ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR / Sec. A
Elsayed et al., 2022, 6 (2), 58-67

Journal of Advanced Pharmacy Research

Section A: Natural Products & Metabolomics

Phenolic Profile and Antioxidant-Related Activities of Astrophytum ornatum (DC) Britton & Rose Flowers Cultivated in Egypt

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Submitted on: 26-01-2022; Revised on: 25-02-2021; Accepted on: 25-02-2022

To cite this article: Elsayed, H. E.; Ebrahim, H. Y.; Elsayed, M. H.; Haggag, E. G.; Choucry, M. A. Phenolic Profile and Antioxidant-Related Activities of *Astrophytum ornatum* (DC) Britton & Rose Flowers Cultivated in Egypt. *J. Adv. Pharm. Res.* **2022**, *6* (2), 58-67. DOI: 10.21608/aprh.2022.115708.1150

ABSTRACT

Objectives: The current study aimed at the phenolic profiling, antioxidant, anti-aging, anti-arthritic *in vitro* screening of the 80% aq. methanol extract of *Astrophytum ornatum* (DC) Britton & Rose flowers for the first time. **Methods:** Phytochemical screening was accomplished using the standard adopted techniques as per constituent. Total phenolic and flavonoid contents were quantified using Folin-ciocalteau and aluminum chloride colorimetric assays, respectively. HPLC, using available reference phenolics, was implemented for the tentative identification of the phenolic metabolites. The extract was subjected to fractionation and isolation of its major components using open column chromatography packed with relevant stationary phases, while the structure of the isolated metabolites was elucidated based on the physical, chromatographic properties and/ or spectroscopic data. Finally, the antioxidant, anti-aging, and anti-arthritic potential of the extract was determined in terms of the DPPH radical scavenging, anti-collagenase, and protein denaturation inhibitory effect, respectively. **Results:** panel of metabolites were revealed from the qualitative phytochemical screening for instance, carbohydrates and/glycosides, flavonoids, tannins, saponins, and essential oils. The quantified total phenolic and flavonoid contents were calculated as 222.51± 0.24 µg GAE/mg and 138.18± 0.10 µg QE/mg, respectively. The HPLC tentatively identified eleven phenolic acids (representing 5.4626 %) and eleven flavonoids (representing 19.5273 %) among which naringin represents the major identified flavonoid glycoside (5.7055 %) while, apigenin (4.1018%) is the major identified aglycon. In the same context, the chromatographic fractionation afforded four metabolites *viz*, mixture of isoquercetrin (**1**), hyperoside (**2**), quercetin (**3**), and kaempferol (**4**). From the pharmacological perspective, the tested extract displayed promising IC_{50} =79.3 ± 3.8, 62.4 ± 2.1, and 54.6 ± 2.7 µg/ml in the DPPH radical scavenging, anti-collagenase, and protein denaturation assays, respectively. **Conclusion:** *Astrophytum ornatum* flowers are valuable source of bio-active flavonoids that may in part displayed a significant role in the management of free radical related disorders just as aging and arthritis.

***Keywords:** Anti-arthritic; Anti-collagenase; Antioxidant; Astrophytum; Flavonoids; HPLC*

ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

INTRODUCTION

Reactive oxygen species (ROS) are molecular species generated in the human body from either intrinsic source as mitochondria metabolic activity or extrinsic ones as radiation and xenobiotics[1]. ROS possess high biological instability due to the availability of unpaired electrons in their outer valency, hence become highly reactive and unstable[2]. Although, ROS can be considered as casual intermediates in biological processes, they mediate homeostatic imbalance by interacting with the biological macromolecules as proteins, DNA, and lipids causing protein and DNA damage along with lipid peroxidation[3]. These changes contribute to the onset of frequent disorders including inflammation, aging, infectious diseases, cardiovascular diseases, and cancer[1]. Homeostasis maintenance needs an effective balance between reactive species production and their removal, a mission that could be achieved by scavenging antioxidants. Antioxidants are stable and safe scavenging compounds that serve as chemical traps for ROS[1]. Accordingly, they suppress the uncontrolled formation of ROS, prevent the consequent damages, and protect against free radical-related diseases[4]. Antioxidants can be classified into two main classes, natural and synthetic[8]. Synthetic antioxidants are thought to cause or endorse negative health effects in humans as mutagenesis[5]. On the other side, natural antioxidants are safe and exert various health benefits[6]. Accordingly, it is worth mentioning that there is an inclination trend to replace the synthetic antioxidants with naturally occurring ones[6]. Natural antioxidants are abundant in various plants extracts represented by various secondary metabolites as, phenolics and their related natural products[10]. They act as a defense mechanism, help in controlling the formation of ROS or inhibit their reaction with biological structures[7], hence protect against some conditions as aging and inflammation.

