Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2015; 7(2): 119-125

ISSN- 0975 1556

Research Article

In vitro Assays of the Antibacterial and Antioxidant Properties of Extracts from *Asphodelus tenuifolius* Cav and its Main Constituents: A Comparative study

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Available Online: 26th February, 2015

ABSTRACT
This study was aimed at investigating the preliminary phytochemical screening, total phenolic and condensed tannins constituents, antioxidant, antibacterial and antifungal by using various in vitro systems and analysis of marker compounds by High performance liquid chromatography (HPLC) of methanolic, ethanolic and petroleum ether extracts of *Asphodelus tenuifolius* Cav (ATC). Antioxidant efficacies of three extracts were estimated by their abilities to scavenge DPPH˙ and total antioxidant activity expressed by reducing Mo (VI) to Mo (V). The antimicrobial activity of the methanolic, ethanolic and petroleum ether extracts of ATC was determined against eleven strains including Gram-positive and Gram negative bacteria as well as yeasts. The phenolic contents of the extracts as vanillin were found to be highest in methanol (68.15%) followed by ethanol (53.8%) and petroleum ether extract (1.56%). the antioxidant and free radical scavenging activities of the extracts assayed through DPPH˙ method were also found to be highest with methanol extracts followed by ethanol and petroleum ether extracts. For the antibacterial and antifungal activity the extracts showed moderately activity, compared to standard antibiotics, inhibiting all tested bacteria except *Pseudomonas aerigunosa*. The most sensitive microorganism was *Staphylococcus aureus* (diameter of inhibition is 16 mm) showed in methanolic extract at 4 mg/ml. To our knowledge, this is the first report on the biological activities of *Asphodelus tenuifolius* Cav extract and our findings suggest the possibility of using the aerial parts as a novel source of natural antimicrobial and antioxidant agents for the food and pharmaceutical industries.

Keywords: *Asphodelus tenuifolius* Cav, phytochemical content, Antioxidant capacity, Antibacterial activity, HPLC analysis.

INTRODUCTION

In developing countries, traditional medicine holds a great promise as a source of readily available effective and safe drugs to the people[1]. Phytochemical components in medicinal plants are of great importance in the manufacture of such drugs[2] and the affectivity of many available drugs is studied by many workers to test folklore medicinal plants for several pharmacological activities[3]. Epidemiological studies have shown that a diet rich in fruits and vegetables is associated with a decreased risk of cardiovascular diseases and certain cancers. These beneficial health effects have been attributed in part to the presence of phenolic compounds in dietary plants, which may exert their effects as a result of their antioxidant properties[4].

Free radicals are chemically unstable atoms that cause damage to lipid cells and proteins as a result of imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzymes[5]. They are known to be the underlying cause of oxidative stress which is grossly implicated in the pathogenesis of various diseases such as cancer, diabetes, cardiovascular diseases, aging and metabolic syndrome[6]. Examples of these radicals include superoxide anions, hydroxyl, nitric oxide and hydrogen peroxide radicals. These radicals can be scavenged by the protective role of natural and synthetic antioxidant agents. Meanwhile, the ingestion of several synthetic antioxidants as has been reported toxic to man[5]. The use of natural antioxidant has gained much attention from consumers because they are considered safer than synthetic antioxidants. *Asphodelus tenuifolius* Cavase (Onion weed) is a native to the Mediterranean region, but it is widespread, extending from Mediterranean region east through the Arabian Peninsula to the Indian Subcontinent, also in Malaysia, Australia, Chile New Zealand, Mexico and United States of America. A very variable species, whose several forms have been given various specific names[7]. These plants are edible and so considered safe. Traditionally, *Asphodelus tenuifolius* Cavase (ATC) used for colds and hemorrhoids, a febrifuge and also used for rheumatic pain. Seeds are also used as diuretic agent, healing wound and they are applied externally to ulcers and for inflamed parts[8]. The extract of ATC showed antibacterial activity against most

