Journal of Advanced Pharmacy Research

Phenolic Content of *Albizia anthelmintica* Leaves and Their Antioxidant and Cytotoxic Activity

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Submitted on: 19-10-2016; Revised on: 28-10-2016; Accepted on: 11-11-2016

ABSTRACT

Objectives: This study aimed to the isolate the polyphenolic constituents from the 80% aqueous methanolic extract of *Albizia anthelmintica* leaves and evaluates the antioxidant and cytotoxic activities of the pure isolates. **Methods:** 80% MeOH leaves extracts of the plant subjected separately to different chromatographic separation techniques. Structures of the isolated compounds were established by different spectroscopic techniques (ESI-MS, $^{1}H/^{13}C$ NMR and 2D NMR experiments). The antioxidant activity was evaluated using DPPH scavenging activity method, the crude extracts and pure isolated compounds form active fractions of *A. anthelimintica* were tested for cytotoxicity against human prostate normal cell line (PNT 2A), human ovarian carcinoma cell line (A2780) and Human Caucasian breast carcinoma cell line (ZR75-1). **Results:** Nine compounds were isolated from *A. anthelmintica* four were identified for first time from the plant ferulic acid **2**, 3`, 7 di-*O*-methylmyricetin**3**, Kaempferol 3-*O*-(6``-E-p-Coumaroyl)-*β*-D-glucopyranoside **6** and Kaempferol 3-*O*-(6``-E-p-Caffeoyl)-*β*-D-glucopyranoside**7**and five were isolated before from the plant methyl gallate **1,** gallic acid **4**, quercetin **5**, Quercetin 3-*O*-(6``-*O*-galloyl)-*β*-D-glucopyranoside **8** and Kaemferol 3-*O*-(6``-*O*-galloyl)-*β*-D-glucopyranoside **9**. Compounds **8**, **9** and **7**showed the most powerful anti-oxidant activity among the compounds tested with IC_{50} 2.75, 3.25 and 5.25 µM, respectively. Compounds **7**, **3** and **6** showed activity against human ovarian carcinoma cell line (A2780) with IC_{50} 2.6, 5.23and 6.11µM, respectively. Compound **8** showed strong cytotoxic activity against Human Caucasian breast carcinoma cell line (ZR75-1) with IC_{50} 3.15 µM. **Conclusion:** *A. anthelmintica* 80% aqueous methanolic leaf extract contains phenolic compounds, some of which were isolated for first time from this species and some have shown strong antioxidant and cytotoxic activity against the tested cell lines. The three active isolated compounds need to be evaluated in animal models to determine their potential as natural cure products.

Key Words: *Albizia anthelmintica; Antioxidant; Cytotoxicity; Polyphenolics*

INTRODUCTION

The Fabaceae or Leguminosae, commonly known as the legume, pea, or bean family, is a large and economically important family of flowering plants. It includes trees, shrubs, and herbaceous plants perennials or annuals, which are easily recognized by their fruit (legume) and their compound, stipulated leaves. The group is widely distributed and is the third-largest land plant family in terms of number of species, behind only the Orchidaceae and Asteraceae, with 630 genera and over 18,860 species.[1,2] The genus *Albizia* (Fabaceae) comprises about 150 species distributed in Africa, Asia, Central and South America. The *Albizia* members in Africa are used in folk medicine for the treatment of rheumatism, cough, diarrhea and injuries.[3] Phytochemical studies carried out on *Albizia* species led to the isolation of several triterpene glycosides, flavonoids, alkaloids and miscellaneous compounds. Many biological activities were studied for *Albizia*

species such as the antioxidant activity of the methanol extract of the stem bark of *Albizia julibrissin*[4]and the cytotoxic effect on Jurkat cells exhibited by triterpene saponins from *Albizia adianthifolia*.[5] *Albizia anthelmintica* is cultivated widely in Africa and Asia. East Africans widely use *A. anthelmintica* to control helminth parasites in human and animal medicine in Sudan[6] and Ethiopia.[7]

MATERIALS AND METHODS

Apparatus

Rotary evaporators (model no: R-110 and R-3) from BÜCHI, Switzerland were used for concentration and drying of extracts and fractions. The laminar flow hood (BioMAT2) was purchased from Medical Air Technology, UK. The stand incubator (Incu-160S) used for the cell culture plates was from SciQuip Ltd., Shropshire, UK. ESI-MS were measured by the aid of High Pressure Liquid Chromatography (UltiMate-3000)-Mass Spectrometry (Exactive) instrument was from Thermo Scientific, Germany. NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on Nuclear Magnetic Resonance spectroscopy machine JNM-LA400 model was from JEOL, Japan and the magnet NMR AS400 model EUR0034 came from Oxford Instruments, England. The δ values are reported as ppm relative to TMS in DMSO-d_6 and *J* values in Hz. The BÜCHI MPLC instrument was the Sepacore (Easy Synthesis) Purification System, consisting of two C-601 pump modules and the C-615 pump manager (BÜCHI, Switzerland). This allowed binary solvent gradients with flow rates of 2.5 to 250 mL/min. The columns and the column stand were purchased from VersaFlash/Supelco, Sigma-Aldrich, Germany. Another system used for purification is The Biotage® Isolera TMSpektra One Flash Purification System ISO-1SV, a product of Biotage, Uppsala, Sweden, was also used. This had a UV detector (wavelength range: 200-400 nm). Biotage® SNAP cartridges (normal and reversed phase silica columns) were used.

Plant material

Leaves of *Albizia anthelmintica* were collected from Alzohria Garden, Giza, Egypt June 2011. Identification of the plant was confirmed by Dr. Terase Labib, Department of Flora and Taxonomy, El-Orman Botanical Garden, Giza, Egypt. Voucher specimens (Reg. no. Aa-7) was kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Egypt.