Aging is a complex process characterized by a progressive decline in physiological function of organs[8]. The entire process is modulated either genetically or environmentally[8]. According to free radical theory, aging is due to accumulation of free radicals that damage the cellular components[9]. During this process ROS leads to the activation of hyaluronidase, collagenase, and elastase enzymes, which support initiation of aging[9]. Hence, the discovery of antioxidant rich plant-extracts which inhibits the forenamed enzymes is a promising strategy to manage skin aging disorders. On the other side, arthritis is a progressive, disabling, inflammatory disorder that affects the joints[10]. It is characterized by pain, swelling and stiffness of the synovial joints. The exact etiology of arthritis is not known, but it is believed to be the result of protein denaturation[11]. Protein denaturation causes the inflammatory and arthritic disorders to proceed to auto-antigens generation, thus resulting in rheumatic disorders[11, 12]. The denaturation possibly implicates variation in hydrogen, electrostatic, disulphide, and hydrophobic bonds[12]. The management of arthritis and other inflammatory disorders involves the use of different classes of drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), and corticosteroids which all correlated to a panel of undesired side effects[11]. Hence, there is a continuous search for alternative, natural products with antiarthritic potential.

Family Cactaceae comprises succulent, flowering plants in the order Caryophyllales with nearly 2000 species and 139 genera native to the Americas[13]. They are considered as one of the common ornamental plants nowadays[13]. Cacti are characterized by being able to survive long periods of drought owing to their anatomical and physiological adaptations[13]. Among the well-known cacti genera is the genus *Astrophytum* aptly named from the Greek word *Astron*, which means a star shaped plant. It comprises only six species[14]. Scarce literature was reported concerning the chemical composition of genus *Astrophytum* except for the detection of few flavonoids from the flowers of its species[15, 16] which are linked to noticeable biological activities as antimicrobial, amoebicidal, antioxidant, and retino-protective potential.[16, 17]

The current study aimed at the characterization of phenolic phytoconstituents in the aqueous methanol extract of *A. ornatum* (DC) Britton & Rose flowers collected from Egypt. Moreover, the *in vitro* antioxidant, antiaging, and antiarthritic potential of the extract was assessed in the available in-house relevant assays.

MATERIAL AND METHODS

General experimental procedures

Reversed phase silica gel (RP_{18}, 25-40 µm, Merck, Germany) stationary phase was exclusively used for packing open column chromatography (CC) and eluted with various ratios of aqueous methanol. Silica gel 60 F_{254} precoated aluminum thin layer chromatography (TLC) sheets (20 x 20, 0.2 mm thickness, Merck, Germany) were used for the examination of the eluted fractions and isolated pure metabolites using Ethyl acetate: Formic acid: Acetic acid: Water (100:11:11:26 v/v/v/v) as solvent system. The chromatograms were examined under UV light (UV lamp, VL-215 LC, Marne La Vallee, France), and after spraying with $FeCl_3$ or freshly prepared *p*-anisaldehyde: methanol: acetic acid: sulfuric acid (2:170:20:10 v/v/v/v) reagents. For structure elucidation of isolated metabolites, 1D- and 2D-NMR spectra were obtained on Bruker APX-NMR spectrometers operated at 400 MHz for ^{1}H NMR, while 100 MHz for ^{13}C NMR. Chemical shifts (δ) were reported in parts per million (ppm) relative to TMS as internal standard. The mass spectrometry was carried out on AB Sciex-3200 QTRAP LC/MS/MS system (Applied

Biosystems, Foster City, CA) in negative ion acquisition mode. Analytes were ionized using electrospray ionization (ESI) interfaced with standard turbo V ion source.

Plant material

The flowers of *Astrophytum ornatum* (DC) Britton & Rose were collected from Helal Cactus Farm, Al Mansoureyah, Giza, Egypt, during December 2018. A specimen was identified by Prof. Dr. Mohamed El-Gebaly, senior taxonomist at Al Orman Botanical Garden, Giza, Egypt. A dried sample was preserved at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Cairo, Egypt and coded as 01Aor/2018.