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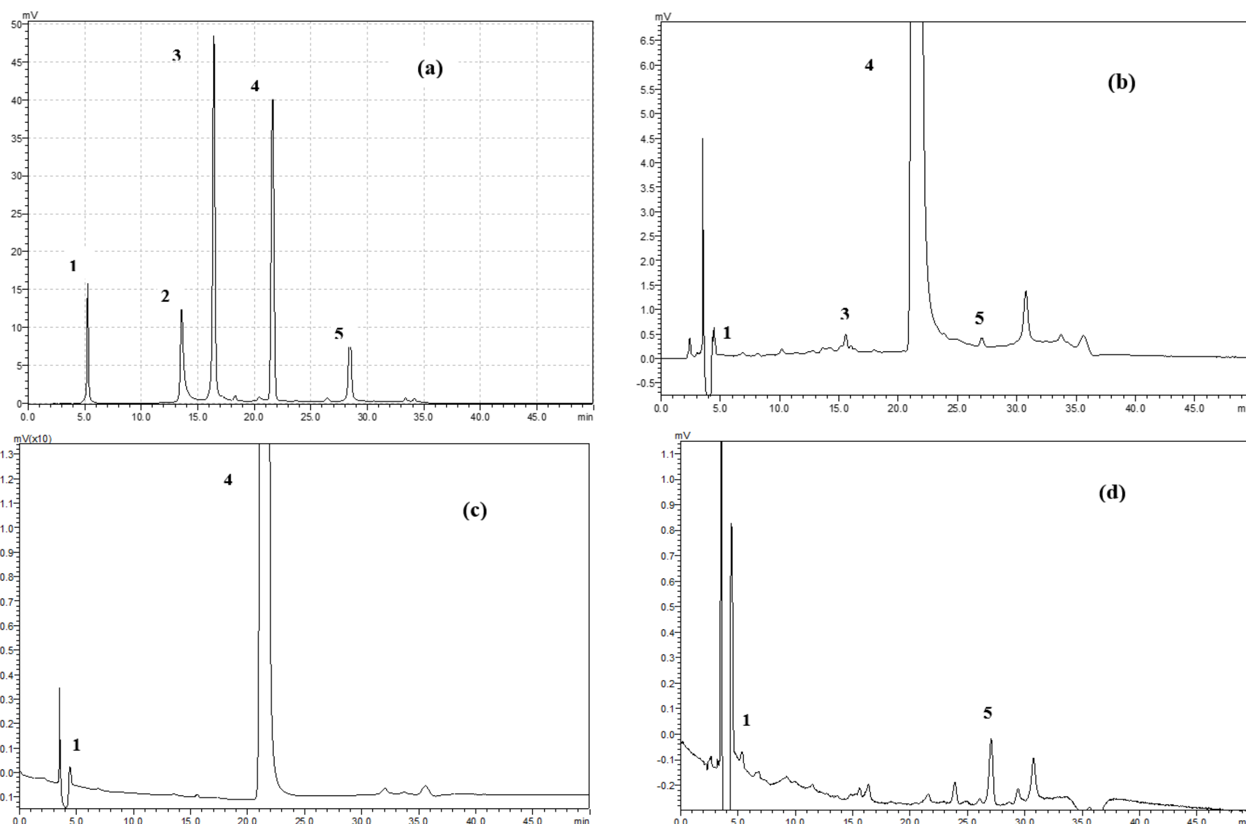

Figure 1: Chromatograms of standard mixture (a), methanolic extract (b), ethanolic extract (c) and petroleum ether extract (d). Peak 1: Ascorbic acid; Peak 2: Chlorogenic acid; Peak 3: Caffeic acid; Peak 4:Vanillin; Peak 5: Rutin