Chemicals

All solvents of HPLC and analytical grade were purchased from Fisher Scientific, UK. The deuterated solvents used for NMR were purchased from Euriso-Top, France or from Sigma-Aldrich Co., Missouri, USA Chemicals used for evaluating the antioxidant activity, DPPH (2,2-Diphenyl-1-picrylhydrazyl) and Ascorbic acid were purchased from Sigma-Aldrich Co., UK. Chemicals used in evaluating the cytotoxicity, Alamarblue BUF012B (BioRAD, Serotec, UK) and Tritone X-100 (Sigma-Aldrich Co., UK).

Authentic reference flavonoid compounds were supplied by Pharmacognosy department, Faculty of Pharmacy, Helwan University. Authentic sugars were purchased from Sigma-Aldrich Co., UK.

Cell line and culture medium

Human prostate normal cell line (PNT 2A), human ovarian carcinoma cell line (A2780) and Human Caucasian breast carcinoma cell line (ZR75-1), All derived from ECACC (Sigma Aldrich, Dorset, UK). All cell lines were cultured in DMEM media supplemented with 10% (v/v) foetal bovine serum, 2 mM glutamine and 50 µg/mL penicillin/streptomycin solution (all from Invitrogen, Paisley, UK).

Extraction and isolation of phenolics

The air dried leaves of *A. anthelmintica* (1 kg) were coarsely ground and extracted thrice with 5L 80% MeOH. Then, the extracts of *A. anthelmintica* were combined and evaporated to dryness under reduced pressure to afford 105gm extracts. The dried extract was then reconstituted with 100 mL H_2O then fractionated with 3x300 ml of hexane and ethyl acetate by liquid-liquid phase separation yielding three subfractions of each extract and the weight of each subfraction was as follow: (17 gm hexane fraction, 32 gm ethyl acetate fraction and aqueous layer). 5 mg of each subfraction was dissolved in the appropriate NMR solvent (Hexane fraction in CD_3Cl, Ethyl acetate in DMSO and Aqueous layer fraction in Deuterium oxide) to run ^{1}H NMR experiment, by comparing the ^{1}H NMR spectrum of each of the three fractions of each plant, it was found that ethyl acetate fraction showed well resolved proton spectrum and characteristic signals in the aromatic region. Therefore, the ethyl acetate fractions were selected for more fractionation work. Purification of compounds from *A. anthelmintica* leaves extract was performed as shown in the flow chart (Figure 1).

Acid hydrolysis for glycosides

Complete acid hydrolysis was performed by addition of 1.5 N HCl in aqueous methanol (50%) over 4-5 mg of each compound and heating for 2 hours at 100°C. Each hydrolzate was then extracted with ethyl acetate. The ethyl acetate extract was subjected to CoPC investigation against authentic aglycones. The aqueous layer was then neutralized with sodium carbonate and subjected to CoPC against authentic sugars for identification of the sugar part.

Antioxidant assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), which possesses an unpaired electron and exhibits a stable violet colour in methanol solution (peak absorbance at 517 nm), is commonly used as a reagent for evaluation of the free radical scavenging activity of antioxidants[8]. The DPPH assay is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form (DPPH-H) in the reaction. This reduction reaction resulting in color change of DPPH from violet to yellow (reduced form) and as much as the intensity of the yellow color increased as the anti-DPPH activity increased[9]. In this method, a microplate reader and 96 well plates were used to carry out the determination of the spectral absorption values. This assay is based on the classic method developed by Blois, 1958[9]. Various forms of this method are widely used[11,12]. Unlike the commonly used methods, which are labour, time-consuming, and reagent and sample-wasting, this microplate assay method is much more rapid, sample-saving and environmentally-friendly. In this method, methanolic DPPH solutions (100 µg/mL, 50 µL) were added to samples of different concentration (200 µL, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL for fractions and µM/mL for pure compounds). These solutions were gently mixed and incubated in the dark for 30 min at room temperature. Then the absorbances of the resulting solutions were measured at 517 nm. The scavenging capability of test compounds was calculated using the following equation:

DDPH· scavenging activity (%) = 1− (λ517-S / λ517-C) X 100

Where λ517-C is absorbance of a control with no radical scavenger and λ517-S is absorbance of the remaining DPPH in the presence of scavenger.

Cytotoxicity Assay

The crude extracts, fractions, and some pure isolated compounds of *A. anthelimintica* were tested for cytotoxicity against human prostate normal cell line (PNT 2A), human ovarian carcinoma cell line (A2780) and Human Caucasian breast carcinoma cell line (ZR75-1), All derived from ECACC (Sigma Aldrich, Dorset, UK). The three cell lines were cultured in DMEM media supplemented with 10% (v/v) foetal bovine serum, 2 mM glutamine and 50 µg/mL penicillin/streptomycin solution (all from Invitrogen, Paisley, UK). All kept in a humidified incubator at 37 °C in the presence of 5% CO_2. Cells were routinely passaged at 90-95% confluence. The cells were then seeded in transparent 96-well flat bottomed plates and allowed to adhere overnight. Column 1 kept untreated serving as control and column 12 treated with triton X 100 as a positive control. Preliminary screening has been done to evaluate which fractions or compounds have activity at concentration of 30 µM/mL for the tested pure compounds and incubated for 48 hours. Viability was assessed using Alamarblue kit according to the manufacturer's instructions. The luminescence was then measured using a Wallac Victor 2. After performing preliminary screening fractions or compounds showed viability (as % of control) less than 40% considered active (cytotoxic). Active fractions or pure compounds were then added at a concentration range of 0.1 nM/mL to 30 µM/mL (in half log units) for determination of The IC_{50} values and all results confirmed microscopically. All experiments were done in triplicates.

Statistical analysis

Alamarblue cytotoxicity assay data were analysed using two-factorial analysis of variance (ANOVA), including first order interaction (two-way ANOVA).

RESULTS AND DISCUSSION

Characterization and identification of isolated compounds

Chromatographic separation of 80% aqueous methanol leaves extracts of *A. anthelmintica* has resulted in nine compounds. They were identified by different spectral techniques including ^{1}H NMR, ^{13}C NMR, 2 D NMR and ESI-MS also by CoPC against standard authentic sugars and aglycones after complete acid hydrolysis.

