Screening of phenolic compounds from *Abelmoschus esculentus* L extract fruits and *in vitro* evaluation of antioxidant and antibacterial activities

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Abstract

Ethanolic fruit extracts collected in August, September and October from Abelmoschus esculentus L were analyzed for their phytochemical profile, the antioxidant capacity to scavenge free radical and antibacterial capacity. The total phenolic content was evaluated in three fruit extracts using Folin–Ciocalteu reagent. Several biochemical assays were used to evaluate their antioxidant properties: 2,2-diphenyl-1-picryl-hydrazyl (DPPH˙), ferric reducing antioxidant power (FRAP) and total antioxidant activities assays. Further, antibacterial activity was screened using disk diffusion method against Escherichia coli, Staphylococcus and Pseudomonas aerigunosa. Three fruits extracts of Abelmoschus esculentus L were found to contain a high total phenolic content (190.11± 5.75 for AFE, 159.64± 4.28 for 138.19± 4.34 mg GAE/g DW for OFE).

The fruit extract exhibited potent antioxidant activity determined by DPPH˙, FRAP and total antioxidant activity assays, the high antioxidant capacity of all extracts has been observed and related to the relative amounts of total phenolic content with good antioxidant properties (DPPH˙ assay, IC_{50}= 14.33±0.55 mg/ml for AFE> IC_{50}= 15.33±0.58 mg/ml for SFE> IC_{50}= 17.43±0.63 mg/ml OFE). All bacteria showed high sensitivity against fruits extract and the AFE had the best antibacterial activity against Staphylococcus aureus. The results suggest that the fruits of Abelmoschus esculentus L can be considered as a good source of natural antioxidant and antibacterial drugs.

Keywords: *Abelmoschus esculentus* L, phenolic content, antioxidant, DPPH˙ antibacterial activity.

Introduction

Polyphenols or secondary metabolites constitute the important party of plants in which they can act as a defense mechanism against parasites and toxic compounds.[1] Phenolic compounds are widely diffused in all plant foods including fruits, vegetables and beverages.[1] These compounds constituted a large class of plants secondary metabolites, ranging in complexity from simple organic acids through complex polyphenolics such as flavonoids, tannins, proanthocyanidins and flavonols.[2] They exert direct and indirect influence on nutrition and functional health, plant physiology and soil processes and thus could play a role in adaptive management strategies.

Phenolic compounds vastly exist in the plants with many biological effects. Recently epidemiological studies showed strong relation between the polyphenols and prevention of many diseases including cardiovascular diseases and cancers.[3]

In addition, high antioxidant capacity[4,5], antibacterial activity[6], antiproliferative[7] and anti-inflammatory properties[8] had the potential to modulate oligomer formation and thus reduce Alzheimer's Disease[9]. These compounds have been suggested to play crucial roles in disease prevention such as reducing the risks of cancer, diabetes and stroke. The phenolic compounds showed other biological activities like anti-hyperlipidemic effects[10], anti-inflammatory[11], antimutagenic[12] and anti-allergic effects[13]. On the other hand, phenolic compounds have also been reported to act as antimicrobials against pathogenic gram-positive and gram-negative[14].

The reaction model of polyphenols against diseases has been primarily attributed to their antioxidant potential, but could also be due to other mechanisms like the absorption of blue light in the retina or actions at the molecular level. These biological activities are postulated to be mediated via the relationship of various class of polyphenols with number of cellular signaling pathways which are principal in maintaining normal cellular function[15].

Moreover, the presence of phenolic compounds in dietary plants may exert strong antioxidant effects and as a result exhibit free radicals.[16] Free radicals formed as a result of an imbalance between the generations of reactive oxygen species (ROS) and the antioxidant enzymes are chemically unstable species that cause damage to lipid cells, proteins and DNA.[17] They are generated in biological systems both as a result of normal cellular aerobic metabolism as well as from abnormal reactions stimulated by some disease processes and xenobiotics.[18] Antioxidant compounds can explain the pathology of many toxicities and disease processes.

Abelmoschus esculentus L. belongs to the family Malvaceae[19] and is one of the most important vegetables widely grown in Algeria for its beneficial fruits and youth

leaves. It is widely distributed from Africa and is now widely cultivated in the tropical and subtropical regions of the world. It plays an important role in the human diet supplying many nutrients[20]. The extract from seed is rich in various phenolic compounds and flavonoids[21]. Traditionally, this plant is useful in the treatment of inflammatory disorders and many diseases[22].

