

Original Article

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *PLANTAGO MAJOR*

SAFFIDINE KARIMA<sup>a</sup>, SAHLI FARIDA<sup>a</sup>, ZERROUG MOHAMED MIHOUB<sup>b</sup>

<sup>a</sup>Department of Medicine, Faculty of Medicine, University Ferhat Abbas, Sétif, Algeria, <sup>b</sup>Department of Microbiology, Faculty of Nature and Life Sciences, University Ferhat Abbas, Sétif 1, Algeria  
Email: safkarima@yahoo.fr

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ABSTRACT

**Objective:** The purpose of this research is to evaluate the antioxidant and antibacterial properties of different leaf extracts of *Plantago major*, using *in vitro* methods.

**Methods:** The antimicrobial activity of different extracts (petroleum ether, ethyl acetate and aqueous fractions) from *Plantago major* leaves and their synergistic effect with standard antibiotic (Gentamicin) were evaluated using the disc diffusion method. The total phenolic and total flavonoid content of these extracts was determined according to the Folin-Ciocalteu procedure and Aluminum chloride colorimetric assay respectively. Antioxidant properties were determined via the DPPH free radical scavenging,  $\beta$ -carotene bleaching assay and ferrous ion chelating activity.

**Results:** The total phenols and total flavonoid content of the extracts ranged from 5.79 to 114.45 mg GAE/g dry extract and from 1.24 to 5.48  $\mu$ g QAE/mg dry extract respectively. The ethyl acetate fraction showed the highest DPPH scavenging capacity ( $IC_{50} = 12.85 \pm 0.27 \mu$ g/ml) and relative antioxidant activity of 70.48% in the  $\beta$ -carotene bleaching assay. While, aqueous and petroleum ether fractions have the lowest activities. On the other hand, only the aqueous fraction has a capacity of chelating iron ( $IC_{50} = 1.02 \pm 0.02$  mg/ml). The findings indicated also that an ethyl acetate fraction was the most active *in vitro* against Gram-negative and Gram-positive bacteria strains. High inhibition zone of  $16.7 \pm 1$  mm and  $14.3 \pm 0.6$  was exhibited on *Staphylococcus aureus* and *Bacillus cereus*. Moderate one of  $13.3 \pm 0.6$  and  $11.3 \pm 0.6$  mm was obtained against *Pseudomonas aeruginosa* and *Acinetobacter bowie*. However, lowest antibacterial activity was obtained against *Klebsiella pneumonia*, *Proteus mirabilis* and *Salmonella typhimurium*. Furthermore, synergistic antibacterial activity was either obtained by the combination of standard antibiotic (Gentamicin) with the tested extracts.

**Conclusion:** Our results showed a potent antioxidant and antibacterial activities of this species. This plant could be exploited as a potential source of natural antioxidant and antimicrobial agents for dreadful human diseases and oxidation prevention.

**Keywords:** Phenolic content, Flavanoid content, Antioxidant activity, Antimicrobial activity.

INTRODUCTION

Medicinal plants are widely used all over the World as folk medicine because they still are the most effective and cheapest alternative sources of drugs [1]. They produce a wide array of compounds (flavonoids, alkaloids, phenols and tannins), most of which are used in plant defense against predators. These compounds of natural origin have been a source of innumerable therapeutic agents with antibacterial, antiviral, antioxidant, antiulcer, anti-inflammatory and anticancer activities. In recent decades, there has been much interest in antioxidant and antimicrobial activities of medicinal plants as the tool in discovering new biologically active molecules. Antioxidants have the capacity for scavenging free radicals, which can damage several proteins and DNA, leading to genomic instability and cancer [2, 3]. The other significant biological action of medicinal plants is their antimicrobial activity against infectious diseases. Conventional antifungal and antibacterial treatment of infections becomes inefficient to cure patients, providing resistance of microorganisms. Many researchers have studied the antimicrobial activities of plants for new compounds that may be effective in drug therapy. Among many popular medicinal plants, *Plantago major* (common plantain) belonging to the Plantaginaceae family, has taken place in many historical uses as a wound healing remedy. This herbaceous plant was traditionally used in North Africa, to treat wounds, burns, abscesses, inflammations, hemorrhoids and fevers [4]. Previous reports have indicated that plantain was also effective against diarrhea, dysentery [5], bronchitis [6, 7], cataracts and conjunctivitis [8, 9]. Recent researches have demonstrated the importance of this species for treating diabetes, increasing male fertility [10] and as anti-cancer [11-13].