Compound 6 was obtained as pale yellow needles (28 mg). Chromatographic properties: R_f-value 0.60 (**S1**); dark purple spot under UV- light, turned to intense yellow color after spraying with P-anisaldhyde spray reagent and heating with air gun at 250 °C. It gave greenish yellow fluorescence after spraying with Naturstoff spray as well as deep green color with $FeCl_3$. Complete acid hydrolysis resulted in glucose in aqueous layer and kaempferol together with coumaric acid in organic layer (CoPC).**^{1}H NMR** (400 MHz, **DMSO-*d*$_6$**), δ ppm 8.00 (2 H, d, *J*= 8.79, H-2`/6`), 7.38 (2 H, *J*= 8.29, H-2```/6```),7.36 (1 H, d, *J*= 15.68, H-7``` *β*), 6.87 (2 H, d, *J*= 8.77, H-3`/5`), 6.80 (2 H, d, *J*= 8.46, H-3```,5```), 6.38 (H, d, *J*= 1.73, H-8), 6.15 (1 H, d, *J*= 1.81, H-6), 6.12 (1 H, d, *J*= 15.95, H-8``` *α*) 5.46 (1 H, d, *J*= 7.31, H-1``), 4.28, 4.16 (1 H, d, *J*= 11.94, 13.67, H-6``).**^{13}C NMR** (100 MHz, **DMSO-*d*$_6$**): δ ppm 177.94 (C-4), 166.73 (C-9```), 164.87 (C-7), 161.71 (C-5), 160.56 (C-4`),160.37 (C-4```), 156.97 (C-2), 156.92 (C-9),145.17 (C-7```-β), 133.69 (C-3), 131.38 (C-2`/6`), 130.72 (C-2```/6```), 125.47 (C-1```), 121.32 (C-1`), 116.32 (C-3```/5```), 115.65 (C-3`/5`), 114.19 (C-8```-α), 104.38 (C-10), 101.53 (C-1``), 99.36 (C-6), 94.24 (C-8), 76.77 (C-3``), 74.79 (C-2``), 74.69 (C-5``), 70.51 (C-4``), 63.53 (C-6``). According to the chromatographic properties of **Compound 6** (R_f-value, fluorescence under UV–light and changes in colour with $FeCl_3$ solution and

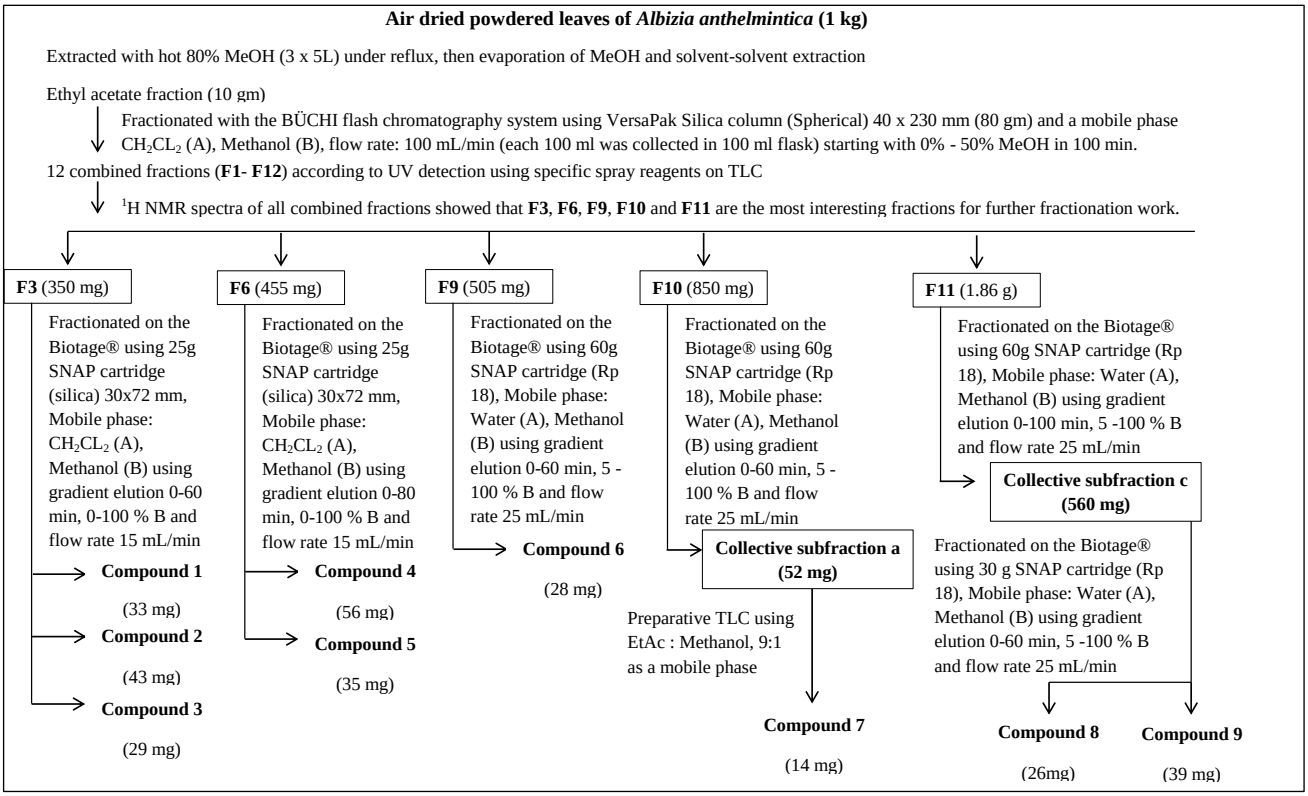

Figure. 1. Flow chart of ecraction, fractionation and purification of phenolic compounds from the leaves of *A. anthelmintica*