The aim of the present study was to investigate the phytochemical profile, free radical scavenging capacity and antibacterial activity of fruits extract from *Abelmoschus esculentus* L collected in different three months.

Material and Methods

Plant material and extraction: The fruits of *Abelmoschus esculentus* L. were collected from southeast of Algeria, the state of El Oued in three different months: August, September and October 2016. Moreover; the fruits (size of fruits less than 5 mm) separated from each other were washed and dried at ambient temperature, then grinded to a powder with a basic electric grinder and stored in the dark at ambient temperature. Depending on the physical characteristics of the samples, the time of drying was 25 days. The bioactive compounds were extracted according to the following method.

50 g powder was added to 250 ml of hexane at room temperature under magnetic stirrer for 24 h, the mixture was separated using a Buchner funnel and Whatmann no. 1 filter paper. The powder obtained was added to the 250 ml ethanol 80% at room temperature for 24 h under magnetic stirrer. The solvent was removed under reduced pressure with a vacuum rotary evaporator at 45 °C and the dried ethanol extracts were stored at -40 °C and used for all investigations.

Plant extract yield: The yield of the extraction was calculated from:

$[(M_f/M_i)\times 100]$

where M_f is the obtained mass of extract after evaporation of the solvent and M_i is the dry powder mass of the fruits.

Total phenolic content: The Folin–Ciocalteu reagent was used to determine the total phenolic content using gallic acid for calibration curve according to the method of Singleton and Rossi[21]. 200 µL of extract (concentration of 0.8 mg/ml) and the standard (a gallic acid at different concentration 0.03 from 0.3 mg/ml) were added to 1 ml of 1N Folin-Ciocalteu reagent (1/10). After 5 min, 0.8 ml of sodium carbonate aqueous solution (7.5%, w/v) was mixed with the above reaction and the reaction was completed in 30 minutes in darkness at room temperature.

The absorbance was read at 765 nm using a spectrophotometer (Shimadzu UV-1800, Japan). The phenolic content was expressed as mg gallic acid equivalent (GAE) per g of dry weight (DW) using the equation obtained from the calibration curve:

Y=3.419x+0.001

All results presented are means (±SEM) and were analyzed in three replications.

Total flavonoid content: The flavonoid content of the plant extracts was determined using the Aluminum chloride method [24]. 1 ml of extract (concentration of 2 mg/ml) and standards (rutin at different concentration 0.02 from 0.1 mg/ml) were added to 5 ml of distilled water. After, 0.3 ml of $NaNO_2$ (5%) and 10% $AlCl_3$ 0.6 ml was mixed with the above reaction and incubated at ambient temperature for 5 min. The reaction finished by the addition of 2 ml of 1 M NaOH and 1.1 ml of distilled water; the mixture was thoroughly vortexed. The absorbance of the pink color developed was determined at 510 nm using a spectrophotometer (Shimadzu UV-1800, Japan). Total flavonoid content was expressed as mg rutin equivalents (RE)/g of dry weight (DW) using the equation obtained from the calibration curve:

Y= 14.493x+0.034

Total flavonols content (TFlC): Total flavonol contents were determined using the method developed by Kumaran and Karunakaran.[23] Aliquot 2 ml of the plant sample (2 mg/mL) and standards (Quercetin of concentration 0.01 at 0.05 mg/ml) were added to 2 ml of $AlCl_3$ (2%) prepared in ethanol. Then, 3 ml of (500 mg/ml) sodium acetate solution was mixed with the above reaction and incubated for 30min at room temperature. The absorbances were measured at 440 nm using a spectrophotometer (Shimadzu UV-1800, Japan). Total flavonol content was calculated as quercetin equivalent (mg/g of dry weight) using the equation based on the calibration curve:

Y= 22.90X+0.041

All tests were conducted in triplicate.

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals: DPPH radical-scavenging activity was determined by the spectrophotometric method [26]. The volume of 1 ml from each extract (various concentration) in methanol was added to methanolic solution containing DPPH radicals at a concentration of 25 mg/ml. The mixture was shaken vigorously and left to stand for 30 min in the dark and the absorbance of the mixture was then measured at 517 nm against a blank. IC_{50} value (mg/ml) is the inhibitory concentration at which DPPH radicals were scavenged by 50% obtained by interpolation from linear regression analysis.

