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Phytochemical Composition, Antioxidant Potential and α-Amylase Inhibitory Activity of Different Extracts from *Aaronsohnia pubescens* (Desf.)

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Abstract

The medicinal plant *Aaronsohnia pubescens* is currently used in the algerian phytotherapy to treat several disorders. In order to investigate the potential influence of *A. pubescens*, six extracts were screened for their antioxidant and anti-amylase capacities. Antioxidant ability was evaluated by different assays as well as scavenging effect of free radicals (DPPH, ABTS, GOR and hydroxyl radical), metal chelating, reducing power, CUPRAC and total antioxidant capacity. Total phenolic and flavonoid contents in these extracts were also measured. Phenolic compounds in different extracts were identified using the HPLC-DAD analysis. The total phenolic contents of these extracts ranged from 70 to 335.9 mg GAE/g. Generally, the highest amounts of total phenolic and flavonoid contents were observed in ethyl acetate and *n*-butanol extracts, which may explain their strong antioxidant activity. All extracts demonstrated a significant inhibitory activity on α-amylase. Based on these findings, *A. pubescens* could serve as a source of natural compounds for oxidative damage and diabetes mellitus management.

Keywords: *Aaronsohnia pubescens*, antioxidant activity, flavonoid content, phenolic content, ethyl acetate extract, *n*-butanol extract.

1. Introduction

Reactive oxygen species (ROS) are constantly produced in cells and involved in many physiological processes such as host defense and cellular signaling (Ray *et al.,* 2012). Cell is equipped with antioxidant systems to eliminate ROS and preserving redox homeostasis. Excessive ROS production can cause cell death and contribute to disease development (Liguori *et al.,* 2018). For a long time, herbal medicine was the main or even the only resource of our ancestors for their health conditions. However, despite the growth of pharmaceutical industries, the use of medicinal plants and plant products has never been totally abandoned and people continue to resort to traditional medicine.

During the last decades, the antioxidant therapy research has increased tremendously with the aim to prevent or remediate oxidative stress damages. Recently, considerable attention has been devoted to naturally occurring antioxidants in medicine industry as an alternative to synthetic antioxidants, which are gradually restricted due to their possible side effects such as the dysfunction of reproductive system (Naresh and Nidhi, 2019). Plant extracts possess pools of biologically active molecules including antioxidants. Natural antioxidants "carotenoids, phenolic acids, flavonoids, coumarins, etc." are generally of low toxicity at their bioavailable dose, and furthermore they can act synergistically (Kancheva and Kasaikina, 2013).

Aaronsohnia pubescens (Desf.) K. Bremer & Humphries, syn. *Matricaria pubescens* (Desf.) called Ouazouaza in arabic, is a medicinal plant that belongs to the Asteraceae family. It is largely distributed in the northern and central Algerian Sahara (Benchelah *et al.,* 2000 and Makhloufi *et al.,* 2015). In Algeria, *A. pubescens* is widely used in folk medicine as an antiseptic, antirheumatic, antiarthritic, antidiarrheal and to treat digestive complications. It is also used as a condiment and as an aromata in the preparation of some traditional dishes (Cherif *et al.,* 2017 and Hammiche and Maiza, 2006). Several studies of *A. pubescens* chemical composition revealed its richness in terpenoids (e. g., *α*-pinine, Z-β-ocimene *α*-curcumene and 6-oxo-cyclonerolido), and phenolic compounds such as apigenin, luteolin, quercetin and herniarin (Boutaghane *et al.,* 2011; Makhloufi et *al.,* 2015 and Sharifi-Rad et *al.,* 2018). Furthermore, there are no previous reports on the antioxidant and enzyme inhibitory potentials of *A. pubescens*.

The main objectives of the present study were (1) to determine the polyphenolic profile (total phenolic, total flavonoid, benzoates, hydroxycinnamates and flavonols) of different extracts from *A. pubescens*; to reveal (2) antioxidant activity (DPPH, ABTS, GOR, ect.) of *A. pubescens*; and (3) to assess α-amylase inhibitory activity

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of the extracts. Six extracts from the aerial parts of *A. pubescens* were evaluated in this study.

2. Materials and Methods

2.1. Chemicals

Folin-Ciocalteu's phenol reagent, 2,2- azino-bis (3-ethylbanzthiazoline-6- sulphonic acid (ABTS), 2,6-Di-tert-butyl-α-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy (Galvinoxyl), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) , 2-deoxyribose, ammonium molybdate ferric chloride, neocuproine, Trichloroacetic Acid (TCA), Thiobarbituric Acid (TBA), iodine potassium iodide, rutin, cafeic acid, ascorbic acid, gallic acid , $AlCl_3$ and porcine pancreatic *α*-amylase (EC 3.2.1.1, type VI-B) were obtained from Sigma-Aldrich Chemicals. Solvents and other reagents were of analytical grade.

2.2. Materials

Aerial parts of *A. pubescens* were collected in March 2018 from Ghardaïa, a region in southern Algeria, (Altitude, 450m). After identification, a voucher specimen (LBE 18/03) was deposited at the Herbarium of the Faculty of Natural and Life Sciences at the University of Frères Mentouri, Constantine 1. Aerial parts of *A. pubescens* were air-dried in the laboratory and stored before use in a dry place.

2.3. Preparation of Aqueous lyophilized extract

Twenty grams of dried raw material was immersed in 300 mL of hot distilled water and agitated for 30 min. The extract solution was filtered and lyophilized (LaBconco, FreeZone Benchtop Freeze Dryer) at −80 °C. The aqueous lyophilized extract (AQL; 3 g, 15% w/w) was stored at −20 °C until analysis.

2.4. Preparation of organic extracts

The dried raw material (200g) was immersed in 1000 mL of methanol for 24h at room temperature then filtered. The filtrate was evaporated at 37 °C until dryness to give the methanol extract (MeOH; 33.55 g, 16.7% w/w) then rinsed with a hot water. As previously described by Kandouli *et al.* (2017), the aqueous solution was treated with petroleum ether, ethyl acetate and *n*-butanol in sequence. In this way, the following extracts were produced: petroleum ether extract (PE; 2.13 g, 1.1 % w/w), ethyl acetate extract (EtOAc; 3.2 g, 1.5% w/w) and *n*-butanol extract (*n*-BuOH; 5 g, 2.5% w/w) and aqueous residual fraction (AR; 4 g, 2% w/w). Each fraction was stored at −20 °C until further analysis.