The present study is the first one on *Plantago major* in Algeria. The objective of our work was to evaluate the potential antioxidant activity of aerial parts of this species growing in the Sétif area, in the North-East of Algeria. The  $\beta$ -carotene bleaching assay and the

ferrous ion chelating test are done for the first time. The phenolic and flavonoids content of this plant was also estimated. On the other hand, the antimicrobial activity of the extracts was assessed against a wide range of different pathogenic microorganisms. Among the tested strains: *Serratia sp.*, *Bacillus cereus*, *Citrobacter freundii*, *Enterobacter faecalis*, *Acinetobacter bowie*, *Salmonella typhimurium* and *Listeria monocytogenese* have not been reported previously. Furthermore, the synergistic effect of the extracts with standard antibiotic (Gentamicin) was either evaluated using disc diffusion method. This alternative approach was not reported in previous research tasks.

MATERIALS AND METHODS

Plant material

The vegetable material consists of leaves collected in May 2011. The fresh plant parts were cleaned and shade dried at room temperature during two weeks. Exposure to sunlight was avoided to prevent the loss of active components. The dried plant material was grounded into powder using the electric blender.

The plant was identified by Doctor Boulachab Nacera "Department of Pharmacy, Faculty of Medicine" university Ferhat Abbas, Sétif 1, Algeria. A voucher specimen of the plant was deposited in "Laboratoire de Valorisation des Ressources Naturelles".

Extraction procedure

The extract was prepared by a maceration technique [14]. 200 g of vegetable powder were mixed with 2000 ml of methanol 70% (methanol/water, 7:3) and put under magnetic agitation at 80°C during 30 min. The mixture was let for maceration during 24 h and then filtered through cotton and Whatman paper n°3. The residue was then re-extracted twice with additional portions (2000 ml) of methanol/water (70:30, v/v). The combined extracts were

evaporated to dryness under reduced pressure at 40°C with a rotary evaporator (BUCHI R215). The crude methanol extract was treated with boiling distilled water to dissolve the flavonoids. It was then let settle in the refrigerator for 24 h and filtered through filter paper. This aqueous solution was firstly extracted with petroleum ether to remove chlorophyll and lipids, then with ethyl acetate. Three fractions were obtained by this treatment:

- Two organic phases: petroleum ether (EP) and ethyl acetate (Ac) fractions.
- Residual aqueous phase (Aq).

Each fraction was evaporated to dryness under reduced pressure to 45°C with a rotary evaporator and each residue was weighed and then dissolved with a minimum volume of methanol to test their antioxidant and antimicrobial activity.

#### Determination of total phenol contents (TPC)

The total phenol in the extracts was estimated by spectrophotometric assay [15]. The protocol of dosage was made as follows: 200 µl of sample (concentration 0.5 mg/ml) or standard was mixed with 1 ml of 1/10<sup>th</sup> dilute Folin-Ciocalteu's reagent. After 4 min, 800 µl of aqueous sodium carbonate solution (7.5%) was added. Samples were vortexed and allowed to stand in the dark at room temperature for 2 h. The optical density of the blue-color solution was read at 765 nm. The data presented were averaged for triplicate measurements. A standard curve of the absorbance of Gallic acid (0 to 250 µg/ml) at 765 nm was plotted. The results were expressed as milligram Gallic acid equivalent per gram of dry extract (mg GAE/g extract).

#### Determination of total flavonoids

The total flavonoid content of extracts was estimated by a colorimetric procedure using aluminum chloride (AlCl<sub>3</sub>) according to the method of Bahorun *et al.* [16]. 1 milliliter of extract (2 mg/ml) or Quercetin standards (0 to 40 µg/ml) was combined with one ml of AlCl<sub>3</sub> solution (2% in methanol). The mixture was kept in the dark for 10 min at ambient temperature and the absorbance was read at 430 nm. The test was carried out in triplicate. The results were expressed as milligram quercetin equivalent per gram of dry extract (µg QAE/mg extract).

#### Antioxidant activity

The antioxidant capacity of extracts from *Plantago major* leaves was evaluated using commonly colorimetric methods, namely, the 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging method and β-carotene bleaching test. While, the metal-chelating assay is based on the ability of extracts to act as antioxidants by binding to iron.

#### DPPH scavenging assay

The antioxidant activity assay employed the inhibition of free radical 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) which is widely used for evaluating natural antioxidants, due to its stability, simplicity and reproducibility. In the DPPH assay, the free radical (DPPH) is reduced to the yellow-colored diphenyl-picrylhydrazine in presence of antioxidant due to the formation of the non-radical form DPPH-H in the reaction [17]. The radical scavenging activity was calculated as a percentage of DPPH discoloration and the decrease of its absorbance is monitored at 517 nm.