Phytochemical Characterization

Phytochemical screening

About 50 g of the air-dried powdered flowers were used for the qualitative phytochemical screening. The detection of the plant's secondary metabolites as alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, saponins, tannins, triterpenes and/ sterols were performed following the standard procedures described per each experiment as previously stated by Elsayed and co-workers[18]. All chemicals were purchased from sigma (Sigma-Aldrich, MO, USA), while the solvents were obtained in analytical grade from El Nasr Pharmaceutical Chemicals Co., Egypt.

Total phenolic content analysis

Total phenolic content (TPC) was estimated using Folin-Ciocalteu colorimetric method[19]. Briefly, about 10 mg of the methanolic extract was dissolved in 10 mL distilled water: methanol (50:50). Thereafter, 1 mL of the prepared stock solution was diluted 10 times with distilled water and mixed with 1.5 mL of Folin-Ciocalteu reagent (LOBA Chemie PVT., Ltd, India). After 5 min, 4 mL of 20% Na_2CO_3 was added, and the volume was completed to 25 mL with distilled water. The samples were further incubated for 30 min at room temperature and the absorbance was measured colorimetrically at 765 nm in triplicates using UV spectrophotometer (Shimadzu UV-1650PC, Shimadzu corporation, Japan). The TPC was calculated based on the regression equation of the calibration curve of gallic acid Sigma-Aldrich (Sigma-Aldrich, MO, USA) and expressed as gallic acid equivalents (GAE)/g sample.

Total flavonoid content analysis

Total flavonoids content (TFC) was determined using the aluminum chloride colorimetric method[20]. Shortly, the stock solution was prepared by dissolving 10 mg of the prepared extract in 10 mL 70 % methanol. Then, to 1 mL of the extract stock (1 mg/mL), was added 1.5 mL methanol, 0.1 mL $AlCl_3$, 0.1 mL 1M potassium acetate, and 2.8 mL distilled water. The absorbance of the prepared samples was recorded after 30 min at 415 nm in triplicates using an UV spectrophotometer (Shimadzu UV-1650PC, Shimadzu corporation, Japan). The TFC was expressed as quercetin equivalent (QE)/ g sample based on the regression equation of the calibration curve of quercetin (Sigma-Aldrich, MO, USA).

HPLC Phenolic Profile

The extract sample for the HPLC analysis was prepared according to the method described by Jakopic and co-workers[21]. Simply, 100 mg of the 80 % aq. methanol extract of the flowers was extracted with 10 ml methanol in an ultrasonic bath for 45 min. Then, the extract was centrifuged for 7 min at 4200 rpm and the supernatant was filtered through polyamide filter Chromafil AO-45/25 and kept in a dry vial. About 1-3 mL was collected in separate vials for injection into HPLC Agilent 1200 series, with auto-sampling injector. HPLC profiling started with linear gradient at a flow rate of 1 mL/min and the injection volume 10 µL with mobile phase of water/acetic acid (98: 2 v/v, solvent A) and methanol/acetonitrile (50:50, v/v, solvent B), starting with 5 % B and increasing B to 30 % at 25 min, 40 % at 35 min, 52% at 40 min, 70% at 50 min, 100% at 55 min. The developed chromatograms were plotted after detection at λ_{max} 280 and 330 nm. The identified compounds were deduced by comparing their retention times with that of the available reference standard-phenolic (Sigma-Aldrich, MO, USA) that were injected under the same conditions. The quantitative estimation of each identified component depends on the relative measurement of area under the peak area %.

Extraction, fractionation, and structure elucidation of isolated metabolites

Astrophytum ornatum (DC) Britton & Rose fresh flowers (40 gm) were air-dried in the shade and grounded to fine powder. The dried powder (20 gm) was macerated at room temperature in 80 % aq. methanol (500 mL x 4). Thereafter, the extracts were pooled together, filtered, and dried under reduced pressure at 50 ^oC using rotary evaporator to yield 2 gm dried, dark yellow extract. The extract was dissolved in least amount of MeOH and adsorbed on 30 g RP_{18} reversed phase silica with subsequent evaporation of the solvent using water bath. The loaded RP_{18} was applied to RP_{18} reversed phase silica open column chromatography (CC) and eluted with a step gradient starting with 10 % MeOH: H_2O followed by decreasing polarity to 100 % MeOH affording three main collective fractions F_1, F_2, and F_3. Collective fraction F_1 (40 % MeOH/H_2O, 0.5 g) was re-chromatographed using RP_{18} reversed phase silica CC eluted with 30 % MeOH/H_2O yielded a mixture of compounds **1** and **2** (50 mg). On the other side, collective

fraction F_2 (70% $MeOH/H_2O$, 200 mg), was re-chromatographed using RP_{18} reversed phase silica CC eluted with 60% $MeOH/H_2O$ affording pure compound **3** (35 mg). Finally, collective fraction F_3 (80% $MeOH/H_2O$, 300 mg) was re-chromatographed using RP-18 reversed phase column chromatography eluted with 60% $MeOH/H_2O$ affording pure compound **4** (28 mg).