of the isolated microbes from the oral cancer cases S. aureus with MTCC 96 strain, S. epidermidis MTCC 435 strain, P. vulgaris MTCC 426 strain, P. mirabilis MTCC 425 strain, E. coli MTCC 443 strain, K. pneumonia MTCC 109 strain, P. aeruginosa MTCC 741 strain, C. albicans 3017 strain and A. fumigates 2550 strain[8]. Although numerous studies have in the past, focused on antimicrobial activity of ATC against various bacteria and fungi Ahmed[9]. There are some reports in the literature showing antioxidative effects of crude extract of ATC and ethanol extracts of have been reported to exhibit antioxidant activity against hydroxyl radical, DPPH$^{\cdot}$, ABTS$^{\cdot+}$ and Peroxynitrite[6]. This paper reports the evaluation of the phytochemical screening, antioxidant properties, antibacterial and antifungal capacity of ethanolic, methanolic and petroleum ether of aerial part extracts from of *Asphodelus tenuifolius* Cav applying different analytical methodologies. We report our findings and relate them to the phytochemical studies of the plant as well as its medicinal uses.

MATERIALS AND METHODS

Plant material and extraction: The aerial parts of *Asphodelus tenuifolius* Cav were collected from southeast of Algeria, state of El Oued on December 2013. The aerial parts of *Asphodelus tenuifolius* Cav were thoroughly washed and reduced into small pieces before being ground and powdered into particles (about 1 mm in size). Then the powder was put in a hot air oven at 60 °C until complete drying. Depending on the physical characteristics of the samples, the time ranged from 18 at 30 h. The bioactive compounds were extracted according to the method described by Bebbar et al[9] and Delgado *et* al[10]. 80 g of the aerial parts were extracted with 400 ml of methanol, petroleum ether and ethanol for 5 h in Soxhlet. The extracts were filtered and evaporated under vacuum at 45°C before being dried and lyophilized for 10 h. the raw extract was stored at -40 °C.

Table 1: Phytochemicals and organic compounds present in extracts of aerial parts from ATC.

phytochemical	Solvents		
	Methanol	Ethanol	Petroleum ether
phenols	+	+	+
tannins	+	+	–
anthraquinones	+	–	–
alkaloids	+	+	–
steroids	+	+	–
flavanoids	–	+	+

+ Presence; - Absence

Test for tannins and phenolics: Dried powdered sample (0.2 g) was boiled in water (8 ml) in a test tube and then filtered. 1 ml of 0.1% ferric chloride was added and observed for brownish green or a blue–black coloration.
Test for alkaloids: Different solvent extracts (0.5 ml) was mixed with picric acid solution (1 ml) in a test tube and observed for the formation of orange coloration[12].

Table 2: constituents content analysed by HPLC

Extracts	Ascorbic acid (µg/mg)	Chlorogenic acid (µg/mg)	Caffeic acid (µg/mg)	Vanillin (µg/mg)	Rutin (µg/mg)
Mthanolic	0.61	ND	0.0063	163.25	1.18
Ethanolic	0.47	ND	ND	84.39	ND
Petroleum ether	0.19	ND	ND	ND	1.74

ND = not detected.

Table 3: Phenolic content[a], condensed tannins[a] and total antioxidant activity[a] of aerial parts extracts from ATC.

	Solvents		
	Methanol	Ethanol	Petroleum ether
Phenolic content mg GAE /g	183.7±3.5	128.5±2.1	109.7±1.5
Condensed tannins mg CE/g	59.8±0.6	49.2±0.5	41.4±0.3
Total antioxidant activity mg ascorbic acid equivalent/g	248.12±8.7	337.35±10.5	381.36±11.6

[a]Values are means±SD of three measurements

Table 4: Antimicrobial activity of ATC methanolic, ethanolic and petroleum ether extracts tested at 2 and 4 mg/ml expressed as zone of inhibition (mm).

Bacteria	Diameter of zone inhibition (mm)						
	Methanolic extract		Ethanolic extract		Petroleum ether extract		polymyxine B
	2 mg/ml	4 mg/ml	2 mg/ml	4 mg/ml	2 mg/ml	4 mg/ml	10 µg/disc
Staphylococcus aureus	11	16	10	14	8	10	19
Bacillus cereus	10	15	9	13	9	12	22
Escherchia coli	10	15	10	13	10	12	18
Salmonella arizona	9	12	8	12	8	9	21
Pseudomonas aerigunosa	10	13	10	11	-	9	17
Agrobacterium tumefaciens	10	14	10	12	-	10	18
Pseudomonas putida	11	14	9	11	-	9	17

(-), indicates zone of inhibition less than 6 mm. Data are presented as the mean ± standard deviation of three determinations.