The method used in this assay was described by Sanchez-Moreno [18] and Agrawal [19]. The test was performed by mixing 50 µl of extract or standard with 1.95 ml of DPPH dissolved in methanol (0.004 %). After shaking, the reaction was placed safe from light during 30 min and the absorbance was read at 517 nm. The extracts were tested at concentrations ranging (0 to 30 mg/ml) for the petroleum ether fraction (EP), (0 to 1.25 mg/ml) for the ethyl acetate fraction (Ac) and (0 to 16 mg/ml) for the aqueous fraction (Aq). The synthetic antioxidant butyl hydroxyl toluene (BHT) and butyl hydroxyl anisole (BHA) were used as antioxidant standards at concentrations of 0 to 5 mg/ml. Triplicate tests were carried out at each dilution of the standard and the extract. The radical scavenging activity (RSA) was calculated using the following formula

$$\%RSA = [(ADPPH - A_{sample})/ADPPH] * 100$$

Where %RSA is the percentage of inhibition, ADPPH is the absorbance of DPPH (t=0 min) and A<sub>sample</sub> is the absorbance of the extract (t=30 min).

IC<sub>50</sub> expresses the concentration of antioxidant that causes 50% loss of activity of DPPH. It was calculated from the plot of the inhibition percentage of extracts and standard [20].

#### Bleaching β-carotene test

Oxidation of aqueous emulsions of β-carotene and linoleic acid is frequently employed as a test for measuring total antioxidant activity of plant extracts. β-carotene bleaching of extracts from *Plantago major* leaves was determined based on the procedure of Tepe [21] with a slight modification. Antioxidant capacities of the samples were compared with those of the synthetic antioxidant butyl hydroxyl toluene (BHT) and the blank. 0.5 mg of β-carotene dissolved with 1 ml of chloroform was placed into a flask containing 25 µl of linoleic acid and 200 mg of Tween 40. Chloroform was removed using a rotary evaporator under reduced pressure at 40°C. Oxygenated distilled water (100 ml) was then added to the emulsion and shaken vigorously. Aliquots of 2500 µl of this mixture were mixed to 350 µl of extracts and standard (BHT) dissolved in methanol at a concentration of 2 mg/ml. The blank was prepared by mixing 350 µl of methanol to 2500 µl of the emulsion. The tests were carried out in triplicate. Readings of all samples were taken immediately t=0, 1, 2, 4, 6, 24 and 48 h respectively, using a spectrophotometer at 490 nm.

Relative antioxidant activities (RAA %) of the extracts were calculated from the equation given below [22].

$$RAA\% = A_{sample}/A_{BHT} * 100$$

Where A<sub>BHT</sub> is the absorbance of the positive control BHT and A<sub>sample</sub> is the absorbance of the extract.

#### Ferrous ion chelating activity

The chelating of ferrous ions by extracts was estimated by the method of Le [23]. This activity was measured by inhibition of the formation of iron (II)-ferrozine complex after treatment of the extracts or standard with Fe<sup>2+</sup>-Ferrous ion. The assay was performed over a range of concentrations: 0 to 50 µg/ml, 0 to 40 mg/ml, 0 to 40 mg/ml and 0 to 5 mg/ml, respectively, for standard (EDTA), petroleum ether fraction (EP), ethyl acetate fraction (Ac) and aqueous fraction (Aq). 500 µl of standard or extract were added to 100 µl of FeCl<sub>2</sub> (0.6 % µmol in water) and 900 µl of methanol. The mixture was shaken and allowed to react at room temperature for 5 min. Then, 100 µl of ferrozine (5 % µmol) was added to this solution and shaken again. After 10 min of incubation, the absorbance of the solution was measured at 562 nm. Each reaction was performed in triplicate. The chelating effect was calculated as a percentage of inhibition of ferrozine- Fe<sup>2+</sup> complex formation, given by the formula:

$$\% inhibition = [(A_c - A_s)/A_c] * 100$$

Where A<sub>c</sub> was the absorbance of the control and A<sub>s</sub> was the absorbance of the sample.

Chelating activity was expressed as ChA IC<sub>50</sub>, the concentration that chelates 50% of Fe<sup>2+</sup> ions. It was calculated from the plot of the inhibition percentage of extracts and standard.