2.5. Determination of phenolic contents

The total phenolic content was determined using Folin–Ciocalteu assay (Singleton *et al.,* 1999). An aliquot of 160 µL extract solution (0.1 mg/mL) was mixed with 800 µL of Folin–Ciocalteu reagent (0.2 N). After 5 min, 640 µL of sodium carbonate (75 %, w/v) was added and incubated for 2h at room temperature. The absorbance was measured at 760 nm against a blank without extract. The TPC was calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per gram of dry weight.

2.6. Determination of flavonoid contents

The total flavonoid content was determined using $AlCl_3$ method (Kandouli *et al.,* 2017). Briefly, 600 µL of extract solution (0.5 mg/mL) was mixed with 600 µL of $AlCl_3$ (2%, w/v) solution in ethanol. The mixture was incubated for 1h at room temperature, and the absorbance was measured at 420 nm. The total flavonoid content was calculated on the basis of the calibration curve of quercetin and expressed as quercetin equivalents (QE), in milligrams per gram of dry weight.

2.7. HPLC Analysis of Phenolic Compounds

The HPLC analysis was conducted on an Agilent Technology HPLC system (Agilent 1260 Infinity Quaternary) and a UV/Vis detector (Diode Array Detector (DAD)) with an analytical column (Stable Bond C18 (Zorbax) (4.6 × 150 mm × 15 µm). The system was controlled by use of Open Lab software (Agilent Technologies). Samples were dissolved in methanol (2 mg/mL) and injected with a volume of 20 µL. The mobile phase was a gradient flow (0.8 mL/min) of Methanol (A) and Water (B) (H3PO4 0.07 %) with multi-step gradient elution conditions and a total run time of 60 min per sample applied as follow: (A) 0 min: 30%; (A) 45 min: 60%; (A) 50 min: 60%; (A) 51-60 min: 30%. For each fraction, amounts of phenolic classes were evaluated on the basis of the calibration curves of standards obtained at the maximum UV absorbances of hydroxycinnamic, flavonols and hydroxybenzoic classes. They were evaluated as caffeic acid equivalents (CAE), rutin equivalents (RUE) and gallic acid equivalents (GAE), in milligrams per gram of dry weight, respectively.

2.8. Antioxidant Activity

2.8.1. DPPH radical scavenging activity

The free radical scavenging activity of *A. pubescens* was assessed using DPPH method (Kandouli *et al.,* 2017). Extracts were dissolved in methanol and water with a range of final concentrations of 0.0125–0.8 mg/mL, then 40 µL of extract solution was added to 160 µL/well of DPPH solution (0.8 mM). The mixture was incubated for 30 min in the dark at room temperature and absorbance was measured at 517 nm using a microplate reader (Perkin Elmer Enspire, Singapore). The ability of the extracts to scavenge DPPH was expressed as a percentage of inhibition calculated using the following equation (1):

$$Inhibition\ (\%) = [100 \times (A_0 - A)/A_0] \quad (1)$$

Where A_0 is the absorbance of the control and A is the absorbance of the test extracts. IC_{50} value (the concentration needed to scavenge 50% of DPPH) was evaluated from a dose-response curve-fitting model.

2.8.2. ABTS radical scavenging activity

The anti-radical activity of different extracts from *A. pubescens* were assessed according to ABTS method reported by (Re *et al.,* 1999). In brief, 160 µL of ABTS (7 mM) was added to 40 µL of each sample prepared in methanol and water at different concentrations (0.0125–0.8 mg/mL, final concentration). The absorbance was measured at 734 nm after 10 min, then the percentage of inhibition was quantified using the previous equation (1). Where A_0 is the absorbance of the control and A is the

absorbance of the test extracts. IC_{50} value (the concentration needed to scavenge 50% of ABTS) was quantified from a dose-response curve-fitting model.

2.8.3. Galvinoxyl radical (GOR) scavenging activity

The Glvinoxyl radical cavenging activity of different extracts from *A. pubescens* were assessed according to the method of (Shi *et al.,* 2001) with slight modifications. In brief, 160 µL of a fresh galvinoxyl radical methanolic solution (0.08 mM) was added to 40 µL of different concentrations of each extract (0.0125–0.8 mg/mL, final concentration). The reaction mixture was kept at ambient temperature for 120 min, then the absorbance was read at 428 nm. The scavenging activity was calculated using equation (1) and IC_{50} value which represents the concentration of the extract needed to scavenge 50% of galvinoxyl radical, it was calculated from a concentration-response curve-fitting model.

2.8.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of AQL, *n*-BuOH and AR extracts was evaluated using the colorimetric assay reported by (Aruoma *et al.,* 1987). For this assay, aliquots (100 µL) of the following reagents (deoxyribose, $FeCl_3$, EDTA or buffer, H_2O_2 and ascorbic acid) were added with 500 µL of tested extract (0.025 to 0.25 mg/mL). After 1h of incubation at 37 °C, TBA and acetic acid were added, and the mixture was heated for 15 min at 100 °C. The absorbance was read at 532 nm, and mannitol (1−10 mM) was used as a reference. The inhibition percentage of hydroxyl radical scavenging activity by the extract in the presence or absence of EDTA was calculated using equation (1) mentioned previously; where A_0 is the absorbance of the control and A is the absorbance of the test extracts.

2.8.5. Metal Chelating Capacity

The chelating activity of samples with ferrous ions Fe^{2+} was determined following the ferrous iron–ferrozine complex method (Santos *et al.,* 2017) with some modifications. Briefly, 840 µL of extract solution (0.0125–0.8 mg/mL) was mixed with 80 µL of $FeSO_4$ (0.3 mM) and 40 µL of 0.8 mM of ferrozine. After 10 min, the absorbance was measured at 562 nm, and the MCC was evaluated using the calibration curve of EDTA. The results are expressed as micromoles of EDTA equivalents (ADTAE) per gram of dry weight.

2.8.6. Reducing power

The reducing power ability of the extracts was evaluated according to the method described by (Oyaizu, 1986). In this assay, 0.2 mL of sample solutions at various concentrations, 0.5 mL of phosphate buffer (0.2 M; pH 6.6) and 0.5 mL of potassium ferricyanide (1%) were mixed and incubated at 50 °C for 20 min. Then, 0.50 mL of trichloroacetic acid (10%) was added. After 10 min, 0.5 mL of the supernatant was mixed with 0.5 mL of distilled water and 0.2 mL of ferric chloride (0.1%). The absorbance was read at 700 nm. Increased values of absorbance indicate a high reducing power of the sample.

2.8.7. Cupric ion reducing antioxidant capacity

Cupric ion reducing antioxidant capacity of the extracts was determined using the method developed by (Apak *et al.,* 2007). The reaction consists of mixing 50 ml of copper chloride (10 mM), 50 ml of Neocuproine (7.5 mM) and 60 ml of ammonium acetate (1 M, pH 7.0) with 40 mL of the sample at different concentrations in a microplate of 96 wells. The microplate was allowed to incubate for 1h, and the absorbance was read at 450 nm. Increased values of absorbance indicate a high cupric reducing power of the sample.