Test for glycosides: Plant material (1 g) was added to 5 ml each of distilled water. H_2SO_4 or water was added in two sets of beakers, heated for 3 min and filtered. To the filtrates, 1 ml of NaOH was added, heated with 5 ml of Fehling's solution for 3 min and observed for the appearance of a reddish-brown precipitate.
Test for anthraquinones: Powdered plant (3 g) was soaked into benzene (10 ml) in a conical flask and allowed to stand for 10 min then filtered. After, we added 5 ml of ammonia solution (10%) to the filtrate, shaken for 1 min. We observed the appearance of a pink, red or violet color in the ammonia phase.
Test for flavonoids: Different extract (5 ml) and 5 ml of distilled water were added to the same volume of NH_3 and filtrate, after mixed with 2–3 drops of concentration (H_2SO_4). The presence of flavonoid indicate by the formation of a yellow coloration.
Test for steroids: Acetic anhydride (2 ml) was added to 0.5 g of extracts followed 2 ml of H_2SO_4. Color change from violet to blue or green showed the presence of steroids[12].

Determination of total phenolic content: The total phenolic contents in the selected varieties were determined by the folin-Ciocalteus method[13,14]. Briefly, 100 µL of both the sample and the standard (gallic acid) of known concentrations were made up to 2.5 ml with water and mixed with 0.25 ml of 1N Folin-ciocalteus reagent. After 5 min, 2.5 ml of sodium carbonate aqueous solution (2%, w/v) was added to the mixture and was completed the reaction for 30 minutes in darkness at room temperature. The absorbance was read at 765 nm using a UV-visible spectrophotometer (Shimadzu UV-1800, Japan). For the blank the same protocol was used but the extract was replaced by solvent. The concentration of total polyphenols in the extracts was expressed as mg gallic acid equivalent (GAE) per g of dry weight using UV-Visible (Shimadzu UV-1800, Japan) and the equation of calibration curve: Y= 0.00778x, R^2 =0.991, x was the absorbance and Y was the gallic acid equivalent. All results presented are means (±SEM) and were analyzed in three replications.

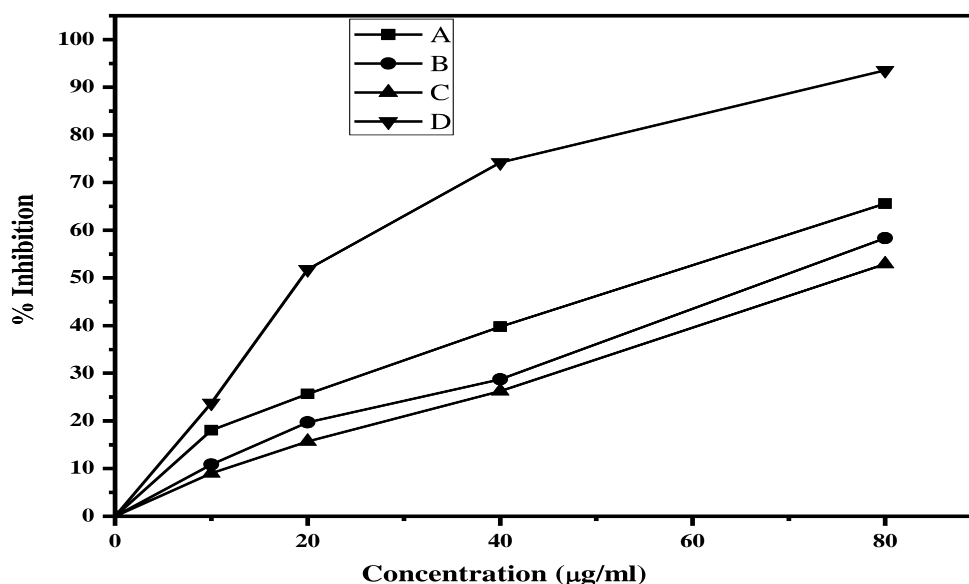

Figure 2: 1-diphenyl-2-picrylhydrazil (DPPH) scavenging activity of methanolic (A), ethanolic (B), petroleum ether extracts (C) and the standard BHT (D).