#### Antibacterial susceptibility test

##### Bacterial strains

The antibacterial tests were carried out using referenced strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and clinical origins strains: *Acinetobacter bowie*, *Serratia sp* and *Candida albicans* obtained from the Laboratory of Bacteriology at Sétif hospital. *Bacillus cereus* (ATCC 10876), *Enterobacter faecalis* (ATCC 49452), *Salmonella typhimurium* (ATCC 13311), *Citrobacter freundii* (ATCC 8090), *Proteus mirabilis* (ATCC 35659) and *Klebsiella pneumonia* (ATCC

700603) were obtained from Laboratory of Natural Substances at the University of Tlemcen Algeria.

### Antibacterial activity

Antimicrobial activity *in vitro* was screened using the disc diffusion and micro-dilution methods [24, 25]. For the antibacterial assay, sterile Muller-Hinton agar plates were inoculated by the method of streak with inoculums of the tested bacterial suspension. The bacterial suspension from young colonies of 18 to 24 h was made in sterile distilled water for each strain and its turbidity was adjusted to 0.5 McFarland ( $10^8$  CFU/ml). Then, sterile paper discs of 6 mm diameter were impregnated with 20  $\mu$ l of plant extract, dried and delicately deposited on the medium. Each extract was tested in triplicate with the presence of sterile disc impregnated with methanol as a negative and standard antibiotic (Gentamicin 10  $\mu$ g) disc as positive control. Plates inoculated with bacteria were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganism and the average of the three measurements in all three replicates was calculated. An inhibition zone of 14 mm or more was considered as a high antibacterial activity [26].

### Determination of minimal inhibitor concentration (MIC)

The MIC was determined using the dilution method for extracts which showed activity on any of the test bacteria and fungus. The test was performed using three dilutions of each extract (1/2, 1/4, 1/8, 1/16 and 1/32) against the tested microorganisms. Each dilution was tested in triplicate with the presence of a sterile disc as a negative control. The same procedure for preparing bacteria cultures was done as described previously. The MIC was the lowest concentration of plant extracts that exhibited no growth of microorganisms.

### Determination of synergistic antibacterial activities of extracts from *Plantago major* leaves and standard antibiotic

The assay was done using the same method of the disc diffusion cited previously. To evaluate the synergistic effect, combinations of extracts of *Plantago major* leaves with standard antibiotic (Gentamicin Himedia SD016) were tested for the sensitive bacteria. The standard antibiotic discs (10  $\mu$ g) were impregnated with 20  $\mu$ l of plant extract: ethyl acetate (100 mg/ml) and aqueous (400 mg/ml) fractions. These discs were let evaporate in an oven and then, delicately deposited on the medium inoculated with the tested bacteria. The plates were incubated at 37°C for 24 h. Results were recorded by measuring the inhibition zone differences around the discs of single standard antibiotic or combined with plant extract [27].

### Statistical analysis

The experiment results were expressed as means  $\pm$  standard deviations. Statistical Analysis was carried out by Graph Pad Prism 5. The analysis of variance was determined by one-way ANOVA and differences among the means were determined for significance at  $p < 0.05$  using a Tukey test.

## RESULTS

### The weight of the different dried extracts

The crude dried methanol of *Plantago major* leaves was suspended in water and subjected to fractionation using petroleum ether and ethyl acetate and gave three fractions:

- Two organic phases: petroleum ether (EP) and ethyl acetate (Ac) fractions have weights of 0.44 and 1.34 g respectively.
- Residual aqueous phase (Aq) had a weight of 4.58 g.

These different weights were determined after evaporation to dryness of the different fractions.

### Total phenolic content and total flavonoid content

Gallic acid was used as a standard compound and the total phenols in extracts from *Plantago major* was expressed as mg GAE/g of extract using the standard curve equation:  $y = 0.012x + 0.073$ ,  $R^2 = 0.997$  (fig. 1). For total flavonoid content, the amount was calculated

using the standard curve equation determined:  $y = 0.031x + 0.019$ ,  $R^2 = 0.998$  (Fig.2), and expressed as milligram quercetin equivalent per gram of dry extract (mg QAE/g extract).