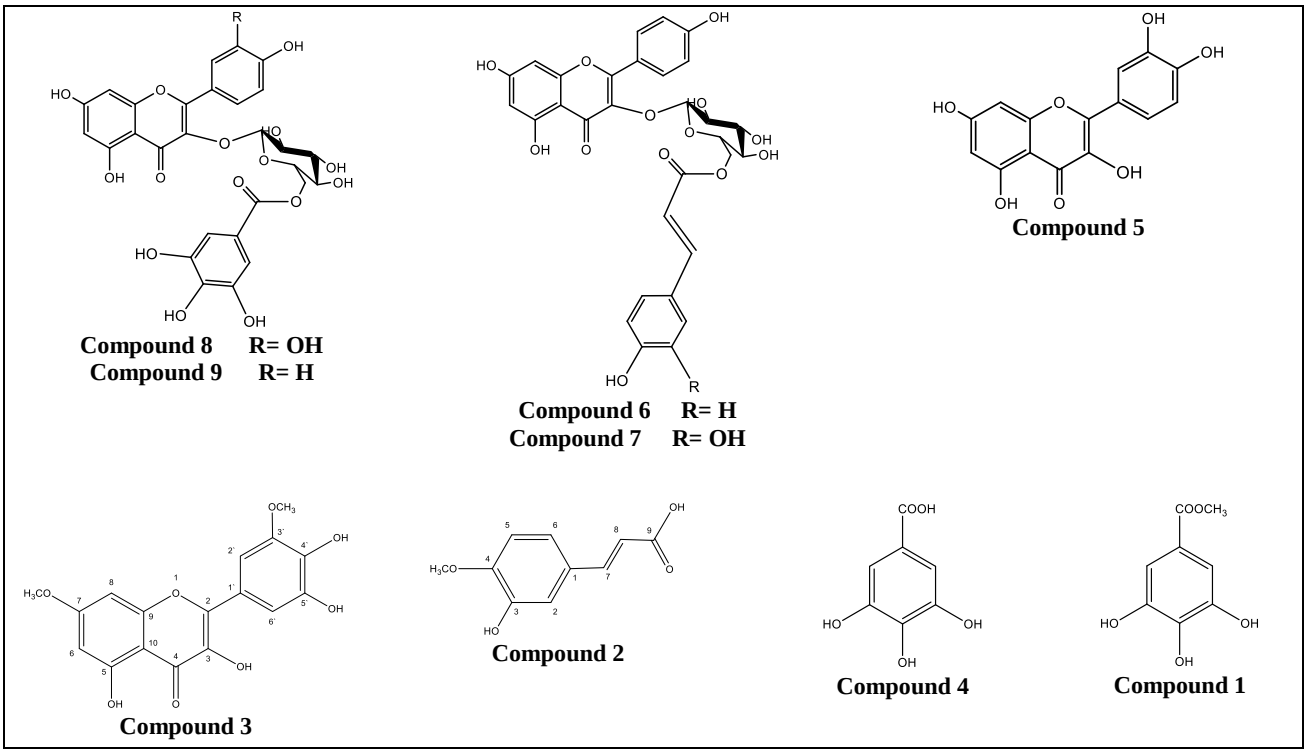

Figure. 2. Structure of compounds isolated from *A. anthelmintica*

Naturstoff reagents and products of acid hydrolysis it was expected to be Kaempferol coumaroyl glucoside[12]. **^{1}H NMR** spectrum of **Compound 6** exhibited an A_2X_2 spin coupling system of two *ortho* doublet, each integrated to two protons, at 8.00 (H-2`/6`) and 6.87 (H-3`/5`) indicated 4`-hydroxyl B-ring together with the two meta doublets, each integrated for one proton at 6.38 (H-8) and 6.15 (H- 6) were intrinsic for kaempferol nucleus. Two ortho doublets each integrated to two protons, at 7.38 (H-2```/6```) and 6.80 (H-3```/5```) together with two doublets with large *J* value each integrated to one proton at 7.36 and 6.12, characteristic for two olefenic protons7```and 8```, respectively suggesting the presence of coumaryl moiety in the structure. In the aliphatic region the presence of a doublet at 5.46 integrated for one proton with large *J*-value of 7.31 Hz indicating *β*-glycosyl moiety. As further confirmation, **^{13}C NMR** spectrum exhibited thirteen ^{13}C resonances of the kaempferol 3-*O*-substituted moiety with key carbon signals of kaempferol nucleus at 177.94 (C-4), 160.56 (C-4`), 131.38 (C-2`/6`), and 115.65 (C-3`/5`). Additionally, the spectrum showed characteristic six carbon signals for *O*- *β*-D-glucopyranoside structure and seven characteristic carbon signals for coumaroyl moiety 166.73 (C-9```), 160.37 (C-4```), 145.17 (C-7```-β), 130.72 (C-2```/6```), 125.47 (C-1```), 116.32 (C-3```/5```), 114.19 (C-8```-α).[13] The downfield and up field shifts of ^{13}C-resonances of C-2 (156.97) and C-3 (133.69) respectively were confirmative evidence for *O*-glycosidation at C-3. The attachment of the coumaroyl group was proved to be at OH-6`` of the glucose moiety from the downfield shift of the two dd of the diasteriomeric $CH_2$-6`` protons at 4.28 and 4.16 that was shown in **^{1}H NMR spectrum** together with the down and up-field locations of C-6`` (63.31) and C-5`` (74.64) resonances respectively in **^{13}C NMR** spectrum.[14,15] Negative and positive **ESI-MS** spectrum showed a molecular ion peak at *m/z* 593.13 $[M-H]^-$ and 595.15 $[M+H]^+$ respectively corresponding to the molecular weight of 594.14 which support the evidence that **Compound 6** is Kaempferol 3-*O*-(6``-E-p-Coumaroyl)-*β*-D-glucopyranoside. A final confirmation of **Compound 6** was achieved by comparison with previously published data.[14, 15, 16]