2.8.8. Total antioxidant capacity

Total antioxidant capacity of the extracts was determined according to the method of (Prieto *et al.,* 1999). In this assay, 0.3 mL of each tested extract or ascorbic acid (standard) was mixed with 3.0 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 90 °C for 90 min, and absorbance was measured at 695 nm. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE) in µmol per gram of dry weight.

2.9. α-Amylase inhibitory activity

The capacity of α-amylase inhibition was performed according to the method proposed by (Zengin *et al.,* 2014). In each well of a 96 microplate, 25 µL of the tested extract at different concentrations (0.00625–0.4 mg/mL, final concentration) was added to 50 µL of α-amylase solution prepared in phosphate buffer (pH 6.9 with 6 mM sodium chloride). The mixture was then incubated for 10 min at 37°C. After incubation, 50 µL of soluble starch (0.1%) was added and plates were incubated for 10 min at 37 ◦C. Then, 25 µL of HCl (1M) was added to stop the reaction. The blank was prepared by adding extract solution to all reagents without enzyme solution. Finally, 100 µL of Iodine Potassium Iodide (IKI) solution were added, and the absorbance was measured at 630 nm. The absorbance of the blank was subtracted from that of the extract, and the inhibition percentage of α-amylase was calculated.

2.10. Statistical Analysis

Data Statistical analysis was carried out using GraphPad Prism software. The results are presented as mean ± SD for the indicated number of independent experiments. Evaluation of statistical significance was conducted by one-way analysis of variance (ANOVA), Newman-Keuls or a Pearson's correlation (r^2) coefficients calculations. A $p< 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Extract Yields, TPC and TFC

Plant polyphenols present a several group of phenolic compounds that possess an ideal structural chemistry for radical scavenging activity. Phenolic compounds have been shown to be responsible for the antioxidant activity of plant materials (Rice-Evans *et al.,* 1996).The comparative data of extraction yield, TPC and TFC of different extracts from *A. pubescens* are presented in Table 1. The Extraction yields varied from 1.1% to 16.7% 9 (w/w) with the following order: MeOH ≥ AQL > *n*-BuOH > AR > EtOAc > EP. Broadly speaking, the extraction

yield was strongly influenced by the used solvent, and has significantly increased with the increase of solvent polarity which is consistent with the results of (Zhang *et al.,* 2014). Accordingly, a significant difference in the extraction yield (p<0.05) was recorded. Zhang *et al.* (2014) has also reported that performing different extraction methods for the same tested sample may lead to a significant difference in the extraction yield and antioxidant activity.

The total phenolics content (TPC) of various *A. pubescens* extracts was performed using the regression equation of the calibration curve ($y = 13.41x$, r^2:0.994). The highest TPC value of 335.9 mg GAE/g was detected in EtOAc extract, while PE and AR extracts recorded the lowest values (~70 mg GAE/g dw) which represent approximately 1/5 of EtOAc extract value. The TPC values in *A. pubescens* extracts ranged from 70 to 335.9 mg GAE/g dw (Table 1) in the following descending order: EtOAc > *n*-BuOH > AQL ≥ MeOH > EP ≥ AR (p< 0.05).

The total flavonoids content (TFC) of different *A. pubescens* extracts was quantified using the regression equation ($y = 24.83x$, r^2:0.993). The TFC ranged from 6 mg QE/ g dw, in AR extract to 165.4 mg QE/g dw, in *n*-BuOH extract (Table 1). There was no significant difference in TFC between AQL and MeOH extracts were registered (p>0.05), while *n*-BuOH and EtOAc extracts had the highest TFC with a significant difference (p< 0.05) compared to the other analysed extracts. Our results are significantly different from the findings of (Metrouh *et al.,* 2015), in which TPC and TFC were around ~26 mg GAE/g and ~9 mg QE/g, respectively. These results possibly reflect differences in plant drying procedures, geographical or the genetic background (Kandouli *et al.,* 2017).

Interestingly, TFC showed a low correlation with TPC (r^2= 0.695, p< 0.05), which is consistent with the work of Zou *et al.* (2011), who recorded a low correlation between TPC and TFC values when conducting a study on phenolic profile and antioxidant properties of lentil. Our results were also in correspondence with the literature of Meda *et al.* (2005), who reported that some chemical groups of proteins and amino acids present in plants are also able to react with Folin–Ciocalteu reagent. Several investigations have focused on the medicinal activities of phenolics as they are considered as powerful antioxidants and free radical scavengers. It has been also shown that the antioxidant capacity of phenolics including flavonoids is mainly attributed to their redox characteristics, acting as reducing agents, hydrogen atom donors or singlet oxygen quenchers (Leopoldini *et al.,* 2011; Kumar and Goel, 2019 and Rice-Evans *et al.,* 1996).

Table 1. Extraction Yield, Total Phenolic, Total Flavonoid and Phenolic compounds Contents in *A. pubescens* extracts.

Extract	Phenolic classes					
	Yield (% ,w/w)	TPC (mg GAE / g)	TFC (mg QE / g)	Hydroxycinnamates (mg CAE / g)	Flavonols (mg RUE / g)	Hydroxybenzoates (mg GAE / g)
AQL	14.9	115.6 ± 2.1*	84 ± 39 ‡	10.41 ± 1.72*	26.60 ± 0.59*	1.64 ± 0.04*
MeOH	16.7	121.5 ± 2.0*	79.3 ± 5.5‡	15.43 ± 1.71*	36.7 ± 0.75*	2.66 ± 0.8*
PE	1.1	73.06 ± 3.2§	15.0 ± 3.2*	1.11 ± 0.07§	9.21 ± 0.9§	5.37 ± 0.26‡
EtOAc	1.5	335.9 ± 8.9‡	150.2 ± 2.9#	50.08 ± 4.8‡	95.40 ± 1.38‡	16.71 ± 0.68§
n-BuOH	2.5	198.3 ± 7.3 #	165.4 ± 6.8#	42.46 ± 4.15#	102.31 ± 10.31‡	9.1 ± 0.41#
AR	2.0	70.1 ± 3.1§	6.0 ± 0.3§	4.25 ± 0.41§	nd	2.35 ± 0.19*

Values are represent the mean ± SD of 4–6 in tetraplicate (n=4). AQL, aqueous lyophilized extract; MeOH, methanolic extract; PE, petroleum ether extract; EtOAc, ethyle acetate extract; *n*-BuOH, *n*-butanolic extract; AR, aqueous residual extract. nd: not detected. Results followed by different symbols in the same column are statistically significant difference (p< 0.05) as measured by Newman-Keuls test.

