n*-butanol and methylene chloride fractions. The total methanol extract and *n*-hexane fractions showed the lowest antioxidant activity. The highest antioxidant activity of ethyl acetate fraction, *n*-butanol and methylene chloride fractions could be attributed to the presence of polyphenolic compounds in these fractions. The major polyphenolics responsible for free radical scavenging activity are flavonoids. The activities are closely related to the chemical structure of their aglycone moieties and also the position of hydroxyl groups. The antioxidant activity of the aglycone is more potent than their corresponding glycosides that are in good agreement with the published data[19-21]. The presence of ortho-dihydroxylation of the B-ring of the flavonoid molecule, C2-C3 double bond and 4-oxo group of the ring C in addition to the presence of both 3-and 5-hydroxyl moiety of the rings C and A, play an important role in radical scavenging activity of the flavonoids[19,21,22].

Table 4. ^{1}H-NMR and ^{13}C NMR spectral data of compound 3 (DMSO-d_6, 600 and 150 MHz respectively)

No	δ_H	δ_C	No	δ_H	δ_C
1	0.99, 1.78 (1H, *m* & 1H,*m*)	36.81	19	0.95 (3H, *s*)	19.09
2	1.47 (2H,*m*)	28.67	20	1.31 (1H, *m*)	35.47
3	3.45 (1H, *m*)	76.86	21	0.89 (3H, *d*, *J*= 6.6)	18.60
4	2.12, 2.36 (1H, *m* & 1H,*m*)	38.28	22	1.30, 1.00 (1H, *m* & 1H,*m*)	33.32
5	-	140.41	23	1.53 (2H,*m*)	23.85
6	5.32 (1H, *m*)	121.20	24	0.90 (1H, *m*)	45.11
7	1.91 (2H,*m*)	31.40	25	1.62 (1H, *m*)	27.78
8	1.38 (1H, *m*)	31.35	26	0.81 (3H, *d*, *J*= 6.6)	11.66
9	0.87 (1H, *m*)	49.58	27	0.87 (3H, *d*, *J*= 7.2)	18.92
10	-	36.20	28	1.80, 1.47(1H, *m* & 1H,*m*)	29.25
11	1.38, 1.47(1H, *m* & 1H,*m*)	20.58	29	0.80 (3H, *t*, *J*= 7.2)	19.71
12	1.13, 1.9 (1H, *m* & 1H,*m*)	40.03	1′	4.2 (1H, *d*, *J*= 7.8)	100.75
13	-	41.84	2′	2.88 (1H, *m*)	73.44
14	1.08 (1H, *m*)	55.40	3′	3.11 (1H, *m*)	76.86
15	1.18, 1.24 (1H, *m* & 1H,*m*)	22.58	4′	3.00 (1H, *m*)	70.06
16	1.14 (2H,*m*)	25.39	5′	3.05 (1H, *m*)	76.74
17	0.98 (1H, *m*)	56.15	6′	3.40, 3.64 (1H, *dd* & 1H, *dd*, *J*=11.4, 1.2 & 11.4, 3.0)	61.06
18	0.64 (3H, *s*)	11.77			

Antimicrobial activity

The obtained results (**Table 6**) showed that the total methanol extract and ethyl acetate fraction of *Phagnalon barbeyanum* aerial parts possess moderate antimicrobial activities against gram positive *Staphylococcus aureus* and *VRSA*, and no activity against *E. coli*, *P. aeruginosa* and *C. albicans*. The methylene chloride fraction and *n*-butanol fraction showed no activity against all tested microbes.

The antimicrobial activity of the active fractions could be attributed to the presence of different classes of active ingredients as Quinones, flavonoids and other polyphenolics. Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive, quinones are known to complex irreversibly with nucleophilic amino acids in proteins, thereby resulting to the inactivation of the protein and loss of cellular function. For that reason, the potential range of quinone antimicrobial effects is great. Flavones are hydroxylated phenolic structures containing one carbonyl group which occur as a C_6-C_3 unit linked to an aromatic ring.