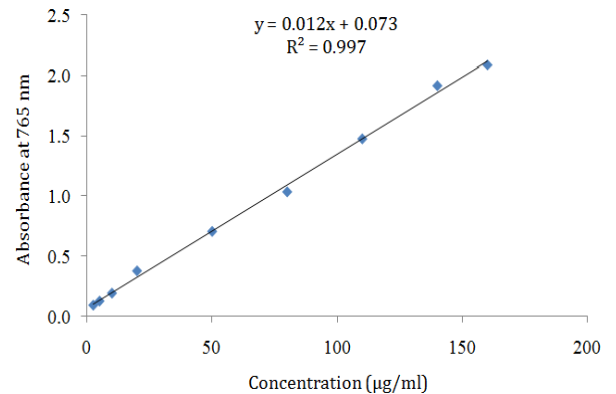


Fig. 1: It shows gallic acid standard curve

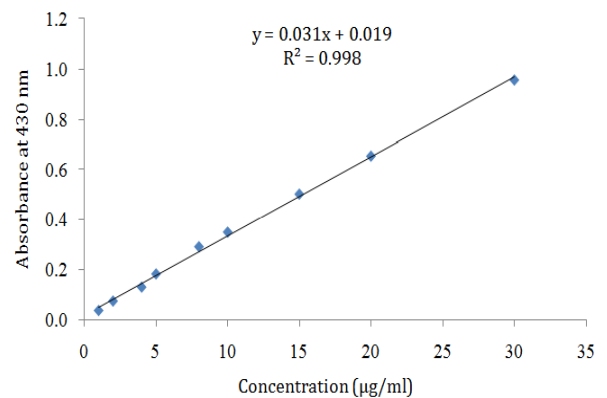


Fig. 2: It shows quercetin standard curve

Total phenolic content and Total flavonoid content of different fractions of leaf extract are represented in table 1. The data indicated that the highest amount of total phenolic was  $114.45 \pm 2.45$  mg GAE/g extract in the ethyl acetate fraction (Ac) and, lowest one of  $26.89 \pm 0.79$  and  $5.79 \pm 0.10$  were evaluated respectively for aqueous (Aq) and petroleum ether (EP) fractions. Whereas, total flavonoid content was found to be highest with values of  $5.48 \pm 0.06$  and  $2.34 \pm 0.19$  mg QAE/g respectively in ethyl acetate and aqueous fractions. The petroleum ether fraction has the lowest level of flavonoids ( $1.24 \pm 0.03$   $\mu$ g QAE/mg).

Table 1: It shows total phenolic and total flavanoid content of leaf extracts from *Plantago major*. EP: petroleum ether, Ac: ethyl acetate and Aq: Aqueous extract

Fractions	Total phenolic content (mg GAE/g)	Total flavonoid content ( $\mu$ g QAE/mg)
EP	$5.79 \pm 0.10$	$1.24 \pm 0.03$
Aq	$26.89 \pm 0.79$	$2.34 \pm 0.19$
Ac	$114.45 \pm 2.42$	$5.48 \pm 0.06$

Each value is represented as means  $\pm$  SD (n=3)

### Antioxidant activity

Polyphenols, such as flavonoids play an important role in antioxidant system in plants. Their anti-oxidative properties are due

to several different mechanisms such as scavenging of free radicals, chelating of metal ions (iron and copper), and inhibition of enzymes responsible for free radical generation [28].

#### DPPH radical scavenging assay

It is well known that the antioxidant effect of the extract on DPPH is due to their ability to scavenge the DPPH free radicals by hydrogen donation. All the extracts were able to reduce the stable radical and exhibited a scavenging activity depending on extract concentration at various degrees. A significant higher scavenging effect against DPPH radical was obtained the ethyl acetate fraction and it increased rapidly at low concentrations ranging from 0.001 to 0.063 mg/ml for. While, a moderate scavenging activity of the aqueous fraction increased from 0.01 to 0.312 mg/ml and the last one was evaluated for the petroleum ether fraction from 0.1 to 1 mg/ml. The

activity was expressed as  $\mu\text{g/ml}$  and represents the concentration of extracts required for 50% of free radical inhibition ( $\text{IC}_{50}$ ). It has been reported that the lower the  $\text{IC}_{50}$  indicates higher activity in this assay. The differences between radical scavenging activities of these fractions were statistically significant, according to Tukey's test at  $p < 0.05$ . The ethyl acetate fraction (Ac) demonstrated a strong antioxidant activity with an  $\text{IC}_{50}$  of  $12.85 \pm 0.27 \mu\text{g/ml}$  (table 2). This fraction was found more effective than the positive control BHA and BHT, which exhibited an  $\text{IC}_{50}$  of  $13.44 \pm 0.3$  and  $20.35 \pm 0.16 \mu\text{g/ml}$  respectively. Low slight activity with an  $\text{IC}_{50}$  of  $109.67 \pm 0.21 \mu\text{g/ml}$  was obtained with the aqueous fraction (Aq), followed by the petroleum ether fraction (EP) with  $439.84 \pm 6.51 \mu\text{g/ml}$ . Thus, phenolic compounds from ethyl acetate extract (Ac) were more efficient antioxidants than aqueous and petroleum ether extracts (Aq and EP).