3.2. Phenolic classes content

A. pubescens major components of its phenolic extracts were separated by analytical HPLC-DAD as described in materials and methods. Representative chromatograms are displayed in Figure 1. Since different phenolic classes comprise a considerable number of compounds, UV absorbance is the most common and convenient parameter for the quantitation of phenolic compounds. Table 1 (right panel) presents the three major classes, benzoates, hydroxycinnamates and flavonols of *A. pubescens*. These phenolic classes were identified by comparing retention times (t_R) and UV spectra of gallic acid (t_R = 4.3 min), caffeic acid (t_R = 11.15 min) and rutin (t_R = 25.6 min) at maximal absorption wavelengths of 280, 325 and 365 nm, respectively (Fig. 1). EtOAc and *n*-BuOH extracts revealed a higher content of total phenols, particularly higher absolute levels of flavonols (95.4-102.31 mg RUE/g), and hydroxycinnamates (42.46-52.08 mg CAE/g), but also still found in good yields in AQL and MeOH extracts, unlike PE and AR extracts which were found in weak amounts or even in traces. Small amounts of benzoates (1.64-16.74 mg GAE/g) were also identified in our tested extracts.

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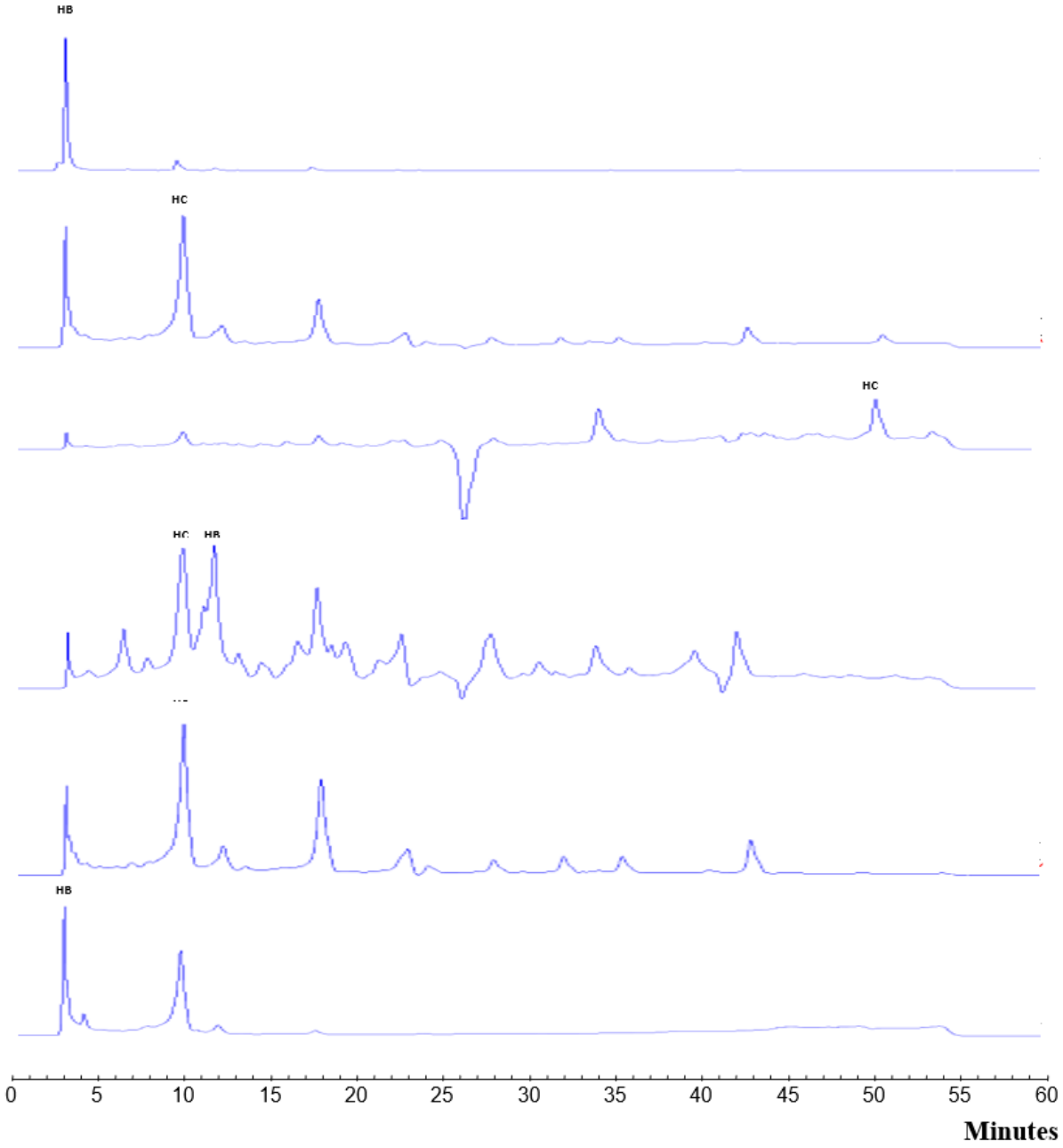

Figure 1. HPLC chromatograms of different extracts from *A. pubescens* recorded at 280 nm. (HB) hydroxybenzoates, (HC) hydroxycinnamates, (F) flavonols.

High correlation levels were registered between TPC and hydroxycinnamates (r^2 = 0.890) and benzoates (r^2 = 0,863), which suggests that hydroxycinnamates and hydroxybenzoates could considerably contribute to the total phenolic content in *A. pubescens*. Hydroxycinnamic acids are important phenolic acids present in plants. Due to their diverse health benefits including antioxidant, antiinflammatory, and anticancer properties, hydroxycinnamic acid derivatives are receiving a growing attention (Zhang *et al.,* 2014). Good correlation values were also rated between TFC and hydroxycinnamic acids (r^2 = 0.870), but also between TFC and flavonols (r^2 = 0.940), whereas benzoates showed an insignificant correlation (p> 0.05).

3.3. Antioxidant activity

A single assay approach is insufficient for assessment of antioxidant activity of polyphenolic compounds. Furthermore, the assay concept and experimental conditions vary between antioxidant assays. Antioxidant capacity may be examined by two main types of assays with various mechanisms, including hydrogen atom transfer (HAT) and electron transfer (ET). In ET-based assays, the capacity of antioxidants to transfer one electron to reduce any oxidant is measurable, while HAT-based assays measure the capacity of antioxidants to quench radicals by hydrogen donation.