Quercetin-3-*O*-*β*-D-glucoside (Isoquercetrin, 1)

Isolated as yellow amorphous powder and shown as dark purple spot under UV light (λ 254/365 nm). It develops green colour with $FeCl_3$ spray reagent and yellow color with *p*-anisaldehyde/H_2SO_4 after heating chromatogram. A molecular ion peak $[M-H]^-$ was displayed at m/z 463.1809. The ^1H- ,^{13}C-NMR data and key HMBC correlations are delineated in **Table 1**.

Quercetin-3-*O*-*β*-D-galactoside (Hyperoside, 2)

Obtained as yellow amorphous powder and exhibited dark purple colour under UV light (λ 254/365 nm), develops green colour with $FeCl_3$ spray reagent, and yellow color with *p*-anisaldehyde/H_2SO_4 after heating chromatogram. It displayed molecular ion peak $[M-H]^-$ at m/z 463.1809. The ^1H-, ^{13}C-NMR data, and key HMBC correlations are outlined in **Table 1**.

Quercetin (3)

Isolated as yellow amorphous powder that shown as dark purple spot under UV light (λ 254 nm/365 nm). It develops green colour with $FeCl_3$ and yellow color with *p*-anisaldehyde/H_2SO_4 spray reagents after heating chromatogram. Compound **3** was elucidated based on its chromatographic properties, negative ESI-MS with $[M-H]^-$ at *m/z* 301.1119, and comparison with reference sample.

Kaempferol (4)

Obtained as yellow amorphous powder that shown dark purple spot under UV light (λ 254 nm/365 nm), develops green colour with $FeCl_3$ spray reagent, and yellow color with *p*-anisaldehyde/H_2SO_4 after heating chromatogram. It possess $[M-H]^-$ base peak at m/z = 285.1220 in the negative mode ESI-MS and elucidated based on its physical, chromatographic properties, and comparison with reference sample.

***In vitro* Biological Activities**

2,2-diphenyl-1-picrylhydrazyl (DDPH) radical scavenging assay

The radical scavenging assay measures the antioxidant capability of metabolites that transfer hydrogen atom to DPPH reagent radical, hence the reagent colour changes from violet to yellow[22]. Simply, the tested extract was prepared at various concentration by two-fold serial dilution (0-1280 µg/mL). Thereafter, 40 mL aliquot of each concentration was added to 3 mL of the freshly prepared DPPH reagent. The samples absorbance was recorded immediately (at T=0 min) for DPPH alone (violet colour) as negative control at $λ_{max}$517 nm on UV-visible spectrophotometer (Milton Roy, Spectronic 1201), then after 16 min time interval (after the reaction colour become stable) for DPPH mixed with the tested extract or with ascorbic acid as positive control drug[21]. All the determinations were performed in three replicates and averaged.

% Inhibition of the DPPH radical was calculated according to the formula:

$$\% \text{ inhibition} = [\{(A_C - A_T)/ A_C\} \times 100]$$

Where A_C = Absorbance of the negative control at t = 0 min, A_T = absorbance of the tested sample and DPPH at t = 16 min. The 50% inhibitory concentration (IC_{50}) is the concentration required to inhibit the DPPH radical by 50% and it was estimated from graphic plots of the dose response curve.

Albumin denaturation assay

The albumin denaturation model was implemented to evaluate the ability of aqueous methanol extract to protect the bovine albumin from heat-induced denaturation[23]. The tested extract was prepared in final concentrations of 1000-70.81 µg/mL. Briefly, in a 96-well plate, 100 µL of TME and 100 µL of 1% bovine albumin solution were mixed. The plate was incubated at 28˚C for 15 min, then at 70˚C for 10 min to induce denaturation. Thereafter, the reaction mixtures were cooled and % turbidity was recorded at 660 nm using microplate reader (Biotek, 800 Ts)[23]. Diclofenac sodium was used as standard drug in the concentration of 1000-7.81 µg/mL and treated similarly as TME. The percentage of bovine albumins denaturation inhibition was calculated compared to control in which no drug was added and calculated as following:

$$\% \text{ Inhibition of protein denaturation} = 1- (A_1/A_2) \times 100$$

Where A_1 is the absorbance of test /standard sample with albumin solution, A_2 is the absorbance of the blank control. The experiment was carried out in triplicate and the IC_{50} was calculated from a dose response curve of % inhibition against various concentrations.