Determination of condensed tannins (proanthocyanidins): Determination of proanthocyanidins content was determined using a spectrophotometric method[15]. A volume of 0.5 ml of different extracts or standard (catechin) was added to the mixture of 3 ml of 4% vanillin- methanol (4%, v/v), 1.5 ml of hydrochloric acid and then vortexed. The resulting mixture was allowed to stand for 15 min at room temperature, the absorbance of each was measured at 500 nm using spectrophotometer (Shimadzu UV-1800, Japan). Total proanthocyanidin content was calculated as mg catechin equivalent (mg CTE/g) using the equation obtained from the calibration curve: Y = 0.5617x, R^2 = 0.985, where x is the absorbance and Y is the catechin equivalent.

Phenolic identification: The composition of the extracts was analyzed by high performance liquid chromatography (HPLC)[16]. We used an Shimadzu (LC 20, Japan). System comprised of a LC-10AD pump, a CTO-10A column oven, a SPD-10A UV-DAD detector, a CBM-10A interface and a LC-10 Workstation was utilized. A LC-18 column (250 mm x 4 mm i.d. x 5 mm) was employed. Samples were injected. The components of the samples were separated by gradient elution at at 30 °C. The mobile phases were: A, 98:2 (v/v) acetic acid, and B, acetonitrile and the elution gradient was: 0–5 min, 5% B; 10 min, 10% B; 11 min, 20% B; 20 min 20% B; 30 min 40% B; 40 min 50% B; 50 min 80% B. The flow rate was 0.8 ml/min and the detection wavelength was 285 nm. Phenolic compound standards gallic acid, Adcorbic acid, Querecetin, Chloregenic acid, Vanillin, Caffeic acid and Rutin were dissolved in solvents extraction and used for identification of the phenolic acids present in different extracts of *Asphodelus tenuifolius* Cav. Peak identification in HPLC analysis was achieved by comparison of retention time and UV spectra of reference standards. Quantification of individual phenolic compounds in the extracts was done using the peak area of reference compounds and reported as mg/g of extract.

DPPH radical scavenging activity[17]: 1 ml aliquot of each extract was added to 0.5 mL of a DPPH ethanolic solution (7.8 mg DPPH in 100 ml of each extraction solvent). The mixture was vigorously shaken and left to stand in the dark for 30 min at room temperature. The antioxidant activity was then measured by the decrease in absorption at 517 nm using UV-Visible spectrophotometer (Shimadzu UV-1800, Japan) and corresponds to the extract ability to reduce the radical DPPH to the yellow-colored diphenilpicryldrazine. The antiradical activity was expressed as IC_{50} (μL/mL) i.e. the antiradical percentage inhibition calculated by the following equation:

DPPH scavenging activity = $(A_o - A_1) / A_o \times 100$ (1)

Where A_o is the absorbance of control test after 30 min, A_1 is the absorbance of the sample extract after 30 min. All results are means (±SEM) and were analyzed in triplicate

Estimation of total antioxidant activity[18]: The total antioxidant capacity of the crude extract was evaluated, based on the reduction of Mo (VI) to Mo (V) by formation of the green phosphate/ M(V). In the appendorf tube, 0.3 mL of different extracst known concentration was added to 2.7 mL mol of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath (Mammert D-91126 Schwabach FRG, Germany) at 95 °C for 90 min. the blank was prepared with the same procedure described above but we replace the volume of simple extract by 0.3

mL ethanol 80%. the absorbance was calculated at 695 nm. The antioxidant capacity was expressed as mg of gallic acid equivalent per gram of dry plant powder (GAE/g DW). All determinations were performed in triplicate.