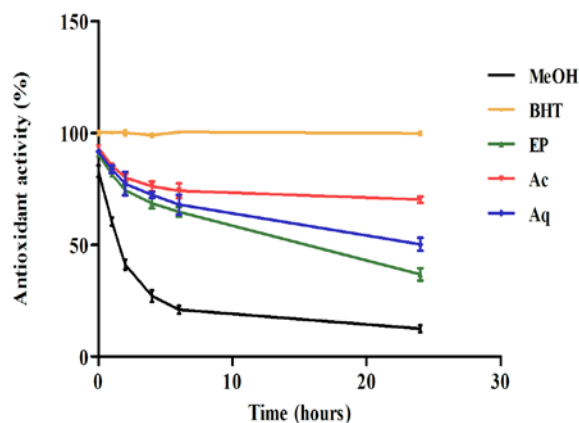
**Table 2: It shows DPPH radical scavenging activity (RSA  $\text{IC}_{50}$ ), antioxidant activity in  $\beta$ -carotene/linoleate test (RAA %) and Ferrous chelating activity (ChA  $\text{IC}_{50}$ ) of leaf extracts from *Plantago major*, and standard**

Sample	RSA $\text{IC}_{50}$ ( $\mu\text{g/ml}$ )	RAA (%)	ChA $\text{IC}_{50}$ ( $\mu\text{g/ml}$ )
EP	439.84 $\pm$ 6.51	36.72 $\pm$ 2.76	
Aq	109.67 $\pm$ 0.21	50.16 $\pm$ 2.9	1020 $\pm$ 20
Ac	12.85 $\pm$ 0.27	70.48 $\pm$ 1.6	
BHA	13.44 $\pm$ 0.3		
BHT	20.35 $\pm$ 0.16	98.88 $\pm$ 0.63	
EDTA			14.52 $\pm$ 0.32

Values are means $\pm$ SD of three replicates (n=3)

#### Bleaching $\beta$ -carotene test

The  $\beta$ -carotene bleaching method is based on the loss of the yellow color of  $\beta$ -carotene, due to its reaction with radicals (hydroperoxide) formed by linoleic acid oxidation in an emulsion. In the absence of an antioxidant,  $\beta$ -carotene undergoes rapid discoloration and the absorbance decreased rapidly. While, the rate of  $\beta$ -carotene bleaching can be slowed down by neutralizing the linoleate free radical in its presence. This retained the color of the emulsion and also absorbance for a longer time. Thus, the degradation rate of  $\beta$ -carotene-linoleate depends on the antioxidant activity of the extracts. The extract which had the lowest  $\beta$ -carotene degradation rate, exhibit the highest antioxidant activity and vice versa [29].



**Fig. 3: It shows the relative antioxidant activity of *Plantago major* extracts, positive control (BHT) and negative control (methanol) in  $\beta$ -carotene/linoleic acid assay**

fig. 3 showed the comparative  $\beta$ -carotene bleaching rates of the standard (BHT), extracts and the control (methanol). There was considerable variation in the antioxidant activities. The control had a rapid oxidation of  $\beta$ -carotene. But, all extracts have the capacity to inhibit the oxidation of  $\beta$ -carotene and their antioxidant activities

were lower than the standard (BHT). The orders of the antioxidant activity are as follows: BHT>Ac>Aq>EP>Methanol. The most active extract in the lipid peroxidation inhibition assay was ethyl acetate fraction (Ac) with relative antioxidant activity (RAA %) of  $70.48 \pm 1.6\%$ , followed by the aqueous fraction (Aq) with  $50.16 \pm 2.9\%$  (table 2). The petroleum ether fraction was the least active extract ( $36.72 \pm 2.76\%$ ). The standard (BHT) has the strongest antioxidant activity of  $98.88 \pm 0.63\%$ , which is statistically higher than the tested extracts when subject to Turkey's multiple comparison test ( $p < 0.05$ ).

#### Ferrous ion chelating activity

The  $\text{IC}_{50}$  value ( $\mu\text{g/ml}$ ), which is the concentration of the extract or standard that chelate 50% of the ferrous ion, was calculated through linear interpolation between values above and below 50% activity. The aqueous fraction was the most active fraction and possessed an  $\text{IC}_{50}$  of  $1.02 \pm 0.02 \text{ mg/ml}$  which was lower than that of the standard EDTA (table 2). No activity was observed for the ethyl acetate and the petroleum fractions.