Compound 7 was obtained as pale yellow amorphous powder (14 mg). Chromatographic properties: R_f-value 0.50 (**S$_1$**) and showed same chromatographic properties like **compound 6**. Complete acid hydrolysis resulted in glucose in aqueous layer and quercetin together with caffiec acid in organic layer (CoPC). **^{1}H NMR** (400 MHz, **DMSO-*d*$_6$**): δ ppm 7.55 (1 H, d, *J*= 2.14, H-2```), 7.52 (1 H, dd, J= 2.14, 8.15, H-6```), 7.36 (2 H, *J*= 8.31, H-2`/6`), 7.33 (1 H, d, *J*= 14.68, H-7``` *β*), 6.83 (1 H, d, *J*= 8.17, H-5```), 6.79 (2 H, d, *J*= 8.57, H-3`/5`), 6.35 (1 H, d, *J*= 1.79, H-8), 6.14 (1 H, d, *J*= 1.24, H- 6), 6.14 (1 H, d, *J*= 15.95, H-8``` *α*), 5.50 (1 H, d, *J*= 7.21, H-1``), 4.27, 4.04 (1 H, d, *J*= 10.54, 11.80, H-6``).**^{13}C NMR** (100 MHz, **DMSO-*d*$_6$**): δ ppm 177.94 (C-4), 166.78 (C-9```), 164.69 (C-7), 161.74 (C-5), 160.33 (C-4`), 156.84 (C-2/9), 149.07 (C-4```), 145.39 (C-3```), 145.13 (C-7```-β), 133.63 (C-3), 130.72 (C-2`/6`), 125.49 (C-1```), 122.06 (C-6```), 121.61 (C-1`), 116.74 (C-2```), 116.31 (C-3`/5`), 115.74 (C-5```), 114.22 (C-8```-α), 104.38 (C-10), 101.25 (C-1``), 99.27 (C-6), 94.04 (C-8), 76.86 (C-3``), 74.87 (C-5``), 74.54 (C-2``), 70.49 (C-4``), 63.66 (C-6``).According to the chromatographic properties **Compound 7** was expected to be Kaempferol caffoeyl glucoside moities[12]. **^{1}H NMR** spectrum of **Compound 7** exhibited the characteristic splitting pattern intrinsic for kaempferol nucleus like that of **Compound 6**, also it showed an ABX spin coupling system of three resonances each integrated for one proton, attributable to H-2```, H-6``` and H-5``` of 3`, 4`-dihydroxy benzene ring, two doublets with large *J* value each integrated to one proton at 7.33 and 6.14 for two olefenic protons which were characteristic for caffoeyl moiety. In the aliphatic region the presence of a doublet at 5.50 integrated for one proton with large *J*-value of 7.21 Hz. **^{1}H NMR** spectrum of **Compound 7** gave evidence that the compound substituted by *β*-*O*-glucosyl and caffeoyl moieties. **^{13}C NMR** spectrum exhibited thirteen ^{13}C resonances of the kaempferol 3-*O*-substituted moiety with key carbon signals of kaempferol nucleus like that of **Compound 6**. Six carbon signals are characteristic for *O*-*β*-D-glucopyranoside and nine characteristic carbon signals for caffeoyl moiety 166.78 (C-9```), 149.07 (C-4```), 145.39 (C-3```), 145.13 (C-7```-β), 125.49 (C-1```), 122.06 (C-6```), 116.74 (C-5```), 115.74 (C-2```), 114.22 (C-8```-α) [14,15,16]. The down-field-shifted carbon signal 156.84 (C-2), together with up-field shift of C-3 at 133.63 indicate *O*-glycosidation at C-3. The attachment of the caffeoyl group was determined to be adjacent to the OH-6`` position of the glucose moiety by the down field shift of the two dd of the diasteriomeric $CH_2$-6`` protons at 4.27 in its **^{1}H NMR** and down and up-field location of C-6`` at 63.66 and C-5`` at 74.87 resonances respectively in **^{13}C NMR** spectrum. Positive and negative **ESI-MS** spectrum of **Compound 7** showed a molecular ion peak at *m/z* 609.13 $[M-H]^-$ and 611.14 $[M+H]^+$ corresponding to the molecular weight of 610.14 which support the evidence that **Compound 7** is Kaempferol 3-*O*-*β*-D-(6``-E-p-Caffeoyl)-glucopyranoside. A final confirmation of **Compound 7** was achieved by comparison with previously published data.[14,15]

Compound 8 was obtained as yellow amorphous powder (26 mg). Chromatographic properties: R_f-value 0.65 (**S$_1$**); dark purple spot under UV- light, turned to intense yellow color after spraying with P-anisaldhyde spray reagent and heating with air gun at 250 °C. It gave orange fluorescence after spraying with Naturstoff spray as well as deep green color with