3.3.1. DPPH, ABTS and GOR

The DPPH radical scavenging activity assay has been used frequently to evaluate natural antioxidant effects. One of the reasons is that this method is simple and very sensitive. This method depends on the capability of antioxidants to provide a proton or an electron to the DPPH radical (purple) to transform it to the non-radical form (yellow) (Apak *et al.,* 2016 and Foti, 2015). The

reduction ability of DPPH induced by antioxidants was evaluated by a decline in its absorbance at 517 nm. The scavenging ability of the *A. pubescens* extracts was found to increase in a dose dependent manner at a final concentration range of 0.0125-0.8 mg/mL. Figure 2 shows that the scavenging effect of samples on DPPH radical was in the following order: EtOAc ≥ *n*-BuOH ≥ AQL > MeOH > AR> EP. Our results demonstrated that the EtOAc and *n*-BuOH extracts exhibited much stronger scavenging activity with IC_{50} values of 43.3 and 62.2 µg/mL respectively, whereas PE and AR fractions have a weaker activity with IC_{50} values of 693.3 and > 800 µg/mL, respectively. Our values were about 17 to 53 times greater than those of methanol and water extracts reported in the study of (Metrouh *et al.,* 2015).

Furthermore, the extraction process performed in this study for preparing various extracts of *A. pubescens* significantly enhanced DPPH scavenging capacity. Similar studies also reported that *n*-butanol and ethyl acetate fractions of *A. radiata* (Kandouli *et al.,* 2017) and *R. anthopogonoides* (Jing *et al.,* 2015) revealed higher DPPH antioxidant activity than other extracts. In our study, the observed differential free radical scavenging effect of the plant extracts against DPPH process seems related to the occurrence of several compounds in the extract with potential antioxidant properties. Accordingly, the activities of EtOAc and *n*-BuOH extracts might be due to the chemical composition of phenolic compounds and the availability of phenolic hydroxyl groups, which have the capability to provide their electron/ hydrogen, thereby producing stable products.

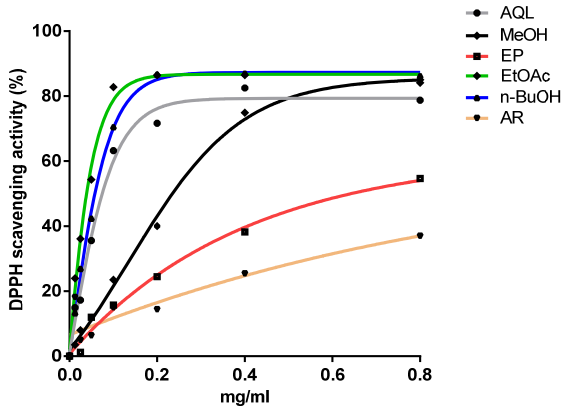

Figure 2. DPPH radical scavenging activity of different extracts from *A. pubescens*. Values represent the mean ± SD made in tetraplicate (*n*=4). AQL, aqueous lyophilized extract; MeOH, methanolic extract; PE, petroleum ether extract; EtOAc, ethyle acetate extract; *n*-BuOH, *n*-butanolic extract; AR, aqueous residual extract

DPPH antioxidant activity of *A. pubescens* extracts correlates significantly in a positive manner with TPC (r^2 = 0.852, $p< 0.01$) and with TFC (r^2 = 0.804, $p< 0.05$), suggesting their contribution in scavenging radicals. Similarly, hydroxycinnamates (r^2 = 0.817, $p< 0.05$) positively correlated with DPPH values. These results are consistent with Mitrevska *et al.* (2020), who demonstrated a good significant correlation between DPPH and phenolic extracts indicating the major contribution of phenolics to DPPH antioxidant activity.

Free radical scavenging process of different extracts from *A. pubescens* was performed using ABTS radical to corroborate the results obtained with DPPH method. ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants (Ozgen *et al.,* 2006). In the absence of antioxidants, the ABTS radical is rather stable, but it reacts actively with an hydrogen atom donor, and by that converted into a noncolored form of ABTS (Sachindra e*t al.,* 2007).

The IC_{50} values of tested extracts varied from 13.2 ± 1.8 to 641.3 ± 11 µg/mL. Figure 3 shows that ABTS radical scavenging activity of different extracts from *A. pubescens* was in the following order: EtOAc > *n*-BuOH ≥ MeOH ≥ AL > EP > AR, with the strongest antioxidant activity for EtOAc fraction and the weaker for the AR fraction. These results suggest that ethyl acetate fraction might contain the most potent free radical scavenger compounds. Our results are in agreement with findings of (Laouini *et al.,* 2016) and our IC_{50} values were 2500 times greater than those of methanol (16.21 mg/mL) and water (15.78 mg/mL) extracts reported in study of (Metrouh *et al.,* 2015). Sachindra *et al.* (2007) and Zou *et al.* (2011), reported that antioxidant compounds scavenging ABTS radical more efficiently compared to DPPH radical scavenging. Tang *et al.* (2010) also reported that ABTS was more sensitive than DPPH for the measurement of antioxidant potential of water-soluble proteins and peptides. These observations may be due to the fact that DPPH radical can be dissolved only in organic media. In contrast, ABTS radical can be solubilized in both aqueous and organic media (Arnao, 2000).

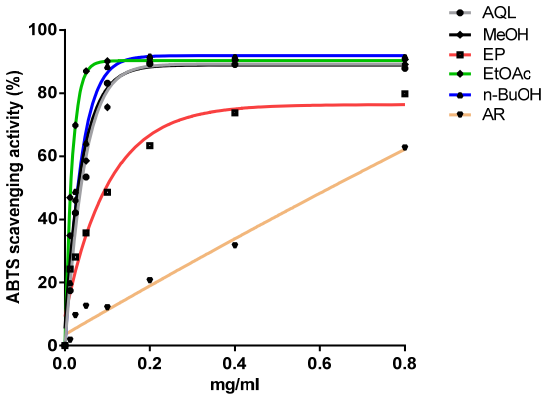

Figure 3. ABTS radical scavenging activity of different extracts from *A. pubescens*. Values represent the mean ± SD made in tetraplicate (*n*=4).

A highly significant correlation was shown between ABTS radical scavenging and TPC (r^2= 0.939, $p< 0.001$), indicating the great contribution of phenolics to radical scavenging capacity, which is consistent with the results of (Metrouh *et al.,* 2015). However, significant correlations were also established between ABTS radical scavenging and TFC, hydroxycinnamates, hydroxybenzoites and flavonols ($0.682 < r^2 < 0.779$, $p< 0.05$), suggesting that the antioxidant activity of tested extracts results mainly from the presence of molecules belonging to different phenolic classes.