DPPH Inhibition % = $[(A_o-A_1)/A_o]x100]$

where A_o is the absorbance of control test after 30 min and A_1 is the absorbance of the sample extract after 30 min.

Measurement of ferric reducing power (FRAP assay): The ability of the extracts to reduce Fe^{3+} was determined by the method described by Bainsi and Strain[25] with some modification[28]. FRAP reagent contained: 2.5 ml of TPTZ (Tripyridyltriazine, 10 mM) mixed with 10 ml of HCl (40 mM) added to 2.5 ml of 20 mM $FeCl_3$. Then, 25 mL of 0.3M acetate buffer (pH 3.6) was mixed with the above reaction. 1.8 ml of freshly prepared solution was added to the volume of 0.2 ml of ethanolic extract (concentration of extract 0.9 mg/ml) or standard (Ascorbic acid) was added to 1.8 ml of freshly prepared FRAP reagent. The absorbance of each sample solution was subsequently measured at 595 nm using a spectrophotometer (Shimadzu UV-1800, Japan).

The results were expressed as mg AAE/g dry weight, using the equation obtained from the calibration curve:

y= 1.9249x+0.0039

All determinations were performed in triplicate.

Determination of total antioxidant activity (TAA): The total antioxidant activity of different fruit extracts was evaluated by the phosphomolybdenum method [29]. In this essay, gallic acid using as standard, 1 ml of reagent solution contained 0.6 M of sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate. 4 mM was mixed with 0.1 ml of extract at a concentration of 0.9 mg/ml. The tubes were incubated in a water bath at 95 °C for 90 min. After the samples were cooled to room temperature, the absorbance of the above mixture was measured at 695 nm against a blank solution. Total antioxidant activity (TAA) was expressed as mg GAE/g plant extract.

Antibacterial susceptibility testing: The antibacterial activity of fruits ethanolic extracts from *Abelmoschus esculentus* L was tested against three bacterial strains including the gram-negative *Escherichia coli* and the gram-positive *Staphylococcus aureus* and *Pseudomonas aerigunosa* using paper disk diffusion inhibition test. The bacteria strain used in the experiment was obtained from the Hospital of El Oued State (Algeria) from laboratory of microorganism. The fruits extracts were dissolved in DMSO to obtain three concentrations for each extract (10 and 15 mg/ml) and sterilized by filtration through 0.45 µm Millipore filters. Antimicrobial tests were then carried out by the disk diffusion method [30] using 0.1 ml of a suspension containing 10^8 colony forming units (CFU).

Wells (6 mm diameter) were cut out from agar plates using sterilized stainless-steel cork borer and filled with 0.1 ml of the fruit extract. Plant-associated microorganisms were incubated at 37 °C for 24 h. The diameter of the resultant zone of inhibition (ZOI), if any, was measured and DMSO was used as negative control. Experiments were run in triplicate for each combination of extract and microbial strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Statistical Analysis: The data obtained in this study were expressed as the mean of three parallel measurements plus or minus the standard deviation (SD). Statistical calculations were carried out by OriginPro 9.2 for Windows. Values of $p < 0.05$ were regarded as significant and values of $p < 0.01$ were regarded as very significant.

Results and Discussion

Extraction yield: The extraction yield of *Abelmoschus esculentus* L from fruit collected in August, September and October obtained by ethanol is given in figure 1. The high value registered for Aout fruit extract (AFE) 15.32± 0.35 g/100 g, followed by September fruit extract (SFE) 10.49± 0.18 g/100 g and October fruit extract (OFE) was found to be 6.06± 0.09 g/100 g of dry powder fruit. The effect of the time collected was significant. August month is usually considered as better collected time for extraction of polyphenols.