$FeCl_3$. Complete acid hydrolysis resulted in glucose in aqueous layer and gallic acid together with quercetin in organic layer (CoPC).**^{1}H NMR** (400 MHz, **DMSO-*d*$_6$**), δ ppm 7.58 (1 H, dd, *J*= 8.49, 2.12, H-6`), 7.43 (1 H, d, *J*= 2.13, H-2`), 6.89 (2 H, s, H-2```/6```), 6.72 (1 H, d, *J*= 8.45, H-5`), 6.37 (1 H, d, *J*= 1.90, H-8), 6.18 (1 H, d, *J*= 2.01, H-6), 5.45 (1 H, d, *J*= 7.29, H-1``),4.25 (1 H, d, *J*= 11.59, H-6``). **^{13}C NMR** (100 MHz, **DMSO-*d*$_6$**), δ ppm 177.84 (C-4), 166.21 (C-7```), 164.75 (C-7), 161.64 (C-5), 156.99 (C-2), 156.86 (C-9),149.04 (C-4`),146.01 (C-3```/5```), 145.37 (C-3`), 138.98 (C-4```), 133.92 (C-3), 122.45 (C-6`), 121.48 (C-1`),119.86 (C-1```), 116.30 (C-2`), 115.86 (C-5`),109.10 (C-2```/6```), 104.45 (C-10), 101.91 (C-1``), 99.28 (C-6), 94.10 (C-8), 76.85 (C-3``), 74.80 (C-5``), 71.91 (C-2``), 70.04 (C-4``), 63.70 (C-6``). According to the chromatographic properties of **Compound 8** (*R*$_f$-value, fluorescence under UV–light and changes in color with $FeCl_3$ and Naturstoff reagents) and products of acid hydrolysis it was expected to be quercetin galloyl glucoside[12]. **^{1}H NMR** spectrum of **Compound 8** exhibited an ABX spin coupling system of three resonances each integrated for one proton, attributable to H-2', H-6' and H-5' of 3', 4'-dihydroxy B-ring and AM spin coupling system of two *meta* doublets, each integrated for one proton at 6.37 (H-8) and 6.18 (H-6) which are characteristic for 5, 7 dihydroxy A-ring, a singlet signal at 6.89 integrated for two protons characteristic for galloyl moiety. In the aliphatic region the presence of a doublet at 5.45 integrated for one proton with large *J*-value of 7.29 Hz which was in good agreement with *β*-glucopyranoside moiety and the down field shift of the two methylene glucose protons (H-6``) at δ ppm 4.25 gave an evidence for galloylation at OH-6``. **^{13}C NMR** spectrum exhibited fifteen ^{13}C resonances of the quercetin 3-*O*-substituted moiety with key carbon signals of quercetin nucleus at 177.87 (C-4), 149.04 (C-4`), 145.37 (C-3`), 122.45 (C-6`), 121.48 (C-1`), 116.30 (C-2`) and 115.86 (C-5`).[13.] The sugar moiety was finally proved to be glucoside in *β*-D-pyranoside structure due to the presence of its typical six carbon resonances. Moreover, the characteristic five resonances of the galloyl moiety were recorded at 166.21 (C-7```), 146.01 (C-3```/5```), 138.98 (C-4```), 119.86 (C-1```) and 109.10 (C-2```/6```). The attachment of sugar to quercetin moiety was deduced to be at the 3- position from the down field-shifted carbon signal 156.99 (C-2), together with up field shift of C-3 at 133.92 indicate *O*-glycosidation at C-3. The attachment of galloyl moiety to OH-6`` proved by the down field shift of C-6`` at 63.70 and up field shift of C-5`` at 74.80 in **^{13}C NMR** spectrum. Supporting evidence was achieved by positive and negative **ESI-MS** spectrum of **Compound 8** which showed a molecular ion peak at *m/z* 615.10 $[M-H]^-$ and 617.11 $[M+H]^+$ corresponding to the molecular weight 616.10 which support the evidence that **Compound 8** is Quercetin 3-*O*-(6``-*O*-galloyl)-*β*-D-glucopyranoside which was in a good agreement with previously published data[13,17,18].

Compound 9 was obtained as yellow amorphous powder (39 mg). Chromatographic properties were similar to that **Compound 6** and **7**: *R*$_f$-value 0.55 (**S$_1$**); Complete acid hydrolysis resulted in glucose in aqueous layer and kaempferol together with gallic acid in organic layer (CoPC).**^{1}H NMR** (400 MHz, **DMSO-*d*$_6$**), δ ppm 7.93 (2 H, d, *J*= 8.83, H-2`/6`), 6.91 (2 H, s, H-2```/6```), 6.76 (2 H, d, *J*= 8.80, H-3`/5`), 6.38 (1 H, d, *J*= 1.84, H-8), 6.18 (1 H, d, *J*= 1.82, H-6), 5.45 (1 H, d, *J*= 7.37, H-1``), 4.25 (1 H, d, *J*= 11.36, H-6``), 3.4-3.2 (m, rest of sugar protons).**^{13}C NMR** (100 MHz, **DMSO-*d*$_6$**),δ ppm 177.86 (C-4), 166.21 (C-7```), 164.88 (C-7), 161.72 (C-5), 160.50 (C-4`), 157.32 (C-2), 156.98 (C-9),146.03 (C-3```/5```), 138.98 (C-4```), 133.69 (C-3), 131.34 (C-2`/6`), 121.20 (C-1`), 119.86 (C-1```), 115.65 (C-3`/5`), 109.10 (C-2```/6```), 104.47 (C-10), 102.00 (C-1``), 99.36 (C-6), 94.31 (C-8), 76.70 (C-3``), 74.64 (C-5``), 71.91 (C-2``), 69.90 (C-4``), 63.31 (C-6``). Negative and positive **ESI-MS** spectrum of **Compound 9** showed a molecular ion peak at *m/z* 599.10 $[M-H]^-$ and 601.12 $[M+H]^+$. According to the chromatographic properties of **Compound 9** (R_f-value, fluorescence under UV–light and changes in colour with $FeCl_3$ and Naturstoff reagents) and products of acid hydrolysis it was expected to be Kaempferol galloyl glucoside[12]. **^{1}H NMR** spectrum of **Compound 9** exhibited an A_2X_2 spin coupling system of two *ortho* doublet, each integrated to two protons, at 7.93 (H-2`/6`) and 6.76 (H-3`/5`) indicated 4'-hydroxyl B-ring and AM spin coupling system of two meta doublets, each integrated for one proton at 6.38 (H-8) and 6.18 (H- 6) characteristic for 5,7 dihydroxy A-ring. It showed the characteristic signals of (6``-*O*-galloyl)-*β*-D-glucopyranoside like that of **Compound 8**. The attachment of sugar was also deduced to be at the 3-position of kaempferol nucleus from the down-field-shifted carbon signal 157.32 (C-2), together with up-field shift of C-3 at 133.69 indicate *O*-glycosidation at C-3. The attachment of galloyl moiety to OH-6`` proved by the down and up-field location of C-6`` at 63.31 and C-5`` 74.64 resonances respectively in **$^{13}$C NMR** spectrum. **Compound 9** was identified as Kaempferol 3-*O*-(6``-*O*-galloyl)-*β*-D-glucopyranoside. A final confirmation of **Compound 9** was achieved by comparison with previously published data.[13,17] **Compound 9** was isolated once before from *A. anthelmintica.*[19]