Galvinoxyl, another stable phenoxyl radical can be reduced by hydrogen-donating free radical scavengers. Like the DPPH and ABTS results, IC_{50} values of Galvinoxyl radical (GOR) scavenging activity ranged widely from 14.1 to 611.6 µg/mL (Fig. 4). The highest

GOR scavenging activity values were observed in EtOAc and *n*-BuOH fractions with the values of 14.1µg/mL and 29.4µg/mL respectively, whereas the lowest value of 611.6µg/mL was observed in AR extract. However, galvinoxyl radical scavenging activity of *A. pubescens* extracts comparison was shown to be in the same order as ABTS and DPPH radical scavenging activity (EtOAc > *n*-BuOH > AQL > MeOH > EP > AR). According to the findings of this study, DPPH, ABTS and GOR were strongly inhibited by extracts which demonstrated moderate inhibition when compared to the standard compounds, BHT (IC_{50} ranges from 1.55±0.26 to 6, 55±0.59 µg/mL) and BHA (IC_{50} ranges from 5.38 ±0.06 to 15.74 ±0.47 µg/mL).

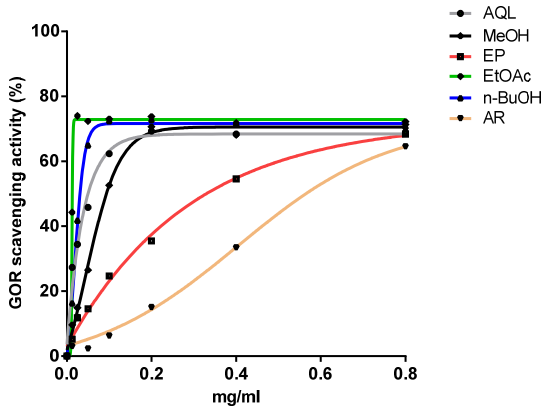

Figure 4. GOR radical scavenging activity of different extracts from *A. pubescens*. Values represent the mean ± SD made in tetraplicate (*n*=4).

This study showed that GOR values of *A. pubescens* extracts correlated significantly with TPC (r^2 = 0.991, p< 0.001), TFC (r^2 = 0.669, p< 0.05) and hydroxycinnamates (r^2 = 0.864, p< 0.01), indicating the contribution of phenolics to galvinoxyl radical scavenging activity and particularly hydroxycinnamates.

3.3.2. Hydroxyl radical scavenging activity

Hydroxyl radical is the most reactive ROS and attacks almost every molecule in the body, resulting in peroxidation of cell membrane lipids and in the formation of malondialdehyde, a mutagenic and carcinogenic product (Basu and Marnett, 1983). The results obtained of hydroxyl radical scavenging from deoxyribose degradation assay are presented in figure 5. In this assay, the radiomimetic method assesses the antioxidant potential of the extracts based on their capacity to compete with deoxyribose for hydroxyl radicals, that were generated free in solution from a Fe^{2+}-EDTA chelate (Halliwell *et al.,* 1987). Our results demonstrated that all of the three soluble extracts showed a concentration dependent radiomimetic hydroxyl scavenging activity. Moreover, the fact that *n*-BuOH (53.51%, 0.5mg/mL), AQL (39.18%, 0.5mg/mL) and AR (33.11%, 0.5mg/mL) extracts demonstrated significantly higher capabilities to prevent deoxyribose degradation compared to D-Mannitol (32.17%) at a concentration of 10 mg/mL (Fig. 5), which suggests the great capacity of *n*-BuOH, AQL and AR extracts to react with ·OH compared to D-Mannitol.

The hydroxyl scavenging activity method performed in the omission of EDTA is important because it evaluates the oxidative deoxyribose damage by site-specific production of hydroxyl radicals and offers information about *A. pubescens* soluble-extracts capability of chelating Fe ions. Thus, Fe chelator reduces the level of thiobarbituric-reactive substances (TBRS) generated from deoxyribose (Aruoma *et al.,* 1987). The activity of *n*-BuOH (30.92%), AR (25.26%) and AQL (24.78%) of inhibiting the site-specific damage to deoxyribose indicates their great capacity of Fe chelation compared to D-Mannitol (19.45%) (Fig. 5). Capacities of extracts in radiomimetic and site-specific indicate their potency as chelating agents as well as their ability to scavenge hydroxyl radicals, which are produced from a Fe^{2+} EDTA chelate (Singh *et al.,* 2007). The strong potency shown by *n*-BuOH extract could be related to its high amount of TPC and TFC. We can suggest that our results might be due to the active hydrogen donating ability of hydroxyl substitutions.

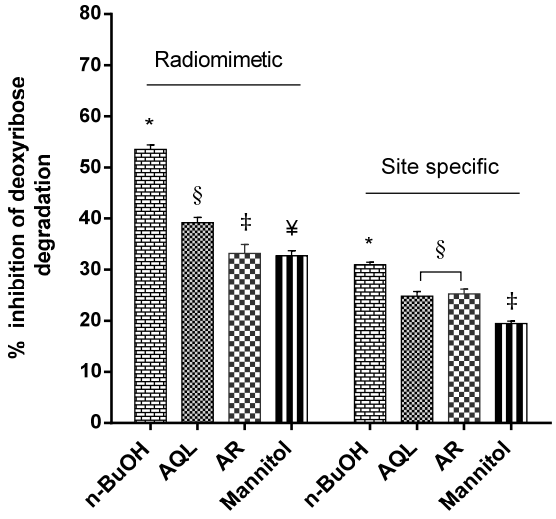

Figure 5. Hydroxyl radical scavenging activity of water soluble extracts of *A. pubescens* in the presence or absence of EDTA. Values represent the mean ± SD made in tetraplicate (*n*=4). Different symbols are statistically significant difference (p< 0.05) as measured by Newman-Keuls test.

3.3.3. MCC, Reducing Power, CUPRAC and TAC

$Fe^{2+/3+}$ and $Cu^{1+/2+}$, are critical biological molecules for normal body function. Dyshomeostasis of these metal ions could increase ROS production via Fenton-like reactions, resulting in increasing oxidative stress. The notion of redox reducing ability as an indicator of antioxidant activity may be used in different methods. There are several tests based on transitional metals (iron and copper), such as those using ferricyanide, ferrozine, or cupric ions. These metal transition assays were widely used in medical investigation, including thiol antioxidants and total antioxidant activity of biological fluids such as plasma, urine, etc (Munteanu and Apetrei, 2021).