Anti-collagenase assay

The antiaging activity of aqueous methanol extract was evaluated using the *in vitro* anti-collagenase assay adopting the method stated in literature with some modifications[24]. Prior to screening, UV spectrum for TME was recorded on a Cary 300 UV-visible spectrophotometer to check for interference and shifts in the absorption. Briefly, 50 mM Tricine buffer (pH 7.5 with 400 mM NaCl and 10 mM $CaCl_2$) and collagenase from *Clostridium histolyticum* (ChC) was dissolved in

Table 1: HPLC phenolic profiling of the 80% aqueous methanol extract of *A. ornatum* flowers compared to the available authentic standards

Peak No.	R_t exp. (min)	Reference standard phenolics	Molecular formula	R_t std. (min)	Area %
1	4.863	Pyrogallol	$C_6H_6O_3$	4.861	0.0616
2	5.397	Gallic acid	$C_7H_6O_5$	5.409	0.0585
3	6.678	Catechol	$C_6H_6O_2$	6.666	0.5861
4	7.102	Catechin	$C_{15}H_{14}O_6$	7.099	0.1310
5	7.701	Chlorogenic acid	$C_{16}H_{18}O_9$	7.700	0.2244
6	7.864	*p*-Hydroxy benzoic acid	$C_7H_6O_3$	7.869	0.6571
7	8.255	Vanillic acid	$C_8H_8O_4$	8.257	0.2021
8	8.392	Caffeic acid	$C_9H_8O_4$	8.393	0.7920
9	10.026	Ferulic acid	$C_{10}H_{10}O_4$	10.003	0.7754
10	10.020	Apigenin-6-*C*-*α*-L-arabinoside-8-*C*-*β*-D-galactopyranoside	$C_{26}H_{28}O_{14}$	10.003	0.5964
11	10.773	Hesperetin-7-*O*-rutinoside (Hesperidin)	$C_{28}H_{34}O_{15}$	10.774	0.4360
12	10.820	Rosmarinic acid	$C_{18}H_{16}O_8$	10.817	0.1891
13	10.915	Salicylic acid	$C_7H_6O_3$	10.911	1.4741
14	10.910	Quercetin-3-*O*-rutinoside (Rutin)	$C_{27}H_{30}O_{16}$	10.907	1.5175
15	11.543	Apigenin-7-*O*-*β*-D-glucopyranoside	$C_{21}H_{20}O_{10}$	11.545	0.5034
16	11.534	Ellagic acid	$C_{14}H_6O_8$	11.535	0.5003
17	11.890	Quercetin-3-*O*-*α*-L-rhamnopyranoside (Quercitrin)	$C_{21}H_{20}O_{11}$	11.889	0.8751
18	13.080	Naringenin-7-*O*-neohesperidoside (Naringin)	$C_{27}H_{32}O_{14}$	13.076	5.7055
19	13.210	Quercetin	$C_{15}H_{10}O_7$	13.209	3.5088
20	13.879	Kaempferol-3-*O*-[2``-*p*-coumaroyl-glucoside)	$C_{30}H_{26}O_{13}$	13.885	1.1678
21	14.507	Kaempferol	$C_{15}H_{10}O_6$	14.508	0.9259
22	14.867	Apigenin	$C_{15}H_{10}O_5$	14.868	4.1018

Highlighted in blue the most abundant phenolic acid, in orange the most abundant flavonoid glycoside, and in green the most abundant aglycone.

buffer at an initial concentration of 0.8 units/ml. Then, the synthetic substrate *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was dissolved in Tricine buffer to 2 mM. Thereafter, the tested extract was prepared at various concentrations (1000-7.81 µg/mL) and incubated with the collagenase for 15 min before adding the substrate to start the reaction. Negative controls were performed using water instead of tested samples. The final reaction mixture (150 µL total volume) contains Tricine buffer, 0.8 mM FALGPA, 0.1 units ChC and 25 µg test extracts. The tested samples absorbance's were measured immediately at λ_{335} nm after adding the

substrate, then continuously for 20 min using a microplate reader (BioTek, 800 TS, USA). Epigallocatechin gallate (EGCG, 250 µM) was used as a positive control. The IC_{50} value was defined as the concentration of sample to inhibit 50% of collagenase under the assay conditions. The percent of enzyme inhibition was calculated as following:

$$\% \text{ Collagenase inhibition} = (1 - S/C) \times 100$$

S is the corrected absorbance of the samples containing collagenase inhibitor (the enzyme activity in the presence of the samples), *C* is the corrected absorbance of negative controls (the enzyme activity in the absence of the samples).