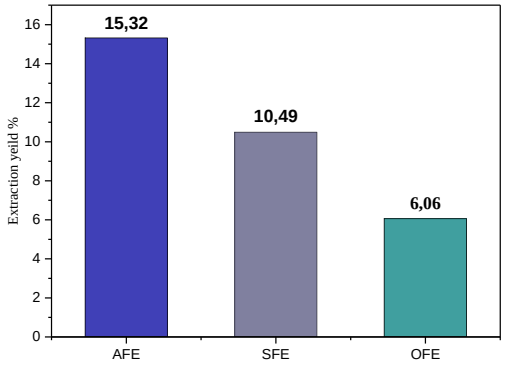

Figure 1: Extraction yields of AEF, SEF and OEF form *Abelmoschus esculentus* L

Total phenolic contents and flavonoids: The contents of total phenolic from *Abelmoschus esculentus* L fruit ethanolic extracts collected in August, September and October are presented in figure 2. The total phenolic contents showed great variation in different fruit extracts. The highest value was found in fruit Aout extract (AFE) 190.11± 5.75 mg GAE/g DW followed by the September fruit extract (SFE) 159.64± 4.28 mg GAE/g DW and the lowest total phenolic content was registered in October fruit extract (OFE) 138.19± 4.34 mg GAE/g DW. Fruit extracts were found to be rich in total flavonoids contents. The total flavonoids content is given in figure 2.

Similar results for phenolic content as the great value were found in AFE (24.21± 0.54 mg RE/g DW) followed by the SFE (19.87± 0.4 mg RE/g DW) and the minimum value in OFE (18.85 ± 0.46 mg RE/g DW). The content of total flavonols (figure 2) was also found to vary significantly ($p < 0.05$). The total flavonols in increasing order were: AFE > SFE > OFE. The values of TPC found in these extracts are comparable to those found in other plants considered as important sources of phenolic compounds[31].

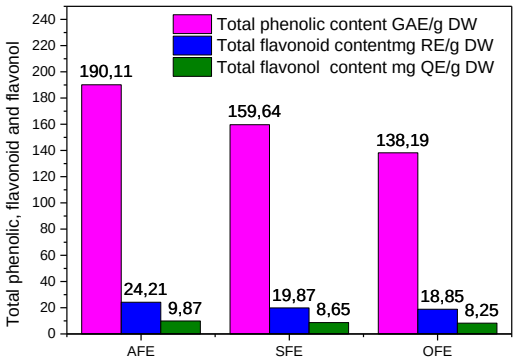

Figure 2: Total phenolic content (mg GAE/g DW), total flavonoid (mg RE/g DW) and total flavonols (mg QE/g DW) of fruit extract from *Abelmoschus esculentus* L collected in August, September and October

DPPH scavenging radical activity: The DPPH free radical can be considered a stable organic free radical and has been generally accepted as a tool for estimating free radical scavenging capacities of antioxidants[32] as very simple and useful assay to detect the presence of antioxidant potential in extracts[33]. The results of the DPPH assays showed the difference between the AFE, SFE and OFE. Regarding the IC_{50} values, three fruit extract depleted the initial DPPH concentration by 50% within 30 min. The lower is IC_{50} value, the higher is free radical scavenging activity of a sample. The free radical scavenging activities of all extracts were in the order: AFE (IC_{50}= 14.33±0.55 mg/ml)> SFE (IC_{50}= 15.33±0.58 mg/ml)> OFE (IC_{50}= 17.43±0.63 mg/ml) (figure 3).

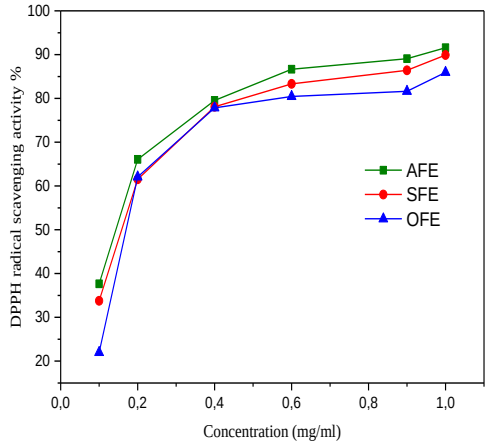

Figure 3: DPPH radical scavenging activity of fruit extract from *Abelmoschus esculentus* L collected in August, September and October.

The results showed that high polyphenol contents in fruit extract had the most potent radical scavenging capacity. Moreover, the concentrations of phenolic compounds were higher in AFE than SFE and OFE which could suggest that the high antioxidant capacity observed in AFE might be attributed mainly to the polyphenols. There is a positive relationship between antioxidant capacity and total phenolic content of the three fruit extracts. Several studies reported a relationship between total phenolic content and antioxidant activity of the fruits, plants and vegetables[34]. Many phenolic compounds are the vital antioxidants which exhibit scavenging efficacy on the free radicals[35].