Table 5. Antioxidant activity assessed by DPPH assay for Scavenging activity (SC_{50}) of total extract and different fractions of *Phagnalon barbeyanum* aerial parts compared to standard antioxidant (Ascorbic acid)

Fraction	Concentration (mg/ml)					SC_{50} mg/ml
	1	**0.5**	**0.25**	**0.125**	**0.0625**	
	% DPPH radical scavenging activity					
Ascorbic acid	90.5±0.06%	86.2±0.06%	81.7±0.05%	75.4±0.05%	63.0±0.08%	0.01±0.00009
Total extract	50.0±0.01%	49.2±0.04%	43.7±0.04%	33.5±0.07%	26.7±0.01%	0.798±0.0008
n-hexane.	24.0±0.04%	20.1±0.01%	17.5±0.01%	13.9±0.03%	9.7±0.05%	2.840±0.004
Methylene chloride	69.1±0.01%	64.6±0.01%	62.7±0.04%	53.9±0.02%	50.5±0.03%	0.0593±0.003
Ethyl acetate	84.5±0.02%	83.0±0.04%	79.1±0.03%	70.4±0.03%	64.5±0.02%	0.080±0.0001
n-butanol.	78.5±0.03%	75.1±0.03%	68.8±0.01%	58.4±0.02%	55.4±0.06%	0.037±0.0022

Sc_{50} = concentration causes 50% Scavenging of free radical
Values are expressed as mean ± SE M; n = 3

Table 6. Antimicrobial activity of total extract and different fractions of *Phagnalon barbeyanum* aerial parts compared to standard antibiotics (Amoxicillin and Cyclohexamide)

Fraction	*S. aureus*	*VRSA*	*E. coli*	*P. aeruginosa*	*C. albicans*
Total extract	16.33±0.66	11.33±0.57	-	-	-
Methylene chloride.	-	-	-	-	-
Ethyl acetate	24.00±1.15	19.00±0.57	-	-	-
n-butanol	-	-	-	-	-
Amoxicillin	36.00±1.00	14.66±0.33	22.00±0.57	25.33±0.66	-
Cyclohexamide	-	-	-	-	22.00±0.57

Values are mean of inhibition zones in mm (± SE), n = 3

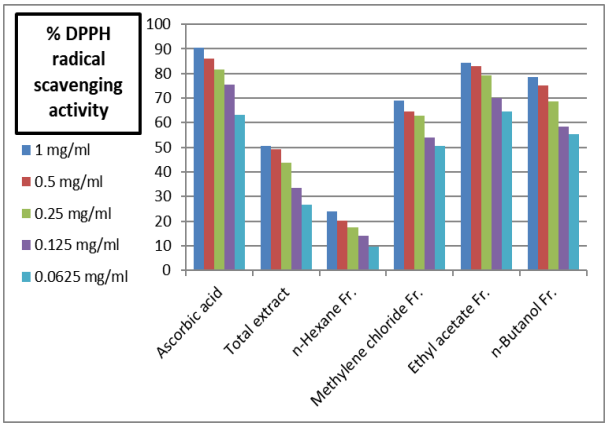

Figure 2. Antioxidant activity of total extract and different fractions of *Phagnalon barbeyanum* aerial parts compared to standard antioxidant (Ascorbic acid).

They are synthesized by plants in response to microbial infection and they have been found to produce *in vitro* antimicrobial action against wide range of pathogens. Their activity is probably due to their ability to form complexes with extracellular and soluble proteins as well as the complexation with bacterial cell walls, thereby inducing microbial cell membrane perturbations[23].

Preliminary phytochemical screening of the total methanol extract of *Phagnalon barbeyanum* aerial parts, showed the presence of carbohydrates and / or glycosides, sterols and / or triterpens, flavonoids, tannins and saponins.

The ethyl acetate fraction of *Phagnalon barbeyanum* aerial parts showed the highest antioxidant activity followed by *n*-butanol and methylene chloride fractions, and *n*-hexane fractions showed the lowest antioxidant activity. The total methanol extract and ethyl acetate fraction showed moderate activity against *S. aureus* and *VRSA* strain.

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Conflict of Interest

The authors declare that they don't have any conflict of interest.

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