Test on antibacterial activity: Antibacterial activity of aerial parts of *Asphodelus tenuifolius* Cav using polymyxine B (10 µg/disc) (Laboratory of Waste Water Treatment, Centre of Research and Technologies of Water, Tunisia)) as positive control was evaluated against *Staphylococcus aureus, Bacillus cereus*, *Escherchia coli, Salmonella arizona, Pseudomonas aerigunosa, Agrobacterium tumefaciens* and *Pseudomonas putida*. All strains were obtained from the Laboratory of Waste Water Treatment, Centre of Research and Technologies of Water (Tunisia). Sensitivity of different bacterial strains to various extracts was measured in terms of zone of inhibition using agar-diffusion assay[19]. The plates containing Mueller–Hinton agar were spread with 0.2 ml of the inoculums. Wells (6 mm diameter) were cut out from agar plates using a sterilized stainless steel borer. Stock solutions (6 mg/ml) of methanolic, ethanolic and petroleum ether extracts were prepared and each well was filled with 100 µl of solution at final concentration of 2 and 4 mg/ml. The plates inoculated with different bacteria were incubated at 37 ℃ for 24 h and diameter of resultant zone of inhibition was measured. The experiment was repeated thrice.

Statistical analysis: Data were expressed as the mean ± standard deviation of at least three measurements. One-way analysis of variance (ANOVA), correspondence analysis and determination of the Pearson correlation coefficient (q) was used during this work to evaluate and correlate results between them. Differences with p-value superior to 0.05 were not considered significant.

RESULTS AND DISCUSSION

Phytochemical screening: Phytochemical and other organic compounds analysis of ATS showed that the solvent extracts of stem bark contain differing classes of compounds. Ethanol extracts contain the highest classes, followed by methanol. Petroleum ether extracts contained the least number of phytochemicals in the aerial parts. Phytochemicals and organic compounds detected include phenols, tannins, anthraquinones, alkaloids, steroids, and flavanoids (Table 1). Solvent extraction is frequently used for isolation of antioxidants and both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarity. The methanol, ethanol and petroleum ether systems were used as extraction solutions in the present studies as they are the most widely employed solvents for hygiene and abundance reasons; also, the solvent is compatible with food. Moreover, in view of the quantitative content of main phenolics, there are some implications for potential biological activity of this species. The phenolic compounds exert anti-inflammatory, antinociceptive and antioxidant activities, in addition to the antioxidant, hepatoprotective and hypoglycemic activity[20,21,22,23].

The content of the total phenolic and condensed tannins compounds of three extracts have been reported as mg of gallic acid and mg of (+)-catechin equivalents per gram of dried extract, respectively (Table 3). The results showed that extracts contained phenolic compounds in the following order: methanolic >ethanolic> petroleum ether extracts. Moreover the methanolic extracts have higher amounts of condensed tannins than methanolic and petroleum ether extracts. Methanol was regarded as the most effective solvent for extracting phenolics from ATC. This could be explained by the possible formation of complexes by a part of the phenolic compounds with carbohydrates and proteins, which are more extractable in ethanol than in methanol or petroleum ether[24]. Values are expressed as means ± SD of triplicate measurements.

HPLC analysis: The constituents in the different extracts were analysed by HPLC. Figure 1 showed the chromatograms of three extracts sample and standard markers mixture. Peaks 1, 2, 3,4 and 5 were ascorbic acid, chlorogenic acid, caffeic acid, vanillin and rutin, respectively. The contents of these components in different extracts were determined according to the calibration curves of ascorbic acid y = 2260.81x (r^2 = 0.998), chlorogenic acid y = 37492.06x (r^2 = 0.999), caffeic acid y = 70429.77x (r^2 = 0.999), vanillin y = 80555.42x (r^2 = 0.989) and rutin y = 3118.94x (r^2 = 0.988), where y was the peak area and x was the concentration of analytes (0–80 µg/ml). The quantitative results are summarized in Table 2. As shown, Vanillin was the most dominant constituent and similar in ethanolic, methanolic extracts and no detected in petroleum ether extract. The methanolic extract contains five compounds and ascorbic acid three extracts were similar, while ethanolic and petroleum ether extract were considered poor from compounds. However, because of its strong polarity, some compounds were not concentrated by methanol and petroleum ether extraction[25].