Statistical analysis

The results are expressed as mean percentage ± SD of at least three independent experiments. Data were analyzed with one-way analysis of variance (ANOVA) and one-tailed unpaired test using GraphPad Prism software (GraphPad Prism 5.0, GraphPad Software, Inc., CA, USA), with $p \leq 0.05$ are considered as statistically significant. IC_{50} were calculated by nonlinear regression analysis using GraphPad Prism software.

RESULTS AND DISCUSSION

Medicinal plants have long been reported as a prospective hub of natural metabolites just as polyphenols which are generated by plant to defend itself or to promote the growth under unfavorable conditions[25]. As well, polyphenols possess various biological activities and health benefits to mankind which almost correlated to their potent antioxidant and anti-inflammatory potential[28]. Herein, *A. ornatum* (DC) Britton & Rose flowers were subjected to detailed investigation of their phenolic constituents using various qualitative and quantitative studies. Primarily, the qualitative phytochemical analysis was accomplished on the dry, powdered flowers of *A. ornatum* as per standard procedures stated before[18]. The results disclosed the presence of variable classes of metabolites as carbohydrates and/or glycosides, saponins, tannins, flavonoids, essential oil, and coumarins. As our major interest focused on phenolic metabolites, hence it was deemed of interest to quantify the total phenolic content (TPC) content in the flower's extract using one of the commonly adopted methods as Folin-ciocalteu colorimetric assay. This assay is one of the widely applied methods to estimate the total phenol/polyphenol content of plant-derived food and biological samples[19, 26]. The colorimetric reaction relies on the transfer of electrons in basic medium from phenolic metabolites to form a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex (the reagent). The absorbance of the reduced reagent is measured spectrophotometry with gallic acid as the reference standard. The results revealed considerable total phenolic content 222.51± 0.24 µg GAE/mg (**Figure 1**). In the same context, total flavonoid content (TFC) was assayed using $AlCl_3$ method which relies on the spectrophotometric detection of colored complexes formed between Al (III) and the carbonyl and hydroxyl groups of flavonoids in alkaline medium[20]. The obtained values were expressed as quercetin equivalent per milligram of the extract (µg QE/mg extract). The results showed that the total extract possessed considerable flavonoid content which almost half fold that of the TFC and measured as 138.18± 0.1 µg QE/mg (**Figure 1**). In all, the total phenolic and flavonoid contents elicited our curiosity to tentatively investigate the phenolic profile for the total extract using HPLC technique synchronously with the available phenolic acids and flavonoids standards. The results (**Table 1**) revealed the identification of eleven phenolic acids (representing 5.46%) and eleven flavonoids (representing 19.52%), among which naringenin-7-*O*-neohesperidoside (Naringin) was the major detected glycoside (5.70%), while apigenin (4.1018%) and quercetin (3.5088 %) were the major aglycones, respectively. It was obvious that some outstanding peaks were kept ambiguous due to the unavailability of some authentic standards, hence the total extract was subjected to fractionation using open column chromatography packed with an appropriate stationary phase (RP-18) and eluted with suitable mobile systems as stated per each experiment aimed at the isolation of the major unidentified phenolic metabolites. Interestingly, four flavonoids (**Figure 2**) were isolated, and their structures were identified as isoquercetrin (**1**) and hyperoside (**2**) based on their physical, chromatographic, ESI-MS spectra, 1D- and 2D-NMR data (**Table 2**) that were in accordance with those previously published in the literature. On the other side, quercetin (**3**) and kaempferol (**4**) were isolated and identified based on their physical, chromatographic, ESI-MS spectra, and comparison with reference authentic sample[27-29].

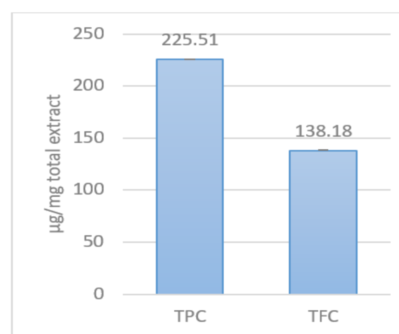
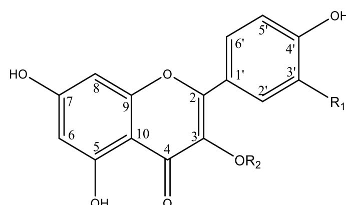


Figure 1. Total phenolic content (TPC) and total flavonoid content (TFC) of the 80% aqueous methanol extract of *A. ornatum* flowers.

Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data of flavonoids 1 and 2[a]

Atom No.	1			2		
	δ_H	δ_C	HMBC correlations	δ_H	δ_C	HMBC correlations
2		157.2			157.6	
3		134.2	**C-1``**		134.4	**C-1``**
4		178.0			178.0	
5		161.5			161.5	
6	6.22, br s	98.5	**C-8, C-10**	6.22, br s	98.5	**C-8, C-10**
7		164.6			164.6	
8	6.40, br s	93.3	**C-6, C-7, C-9**	6.40, br s	93.3	**C-6, C-7, C-9**
9		157.0			157.0	
10		104.1			104.1	
1`		121.5			121.6	
2`	7.89, br s	116.2		7.91, br s	116.4	
3`		144.3			144.3	
4`		148.4			148.5	
5`	6.91, d (7.8)	114.7	**C-6`, C-3`, C-4`**	6.91, d (7.8)	114.7	**C-6`, C-3`, C-4`**
6`	7.73, d (7.9)	122.8	**C-1`, C-4`**	7.73, d (7.9)	122.2	**C-1`, C-4`**
1``	5.23, d (7.0)	103.0	**C-3**	5.28, d (7.1)	103.4	**C-3**
2``	3.90- 3.60, m	74.3		3.86- 3.62, m	74.2	
3``		76.9			73.7	
4``		69.8			68.6	
5``		76.7			75.6	
6``		61.1			60.5	

[a] *data was recorded in CD_3OD*



1: R_1=OH, R_2=Glu
2: R_1=OH, R_2=Glc
3: R_1=OH, R_2=OH
4: R_1=H, R_2=OH

Figure 2. Chemical structures of flavonoids 1-4 isolated from the 80% aqueous methanol extract of *A. ornatum* flowers

The supplementation with natural antioxidants, as flavonoids and phenolic acids, is traditionally considered by many scholars and medical practitioners as a reasonable strategy to delay or prevent oxidative stress-related pathological conditions[30]. According to our promising findings concerning the phenolic investigation of the 80% aq. methanol extract of *A. ornatum* flowers, it was interesting to investigate antioxidant capacity of the total extract for the first time in terms of DPPH scavenging potential. The results (**Figure 3**) are shown in comparison to the reference standard drug, ascorbic acid. It was observed that the tested extract possessed significant DPPH scavenging potential with IC_{50}=79.3 µg/ml compared to the standard, ascorbic acid (IC_{50}=14.2 ± 1.31 %). While at the maximum tested concentration for the extract (1.28 mg/ mL), it exerted 93.47 ± 1.33% DPPH scavenging. The activity may be correlated in part to the estimated phenolic metabolites[30-33] which were appreciated by many researchers for their antioxidant potential. An effect by virtue to their unique SAR as singlet oxygen scavengers, reducing agents, and hydrogen atom donators[34], hence they could serve to protect against several ailments, examples as, but not restricted to cancer, metabolic, and inflammatory disorders. Consequently, the total extract was screened in antiaging and anti-arthritic appropriate assays to point out their antioxidant related profits in the management of the previously mentioned disorders. For the investigation of the anti-arthritic activity of the tested extract, protein denaturation assay was authorized using 1% bovine serum albumin (BSA). Upon exposure to heat BSA undergoes denaturation which is a well-documented