#### Antibacterial activity

Antimicrobial activities results of all fractions are shown in (table 3) which indicated that the ethyl acetate (Ac) and aqueous (Aq) fractions were more active against gram-positive than gram-negative bacteria. Similar results were obtained with these fractions against three strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter bowie*). On the other hand, no inhibitory effects of the same fractions were found on *Citrobacter freundii*, *Enterobacter faecalis*, *Serratia sp*, *Listeria monocytogenes* and *Candida albicans*. Analysis of the data also showed that the petroleum ether extract (EP) did not show any activity against the test bacteria. The ethyl acetate fraction was more active than aqueous fraction and various antimicrobial effects have been obtained against different strains with inhibition zone ranging from 8.3 to 16.7 mm. *Staphylococcus aureus* showed high sensitivity with a maximum inhibition zone of 16.7 and 15.3 mm respectively to the investigated fractions (Ac and Aq). A maximum inhibition zone of 14.3 mm was exhibited on *Bacillus cereus* for the ethyl acetate fraction (Ac). While moderate activities of 13.3 and 11.3 mm for the same fraction were obtained respectively against *Pseudomonas aeruginosa* and *Acinetobacter bowie*. *Klebsiella pneumonia*, *Proteus mirabilis* and *Salmonella typhimurium* exhibited low susceptibility in comparison with the other strains. The antibacterial activities registered were weaker than the positive control used (Gentamicin).

**Table 3: It shows antibacterial and synergistic activity (zone of inhibition in mm) of extracts from *Plantago major* leaves. Ac: ethyl acetate and Aq: aqueous extract, and Gen: positive control (Gentamicin)**

Test microorganisms	Inhibition zone in mm					
	Ac	Aq	C-	Gen	Gen+Ac	Gen+Aq
<i>Staphylococcus aureus</i>	16.7±1	15.3±1	-	27±0.00	36±0.00	36±0.00
<i>Escherichia coli</i>	-	-	-	nt	nt	nt
<i>Pseudomonas aeruginosa</i>	13.3±0.6	12±1	-	21±0.00	40±0.00	32±0.00
<i>Serratia sp.</i>	-	-	-	nt	nt	nt
<i>Bacillus cereus</i>	14.3±0.6	-	-	30±0.00	33±0.00	nt
<i>Citrobacter freundii</i>	-	-	-	nt	nt	nt
<i>Enterobacter faecalis</i>	-	-	-	nt	nt	nt
<i>Klebsiella pneumoniae</i>	8.7±0.6	-	-	20±0.00	20±0.00	nt
<i>Acinetobacter bowie</i>	11.3±0.6	10.3±0.6	-	23±0.00	23±0.00	22±0.00
<i>Proteus mirabilis</i>	8.3±0.6	-	-	27±0.00	30±0.00	nt
<i>Salmonella typhimurium</i>	9.5±0.5	-	-	31±0.00	35±0.00	nt
<i>Listeria monocytogenese</i>	-	-	-	nt	nt	nt
<i>Candida albicans</i>	-	-	-	nt	nt	nt

C-, Negative control (Methanol), - indicates no inhibition and nt: not tested. Are the results mean±SD of triplicate determinations.

The fractions whose showed activity, were tested further on the same bacteria to determine the Minimum Inhibition Concentrations (MIC). Table 4 shows the MIC values of the extracts against the tested microorganisms. The MIC values for the ethyl acetate fraction (Ac) were 16.7 mg/ml against *Staphylococcus aureus* and 33.5 mg/ml exhibited on *Pseudomonas aeruginosa* and *Bacillus cereus*. A MIC of 67 mg/ml was obtained by the same fraction on *Klebsiella pneumoniae*, *Proteus mirabilis* and *Salmonella typhimurium*.

Kamal Metiner et al. [30] have reported that acetone extract demonstrated the highest effective ness against *Bacillus Cereus* with

a MIC of 3.562 mg/ml. A MIC of 14.25 mg/ml was found with the same extract against *Staphylococcus aureus* and *Klebsiella pneumoniae*, and MIC of 28.5 mg/ml against *Pseudomonas aeruginosa*. These results are almost similar to those that we had obtained regarding *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

For the aqueous fraction (Aq), a MIC of 57.25 mg/ml was obtained on *Staphylococcus aureus*, and 114.5 mg/ml against *Pseudomonas aeruginosa* and *Acinetobacter bowie*.

This data indicate that ethyl acetate (Ac) fraction was highly active as an antimicrobial agent than the aqueous fraction (Aq).