Ferrous ion is a key transition metal ion responsible for the initiation of peroxidation in food and biological systems. In MCC assay ferrous ions form a complex with ferrozine, and the intensity of this complex purple color decreases in the presence of chelating agents. Lower absorbance indicates higher metal chelating activity. The MCC of the extracts was determined using the regression equation of the calibration curve ($y = 4.56x$, r^2:0.993), and the results were expressed as µmol EDTA equivalents per gram dry weight (Fig. 6). MCC values varied widely from

26.1 to 78.54 µmol EDTAE/g dw. The highest MCC values of 78.54, 61.93 and 62.8 µmol EDTAE/g, were detected in EP, AL and MeOH extracts respectively, whereas the lowest value of 26.1 µmol of EDTAE/g was observed with *n*-BuOH extract.

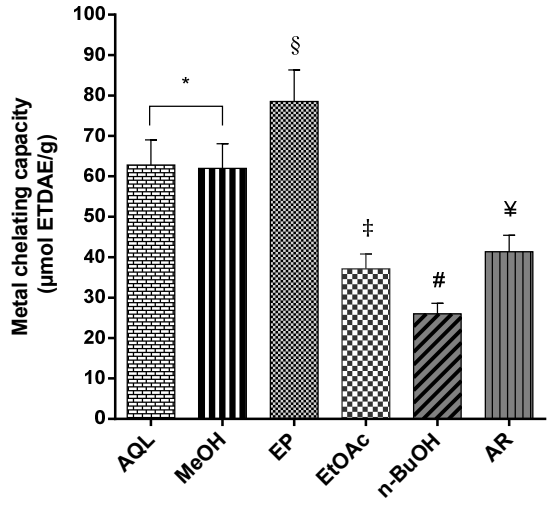

Figure 6. Metal chelating capacity (MCC) of different extracts from *A. pubescens*. Values represent the mean ± SD made in tetraplicate (n=4). Different symbols are statistically significant difference (p< 0.05) as measured by Newman-Keuls test.

Interestingly, MCC of *A. pubescens* correlated neither with TPC and TFC nor with DPPH, ABTS, and GOR (r^2 ranges from 0.181 to 0.404), which is consistent with previous findings which reported that a poor correlation existed between MCC, TPC and other antioxidant activities such as DPPH, ABTS and reducing power (Kandouli *et al.,* 2017 and Zhao *et al.,* 2008). Metal chelating potency of polyphenolic compounds are dependent upon their unique phenolic structure and the number and arrangement of hydroxyl groups (Khokhar *et al.,* 2003). The study conducted by Saiga *et al.,* (2003) showed that some peptides as well as proteins have also been reported to possess the ability to chelate metal ions, which is also believed to be the reason for EP and AQL extracts chelating capacity.

In the reducing power assay, the antioxidants present in a sample reduce the Fe^{3+} to Fe^{2+} by donating an electron and a blue colored Iron (II) complex is formed. We demonstrated that the reducing power ability of different extracts from *A. pubescens* increased in a concentration dependent manner. Values for fractions were in the following order: EtOAc > *n*-BuOH > AQL > MeOH> EP> AR. The increase in absorbance indicated an increase in reducing power due to the high antioxidant potential. The EtOAc and *n*-BuOH fractions showed a good reducing power with values of 0.314 ± 0.004 and 0.256 ± 0.001 at 0.2 mg/mL, respectively (Fig. 7). However, the EP and AR fractions demonstrated lower values. These findings may be explained by the high content of phenolics present in various fractions of *A. pubescens*. It has also been reported that reducing power is associated with antioxidant activity, and may serve as a significant reflection of the antioxidant potential (Oktay *et al.,* 2003).

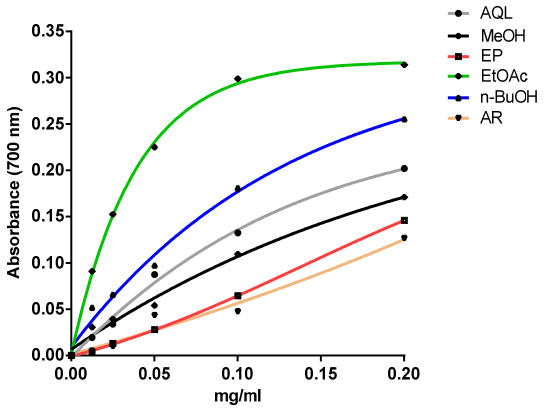

Figure 7. Reducing power activity of different extracts from *A. pubescens*. Values represent the mean ± SD made in tetraplicate (n=4).

The antioxidants donate their electrons in order to stabilize the radicals and also break the free radical chain reaction in the reducing power assay (Shabbir *et al.,* 2013). A positive relationship existed between reducing power and the different phenolic classes of TPC, TFC, hydroxycinnamates, hydroxybenzoites and flavonols ($0.749 < r^2 < 0.929$, p<0.01), suggesting their contribution in reducing power.

CUPRAC antioxidant capacity assay is a stable, rapid, selective and suitable for a wide variety of antioxidant types including both lipophilic and hydrophilic. It utilizes the copper (II)-neocuproine [Cu(II)-Nc] reagent as a chromogenic oxidizing agent at the basis of cupric reducing ability of reducing compounds to cuprous. Our obtained results in CUPRAC assay demonstrated a trend of antioxidant activity similar to the previous tests. As shown in figure 8, CUPRAC antioxidant activity of extracts is in the following order: n-BuOH > EtOAc > AQL≈ MeOH ≈ EP > AR. In this assay, a high absorption indicates high reducing power of cupric ions. The highest CUPRAC antioxidant activity values of 4.27 ± 0.04 and 3.96 ± 0.03 at 0.2 mg/mL, were observed in EtOAc and *n*-BuOH fractions respectively, whereas AR extract showed the lowest value of 0.43 ± 0.02 at 0.8 mg/mL, (Fig 8). Similar findings that reported an increase of cupric reducing ability in a dose dependant manner were demonstrated in different studies (Sharma and Adarsh, 2014).

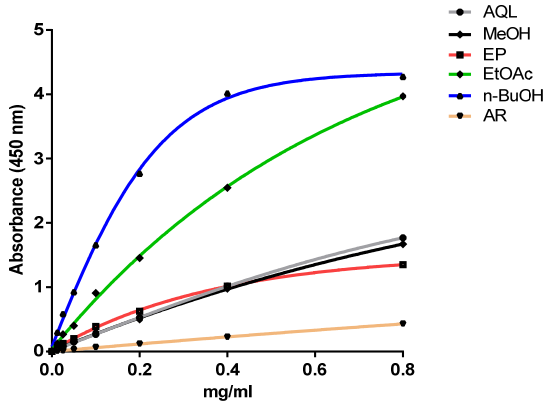

Figure 8. CUPRAC capacity of different extracts from *A. pubescens*. Values represent the mean ± SD made in tetraplicate (n=4).