Compound 3 was obtained as a light brown amorphous powder (29 mg). Chromatographic properties: *R*$_f$-value 0.75 (**S$_1$**); dark purple spot under UV- light, turned to intense yellow color after spraying with anisaldhyde and heating with air gun at 250 °C. It gave red fluorescence after spraying with Naturstoff spray reagent as well as deep green color with $FeCl_3$. **^{1}H NMR** (400 MHz, **DMSO-*d*$_6$**), δ ppm 7.04 (1 H, d, *J*=

1.87 Hz, H-6`), 6.67 (1 H, brs, H-2`), 6.26 (1 H, brs, H-8), 5.48 (1 H, brs, H-6), 3.79 (3 H, S, OCH_3), 3.69 (3 H, S, O-CH_3). **^{13}C NMR** (100 MHz, **DMSO-*d*$_6$**), δ ppm 175.03 (C-4), 166.49 (C-7), 160.10 (C-9/5), 148.51 (C-3`), 147.15 (C-2), 146.15 (C-5`), 136.63 (C-4`), 134.58 (C-3), 122.78 (C-1`), 108.99 (C-6`), 105.42 (C-10/2`), 95.93 (C-6), 91.77 (C-8), 56.63 (OCH_3), 52.51 (OCH_3). **Compound 3** was expected to be myricetin on the basis of its chromatographic properties and complete acid hydrolysis.[12] **^{1}H NMR** spectrum of **Compound 3** showed the characteristic splitting pattern of myricetin nucleus, it exhibited AM spin coupling system of two meta doublets each integrated for one proton at δ ppm 7.04 (H-2`) and 6.67 (H-6`), characteristic for 3`-substituted B-ring of myrecetin. Also showed AM spin coupling system of two meta doublets, each integrated for one proton at δ 6.26 (H-8) and 5.48 (H- 6) gave an evidence for 5,7-dihydroxy A-ring. In the aliphatic region it showed a characteristic two singlet signals each integrated to three protons at δ 3.79 and 3.69 which indicative for the presence of two methoxy groups. **^{13}C NMR** spectrum exhibited fiften^{13}C resonances with key carbon signals of myricetin nucleus at δ 175.03 (C-4), 166.49 (C-7), 160.10 (C-9/5), 148.51 (C-3`), 147.15 (C-2), 146.15 (C-5`), 136.63 (C-4`), 134.58 (C-3), 122.78 (C-1`), 108.99 (C-6`), 105.42 (C-10/2`), 95.93 (C-6), 91.77 (C-8),**[13]**. Two characteristic methoxy signals displayed at δ 56.63 and 52.51. **Cosy** spectrum of **Compound 3** showed the characteristic correlations of myricetin nucleus between 7.04 (H-2`) and 6.67 (H-6`) and a correlation between 6.26 (H-8) and 5.48 (H- 6). Attachment of the two methoxy moieties was assigned to be at C-3` due to up-field shift of C-2` at δ (105.42) and C-7 due to up-field shift of C-8 at δ (91.77). The appearance of C-2 signal at δ (147.15) is indicated that C-3 is not substituted. Accordingly **Compound 3** was identified as 3`, 7-di-*O*-methylmyricetin. A final confirmation of **Compound 3** was achieved by comparison with previously published data.[20]

Compound 5 showed the characteristic chromatographic properties of Quercetin aglycon and also showed the characteristic NMR signals of quercetin.[12,13] **^{1}H NMR** (400 MHz, **DMSO-*d*$_6$**), δ ppm 7.69 (1 H, d, *J*= 2.02, H-2`), 7.55 ( H, dd, *J*= 8.51, 2.05, H-6`), 6.89 (1 H, d, *J*= 8.52, H-5`), 6.41 (1 H, d, *J*= 1.95, H-8), 6.19 (1 H, d, *J*= 1.86, H-6).**$^{13}$C NMR** (100 MHz, **DMSO-*d*$_6$**), δ ppm 176.40 (C-4), 164.44 (C-7), 161.28 (C-5), 156.69 (C-9), 148.25 (C-4`), 147.35 (C-2), 145.61 (C-3`), 136.30 (C-3), 122.52 (C-6`), 120.54 (C-1`), 116.17 (C-2`), 115.63 (C-5`),103.58 (C-10), 98.74 (C-6), 93.91 (C-8).

In addition to the isolated flavonoid compounds, 2 phenolic acids ferulic acid **2**, gallic acid **4** and it's methyl ester methyl gallate **1**. They were identified by CoPC against authentic samples and comparison with previously published data.[17]

Antioxidant Activity

All of the main fractions of the plant (Ethyl acetate, hexane and aqueous layer) have been tested for their antioxidant activity and compared to ascorbic acid. The tested concentrations were (3.125-100 µg/mL). (Figure 3) showed the scavenging activities of different concentrations of *A. anthelmintica* fractions (3.125-100 µg/mL) against ascorbic acid. From the figure presented below it was observed that the ethyl acetate fraction exhibited a good anti-oxidant activity more than that of hexane and aqueous layer, so the ethyl acetate fraction was promising for more fractionation work.

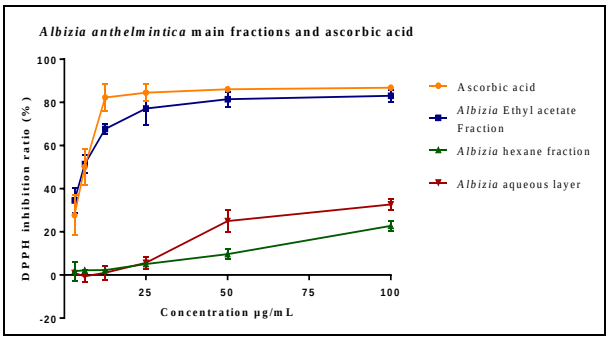

Figure. 3. The scavenging activities of different concentrations of *A. anthelmintica* fractions (3.125-100 µg/mL) against ascorbic acid.

After fractionation, purification and Isolation of the polyphenolic constituents of the ethyl acetate extract of the plant, the pure compounds (**6**, **7**, **8** and **9**) were tested for their scavenging activity and results are compiled in **Fig. 4.** From results showed in (**Fig. 4.**) it is clear that **Compound 8** (IC_{50} =2.75 µM/mL) is the most active one followed by **Compound 9** (IC_{50} = 3.25 µM/mL), **Compound 7**(IC_{50} =5.25 µM/mL) then **Compound 6**(IC_{50} =25 µM/mL).