**Table 4: It shows the minimum inhibitory concentration (MIC) of leaf extracts of *Plantago major* against test bacteria. Ac: ethyl acetate and Aq: aqueous extract**

Test microorganisms	MIC (mg/ml)	
	Ac	Aq
<i>Staphylococcus aureus</i>	16.75±0.00	57.25±0.00
<i>Pseudomonas aeruginosa</i>	33.5±0.00	114.5±0.00
<i>Bacillus cereus</i>	33.5±0.00	nt
<i>Klebsiella pneumoniae</i>	67±0.00	nt
<i>Acinetobacter bowie</i>	33.5±0.00	114.5±0.00
<i>Proteus mirabilis</i>	67±0.00	nt
<i>Salmonella typhimurium</i>	67±0.00	nt

nt: not tested

#### Evaluation of synergistic effect of plant extracts and standard antibiotic

Sometimes the use of a single antibiotic does not produce the desired or effective antimicrobial activity, and to overcome this, treatment with a combination of drugs may be attempted. In this study, the inhibitory effect of Gentamycin was evaluated individually and in combination with ethyl acetate and aqueous fractions from the leaves of *Plantago major*. Synergistic antibacterial activity was obtained by the combination of standard antibiotic with leaf extracts of *Plantago major* and showed greater inhibition zones than a single antibiotic against the tested bacteria (table 2). The maximum synergistic effect had average zones of inhibition from 30 to 40 mm and 27 to 36 mm respectively against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Moderate synergistic activity was obtained against *Bacillus cereus* (from 30 to 33 mm), *Proteus mirabilis* (from 27 to 30 mm) and *Salmonella typhimurium* (from 31 to 35 mm). There were no significant differences between Gentamycin and combination of this antibiotic with plant extract on *Klebsiella pneumoniae* and *Acinetobacter bowie*.

The synergy found in this study, suggests that the ethyl acetate and aqueous extract of *Plantago major* contain bioactive compounds that can enhance the activity of antibiotic.

#### DISCUSSION

In the present investigation, high amount of phenolic and flavonoids content was obtained in the ethyl acetate extract than that of petroleum ether and aqueous extracts. Among the tested extracts from leaf *Plantago major*, the ethyl acetate extract has a strongest scavenging activity on DPPH as compared to the standards (BHA and BHT). The IC<sub>50</sub> of this fraction was similar to that of 10.7±0.2 mg/ml found in the study of Özge Kaya [31], and higher than inhibitory concentration (IC<sub>50</sub>: 1369.31µg/ml) scavenged by methanol leaf extract of the same species [32]. An IC<sub>50</sub> of 16.77±1.56 g/ml recorded with seed extract was 1.3 fold lowest compared to inhibitory concentration evaluated in our findings [33]. On the other hand, the β-carotene bleaching method was either used to evaluate the antioxidant capacity of this genus and showed that the ethyl acetate extract was also more active than the other extracts. The ethyl acetate exhibit higher antioxidant activities and this might be due to the presence of high amount of phenolic and flavonoids content. The phenolic compounds contain hydroxyls that are responsible for the radical scavenging redox properties [34].

The ethyl acetate extract showed better antibacterial activity as compared to aqueous extract. This could be due to the presence of greater amount of active antimicrobial components which are more

soluble in organic solvent (ethyl acetate) than water. Our results indicated a high sensitivity of *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* than the antibacterial activity obtained from the same species by Victor et al. [35] and Sharifa et al. [36] (2008); and that of reported for *Plantago mediastepposa* by Marwan et al. [37]. Similar antibacterial activity was obtained in comparison with other species of the same genus such as *Plantago asiatica* [38], *Plantago ovata* [39] and *Plantago lanceolata* [40]. Our results showed a significant synergistic effect against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus mirabilis* and *Salmonella typhimurium*. The ability of plant extracts to act synergistically with antibiotics could be a new approach to solve the problem of bacterial resistance. Thus, phytochemical studies are required to establish the structure of the active compounds of leaf extracts from *Plantago major*.

## CONCLUSION

*Plantago major* contained significant amounts of phenolic compounds and has a potential antioxidant and antibacterial activities. The synergistic effect of combining antibiotics with bioactive plant extracts against multidrug-resistant microorganisms can lead to new forms of treatment of infectious diseases. There is a great promise for development of novel antiradical compounds and antibacterial agents from this plant for dreadful human diseases and oxidation prevention. Further, studies are being conducted to elucidate the chemical structure of bioactive compounds and will be tested to determine their antioxidant and antimicrobial activities.

## CONFLICT OF INTERESTS

The authors have no conflict of interest directly relevant to the content of this article.

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