Scavenging activity of DPPH radical: The inhibitory concentrations of each extract and reference compound (BHT) required to scavenge 50% of the DPPH radical, the IC_{50} values in antioxidant activity of three extracts were presented in figure 2. It shows that methanol, ethanol and petroleum ether extracts and BHT have important antioxidant potencies with IC_{50} values of 55.62, 69,08, 75.91 and 28.34 µg/ml, respectively . Furthermore, methanol extracts showed to be the most potent free radical scavenger (IC_{50}=55.62 µg/ml).The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants[26]. We can conclude that the methanol extract was the most effective in this respect. The polyphenolic compounds are usually the major antioxidants in plant extracts[27,28]. Furthermore, a dietary intake of phenolics has been associated with reduced risk of different diseases, such as cancer, cardiovascular disease, diabetes, or atherosclerosis, probably due to their potent antioxidant properties[29,30,31]. Thus, the estimated antioxidant activity of ACT can contribute to the benefits of this species.

Total antioxidant activity: The phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compounds and the formation of green Mo(V) complexes with a maximal absorption at 695 nm[32,33]. Using this method, the result indicated that the methanolic extract of ATC had the highest antioxidant capacity with a value of 248.12±8.7 mg ascorbic acid equivalent/g dried extract, this activity may be due to the presence of phenolic compounds[34]. The ethanolic and petroleum ether extracts showed lower activity with values of 337.35±10.5 and 381.36±11.6 mg ascorbic acid equivalents/g dried extract, respectively (Table 3).

Antibacterial activity: The results of antibacterial effect of methanolic, ethanolic and petroleum ether extracts of ATC in terms of zone of inhibition (mm) were presented in Table 4. The methanolic extract had maximum zone of inhibition (16 mm) against *Staphylococcus aureus* and minimum zone of inhibition (12 mm) for *Salmonella arizona* at concentration of 4 mg/ml. Similarly, ethanolic and petroleum ether extracts had maximum zone of inhibition (14 mm) and (10 mm) for *Staphylococcus aureus* respectively. The ethanolic and petroleum ether extracts had minimum zone of inhibition (12 mm) and (9 mm) for *Salmonella arizona* at 4 mg/ml. Antibacterial activity of methanolic , ethanolic and petroleum ether extracts of ATC was compared with polymyxine B as a standard antibiotic. All extracts had shown marked antibacterial activity against *Staphylococcus aureus, Bacillus cereus*, *Escherchia coli, Salmonella arizona, Pseudomonas aerigunosa, Agrobacterium tumefaciens* and *Pseudomonas putida*, however extracts were less potent than polymyxine B. Phenolic constituents of the plant extracts have shown potent antimicrobial properties[35,36]. The active compound for the inhibition of bacteria was identified as may be the vanillin. The antimicrobial activity of vanillin against a broad variety of bacteria and yeasts is well documented[34]. Yet, the inhibitory activity of vanillic acid against Listeria spp. has been studied by Moon et al[36]. and it was found to be the hight antilisterial activity. In the same work, it was reported that, the aqueous solutions of vanillic acid had stronger inhibitory activities than had vanillin solutions. also, reported a dose-dependent inhibitory activity of vanillin against *Escherchia coli*, *Enterobacter aerogenes*, *Salmonella enterica* subsp[33].

CONCLUSION

The present research provides new understanding of phytochemical screening, antioxidant properties and antibacterial activity of ethanolic, methanolic and petroleum ether extracts of ATC. The results indicated that all of the extracts of ATC possessed remarkable antioxidant and antibacterial activities. Moreover, with the polarity of extracts increasing, the antioxidant and antibacterial activities increased. This study suggested the extracts would be very promising to be natural antioxidant and antibacterial agents in food industry field. Further investigation on the isolation and identification of active component(s) may lead to chemical entities with potential clinical use.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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