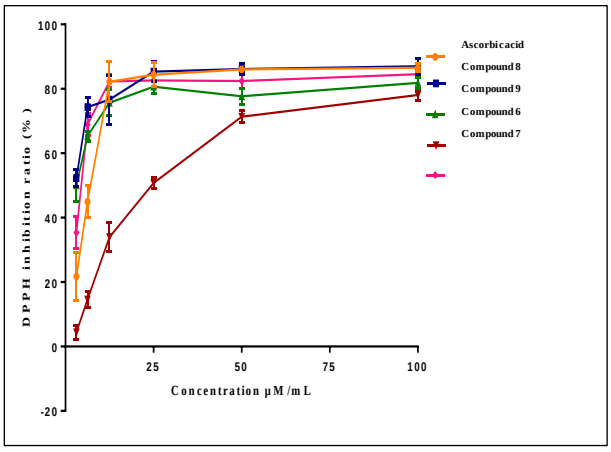

Figure. 4. The scavenging activities of different concentrations (3.125-100 µM/mL) of *A. anthelmintica* pure compounds (Compound 6 to 9) compared to ascorbic acid.

Cytotoxic Activity

Evaluating cytotoxicity against normal human prostate cell line (PNT 2A)

After reading plates, the viability as (% of control) has been calculated for each sample at the preliminary screening dose. Samples which showed viability less than 40% are considered to be cytotoxic. (Figure 5) showed cytotoxicity of some of the pure isolated compounds. From the figure presented below it was observed that pure compounds tested are non-toxic to the normal cell line (**PNT 2A**)

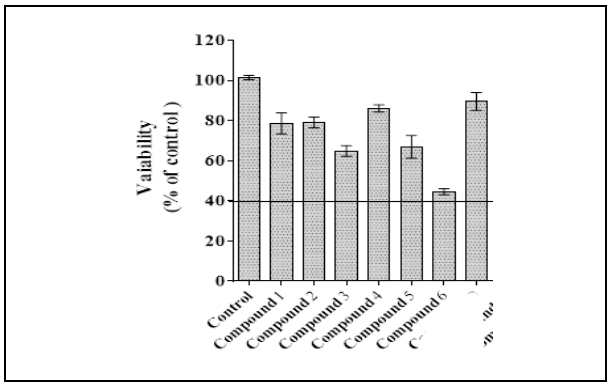

Figure. 5. Cytotoxic activity of some pure compounds isolated against normal human cell line (PNT 2A). Sample concentration 30 µM/ml, n=3

Dose response curve for those active compounds following the concentration range 0.1 nM/ml or ng/ml to 30 µM/ml or µg/ml (in half log units) for determination of The IC_{50} values. **Compound 7** was found to be the most active showing IC_{50} (2.6 µM/ml) then **Compound 3** (5.23 µM/ml) and finally **Compound 7** with IC_{50} (6.11 µM/ml). Figure 6 is showing the dose response curve of these compounds.

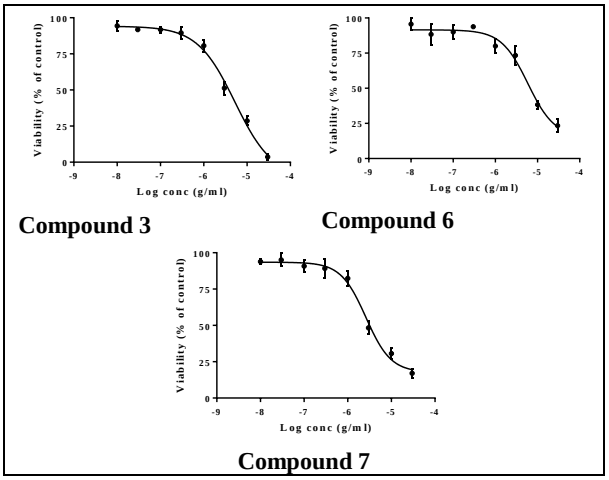

Figure. 6. Dose response curve of compound 3, 6 and7 against human ovarian carcinoma cell line (**A2780**) (n=3)

Evaluating cytotoxicity against Human Caucasian breast carcinoma cell line (ZR75-1)

All compounds were tested for activity at a preliminary screening dose of 30 µM/mL, only **Compound 8** exhibited viability less than 40 %. A dose reponse curve has been done using the concentration range 0.1 nM/mL or ng/mL to 30 µM/mL or µg/mL (in half log units) for determination of The IC_{50} values. Figure 7 is showing the dose response curve of **Compound 8**, it was clear that **Compound 8** is very active against Human Caucasian breast carcinoma cell line (**ZR75-1**) at different doses having low IC_{50} (3.15 µM/mL)

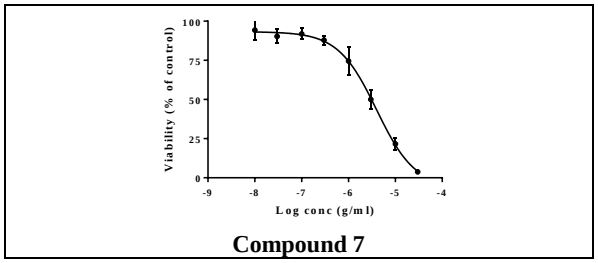

Figure. 7. Dose response curve of F11 and compound 1 against Human Caucasian breast carcinoma cell line (**ZR75-1**). (n=3)

CONCLUSION

In conclusion the methanolic extract of A. *anthelmintica* leaves contains phenolic compounds, some of which isolated for first time from this species. Some of the isolated compounds showed strong antioxidant activity and strong cytotoxic activity against both human ovarian carcinoma cell line (A2780) and human Caucasian breast carcinoma cell line (ZR75-1), thus thee active compounds needs to be evaluated in animal models to determine it's potential as natural cure products.

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

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