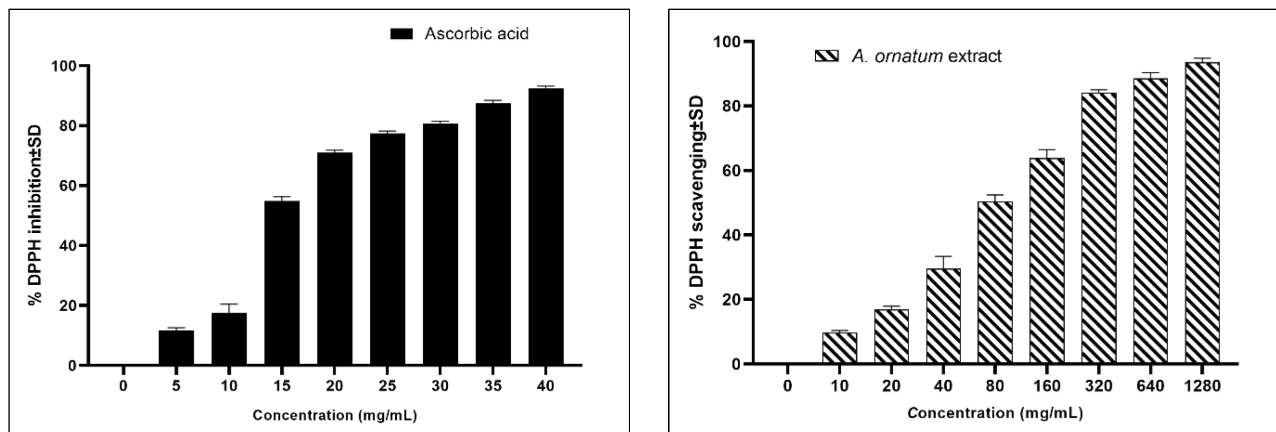


Figure 3. Antioxidant activity of the 80% aqueous methanol extract of *A. ornatum* flowers measured by % DPPH scavenging potential compared to the standard ascorbic acid

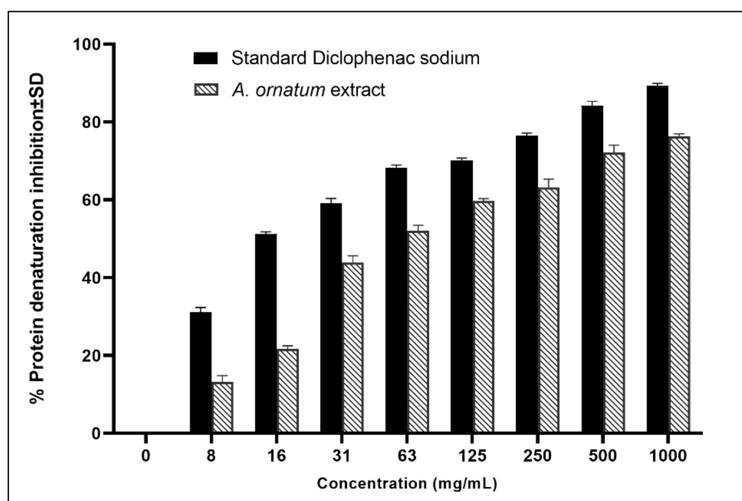


Figure 4. Anti-arthritic activity of the 80% aqueous methanol extract of *A. ornatum* flowers in protein denaturation assay compared to the standard drug, diclofenac sodium.

cause for inflammatory disorders[35]. Ultimately, the findings showed that the extract of *A. ornatum* flower's exhibited promising, significant dose-dependent inhibition for the protein denaturation (**Figure 4**) with IC_{50}=54.6 ± 2.7 µg/ml which is nearly three and half-fold less potent than the reference standard drug, diclofenac sodium (IC_{50}=15.12 µg/mL). The observations may be correlated to the presence of various phytoconstituents as saponins, flavonoids, and tannins which were reported in the literature to reduce inflammatory markers through an antioxidant mechanism and reduction of arthritic indexes[36]. On the other side, the inhibitory activities of various flavonoids on collagenase from *Clostridium histolyticum,* to establish their therapeutic potential against skin inflammation and photoaging, is well documented[35]. Prior data demonstrated that flavonols were stronger inhibitors than the other flavonoid classes which shows up the significance of the C-3 hydroxyl substitution, a pharmacophore represented in most of the identified flavonoids. Subsequently, the collagenase inhibitory activity of the flower's extract was estimated for the first time and the results are shown in (Figure **6**) compared to the positive control, epigallocatechin gallate (EGCG). From the analysis of the experimental data, *A. ornatum* extract apparently inhibit the *α*-collagenase enzymatic activity with IC_{50} =62.4 µg/mL which is almost half fold less potent than EGCG (IC_{50} =40.3 µg/mL). Hence the total extract which is enriched with various flavonols may linked to the prevention of collagen breakdown by inhibiting collagenase in inflamed skin as well as photoaged skin. Although there is hardly an "all in one" component that exhibits the

previously mentioned bioactivities, but we could correlate the findings in part, to the presence of various array of phenolic secondary metabolites.

CONCLUSION

Our findings provide evidence that *A. ornatum* flowers are potential source of various natural antioxidants. HPLC is a significant tool in revealing the major classes of bioactive phytoconstituents with their subsequent quantitative estimation. The synergistic interactions among different bioactive classes of antioxidants, could be useful to develop adjuvant therapeutic agents for the management of aging and arthritic disorders.

Funding Acknowledgment

No external funding was received.

Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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