In this study, CUPRAC assay results showed a significant positive correlation with TPC and TFC, hydroxycinnamates and flavonols ($0.737 < r^2 < 0.974$, $p < 0.01$), which is consistent with the results of (Jin *et al.,* 2012), suggesting that a significant correlation is detectable between CUPRAC and phenolic content levels in plant extracts. A recent report (Apak *et al.,* 2016) demonstrated that some bioactive compounds present in medical plants possessed high antioxidant capacity in the CUPRAC, that was due the number and position of the hydroxyl groups as well as the degree of conjugation of the whole molecule, which are important for easy electron transfer.

Total antioxidant capacity (TAC) of the extracts, expressed as the number of equivalent of ascorbic acid (AA), was obtained from the calibration curve ($y = 0.008x$, r^2:0.994). In this assay, Mo (VI) was reduced to Mo (V) by antioxidant effect of extracts in a concentration dependent manner. Results of TAC of the extracts are shown in figure 9. Total antioxidant capacity of different extracts of *A. pubescens* was in the order of EtOAc ≈ *n*-BuOH > AQL ≈ MeOH > EP ≈ AR. In this assay, high µmol AAE/g value indicates a high total antioxidant capacity. Accordingly, EtOAc and *n*-BuOH extracts demonstrated the highest values of activity (~455µmol AAE/g), while PE extract was 2 times less potent (~240 µmol AAE/g). Similar findings highlighting the potency of EtOAc and *n*-BuOH over methanol extracts in term of TAC have been already observed (Ahmed *et al.,* 2014 and Khatoon *et al.,* 2013).

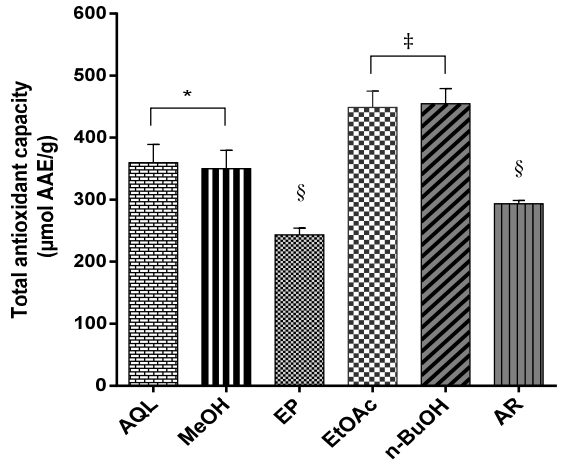

Figure 9. Total antioxidant capacity (TAC) of different extracts from *A. pubescens***.** Values represent the mean ± SD made in tetraplicate (n=4). Different symbols are statistically significant difference ($p < 0.05$) as measured by Newman-Keuls test.

Moreover, a good relationship existed between total antioxidant activity and TPC, TFC, hydroxycinnamates and flavonols ($0.711 < r^2 < 0.943$, $p < 0.01$). Many flavonoids and polyphenolics present in medicinal plants were reported to contribute significantly to the total antioxidant capacity (El Kamari *et al., 2021* and Afsar *et al.,* 2016). Our results are consistent with the research of Tung *et al.,* (2009) who reported that gallic acid, catechin, myricetin along with other polyphenols in *A. confusa* leaves extracts were responsible for the significant antioxidant potential.

3.4. α-Amylase inhibitory activity

Diabetes mellitus is mainly associated with hyperglycemia, which is characterized by high circulating blood glucose levels. α-Amylase inhibitors could retard the rate of maltose released from starch, resulting in delaying maltose conversion to glucose and lowering postprandial plasma glucose concentrations. The inhibitory activity of *A. pubescens* extracts against porcine α-amylase was evaluated in this study. As seen in figure 10, all extracts exhibited α-amylase inhibitory activity at the same concentration (12.5µg/mL). The highest α-amylase inhibitory activity values of 89.8 ± 0.7, 81.7 ± 3.2 and 72.3 ± 1.6 were observed in AQL, *n*-BuOH and EtOAc fractions respectively, whereas the lowest value of 36.8 ± 2.1 was observed with AR extract (Fig. 10). A 12.5 µg/mL concentration of acarbose inhibited enzyme activity by 8.08± 0.3%. α-amylase inhibitory activity of *A. pubescens* extracts was higher than the activity of acarbose at the same concentration. This study suggested that the plant has a very strong inhibition on α -amylase which may contribute to its demonstrated *in vivo* antidiabetic effect. Polyphenolic compounds have been discovered to form complexes with a wide range of proteins. Notably, previous research suggests that polyphenolic compounds, including flavonoids, may interact with amino acid residues in enzyme active sites or interact with amino acid residues near the active site, thereby closing the channel to the active site and inhibiting enzyme activity (Zhu *et al.,* 2020). It has been reported in different studies that phenolic compounds are effective α-glucosidase and α-amylase inhibitors (Dehimat *et al.,* 2021 and Zhu *et al.,* 2020).

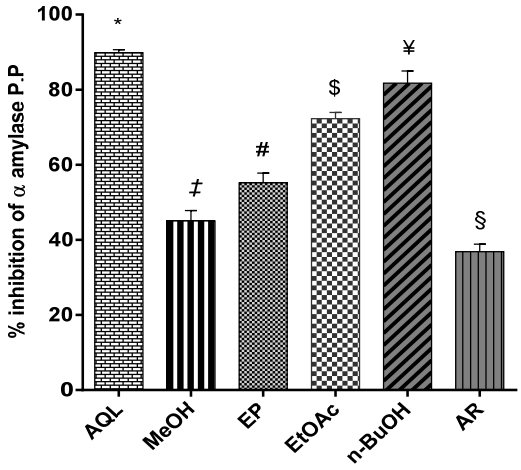

Figure 10. Inhibitory effect of different extracts from *A. pubescens* on α-Amylase**.** Values represent the mean ± SD made in tetraplicate (*n*=4). Different symbols are statistically significant difference ($p < 0.05$) as measured by Newman-Keuls test.

4. Conclusion

In conclusion, it should be emphasized that in the present study, chemical and biological examinations of six *A. pubescens* extracts were undertaken for the first time. The radical scavenging activity of the tested samples is due to flavonoids, phenolic acids and their derivatives. Therefore, *A. pubescens* may be an interesting alternative for the treatment of diabetes mellitus not only by inhibiting

an enzyme that is related to the disorder, but also by improving the antioxidant defenses of patients. The highest antioxidant activity and phenolic contents were exhibited by the EtOAc and *n*-BuOH extracts. These results suggest the use of EtOAc and *n*-BuOH fractions as primary antioxidant therapeutic sources. However, further investigation would be required to study such potential capacities, and to better understand their mechanisms of action related to the chemical composition of these extracts

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