FRAP assay: Similarly, the FRAP antioxidant activity of fruits extract from *Abelmoschus esculentus* L collected in August, September and October also showed a wide range; the highest activity was observed for AFE (473.54 ± 7.05 mg AAE/g) and the lowest for OFE (249,03 ± 5.42 mg AAE/g). The SFE showed moderate antioxidant activity with a value of 462.41 ± 7.28 mg AAE/g. The antioxidant scavenging activities of the three extracts for FRAP assay were shown in table 1.

Total antioxidant activity: The total antioxidant activity is characterized by the reduction of Mo (VI) to Mo (V) and formation of dark bluish-green phosphate Mo (V) complex under acidic conditions[32]. Using this method, the results indicated that the AFE of *Abelmoschus esculentus* L had the highest antioxidant capacity (210.36 ± 3.74 mg GAE/g dried extract) followed by the SFE (203.37 ± 3.21 mg GAE/g dried extract) and the lowest activity for OFE (172.88 ± 2.59 mg GAE/g dried extract). The order of the TAA in extracts was AFE > SFE > OFE (Table 1). It is extremely important to point out that the most scavenging radical activities from plant extract are correlated with phenolic compounds. The correlation between antioxidant activity and phenolic compounds was consistent with the previous studies[33].

Table 1
Total antioxidant activity (TAA) and FRAP assay of AFE, SFE and OFE from *Abelmoschus esculentus* L

	Antioxidant activity	
	TAA (mg AGE/g DW)	**FRAP (mg/g)**
AFE	210.36 ± 3.74	473.54 ± 7.05
SFE	203.37 ± 3.21	462.41 ± 7.28
OFE	172.88 ± 2.59	249,03 ± 5.42

Values are expressed as means ± SD of triplicate measurements.

***In vitro* antibacterial activity:** The antimicrobial activities of the three extracts were screened against three common pathogens including *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aerigunosa*, by disk diffusion method (table 2). The OFE extract had a maximum zone of inhibition (12 mm) against *Staphylococcus aureus*[9] and minimum zone of inhibition (8 mm) for *Pseudomonas aerigunosa* at a concentration of 15 mg/ml. Similarly, SFE had a maximum zone of inhibition 9 mm and 8 mm for *Escherichia coli and Staphylococcus aureus* respectively at concentration of 15 mg/ml. Moreover, OFE showed similar results for three bacteria.

Table 2
Antimicrobial activity of tested at 10 and 15 mg/ml expressed as a zone of inhibition (mm). (-), indicates the zone of inhibition less than 6 mm.

Bacteria	Diameter of zone inhibition (mm)						
	AFE		SFE		OFE		
	10 mg/ml	15 mg/ml	10 mg/ml	15 mg/ml	10 mg/ml	15 mg/ml	
Escherchia coli	-	8	8	9	8	9	
Staphylococcus aureus	9	12	9	8	7	8	
Pseudomonas aerigunosa	12	8	-	9	7	11	

In contrast, the antimicrobial activity of the AFE and SFE against *Escherichia coli* and *Pseudomonas aerigunosa* was not detected at a concentration of 10 mg/ml. The extract from *Abelmoschus esculentus* L showed the highest inhibitory effect on gram negative and gram-positive bacteria which was contrary to some reports published in the literature[36]. The antibacterial effect observed in this plant may be caused by some bioactive compounds such as polyphenols, flavonoid and flavonols which are considered to be antibacterial compounds. This may be due to the important content of phenolic compounds (flavonoids and flavonol) in the month of August during this stage of maturation[37].

Conclusion

The primary objective of this study was to determine the phytochemical profile, antioxidant and antibacterial activity of fruit extracts from *Abelmoschus esculentus* L collected in three different months August, September and October. The results showed that fruit extract of *Abelmoschus esculentus* L contained important amounts of polyphenols including flavonoid and flavonols and can be considered as good source of these compounds for medicinal and food applications. AFE were those that had the highest polyphenols content and promoted the highest free radicals scavenging and antibacterial inhibition against SFE and OFE.

Phenolic compounds may play important role in the antioxidant activity of *Abelmoschus esculentus* L. Based on good correlation found between activity and phytochemical contents, it indicates that effects observed could be attributed to phenolic compounds. The fruit extract from *Abelmoschus esculentus* L may be used in many applications in the future.

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