

Full Length Research Paper

Antioxidant and antibacterial activities of extracts from *Artemisia herba alba* Asso. leaves and some phenolic compounds

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***Artemisia herba alba* Asso. (Compositae)** is widely used in the Algerian folk medicine. Phenolic compounds present in this plant could be candidates for some of its biological activities and therefore for its therapeutic use. In this study, polyphenolic compounds were extracted, identified and quantified in *A. herba alba* leaves extracts. High flavonoids contents (apigenin and luteolin) were present in the ethyl acetate phase, in addition to phenolic acids (protocatechic acid, caffeic acid, gallic acid and ferulic acid derivatives). The aqueous phase contains smaller amounts of phenolic acids while the chloroform phase contains phenolic acids and aglycon flavonoids. The antioxidant properties of these extracts and some phenolic compounds were estimated by measuring the capacity of these extracts and compounds in scavenging the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation and inhibiting lipid peroxidation. The antibacterial activity of *Artemisia* extracts and phenolic compounds was also estimated against some bacterial strains.

Key words: *Artemisia herba alba*, phytotherapy, polyphenols, antioxidant activity, antibacterial activity, free radicals scavenging activity.

INTRODUCTION

Artemisia herba-alba Asso. (Compositae) (Shih) is grown in North Africa and certain parts of Asia and Middle East. It is one of the most widely used plants in the Algerian folk medicine. This plant is used for treatment of gastric disturbances, such as diarrhea, abdominal pain and for healing external wounds. It is also used as remedy for gastritis and inflammation of the gastrointestinal tract (Feuerstein et al., 1986). *Artemisia* is used for diabetes mellitus and other conditions (Marrif et al., 1995) for diabetes in Iraq (Al-Shamaony et al., 1994), and for

hypertension and diabetes in oriental Morocco (Ziyyat et al., 1997).

The aqueous extracts of *A. herba alba* increased gastro-intestinal transit time (Marrif et al., 1995), and protected the rat stomach from 70% ethanol induced gastric lesions (Gharzouli et al., 1999). The aqueous extract and essential oils of this plant were reported to possess a leishmanicidal activity (Hatimi et al., 2001) and used against the intestinal parasite *Enterobius vermicularis* (al-Waili, 1988).

Oxidative damage caused by free radicals is related to various diseases (Halliwell and Gutteridge, 1984). Almost all organisms are well protected against free radical damage by enzymes, such as superoxide dismutase and catalase, or compounds such as ascorbic acid,

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tocopherols and glutathione (Mau et al., 2002; Niki et al., 1994). Medicinal plants are good sources of antioxidants, such polyphenols which scavenge free radicals and limit the harmful effect of these radicals (Burits and Bucar, 2000).

The resistance of pathogenic bacteria to various antibiotics has been reported. Most important resistant bacteria include Gram-positive like *Staphylococcus aureus* and Gram-negative bacteria like *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* (Eloff et al., 2005). Essential oils of *Artemisia* were reported to have antibacterial activity against some bacteria (Yashphe et al., 1979). However, Marri et al. (1995) reported that the extract of this plant possesses relatively weak antibacterial activity and little or no inhibitory activity against the yeast *Saccharomyces cerevisiae*. Other species of *Artemisia* have antimicrobial and antimalarial activities (Kaur and Shina, 1982; Yang et al., 1982).

Several types of flavonoids were identified in *A. herba alba* among them Quercetin-3'-glucoside, Quercetin-3-O-Rutinoside and 5, 4'-dihydroxy-6, 7, 3'-trimethoxy-flavone (Duke 1992). Two other methylated flavones, Dinatin (4', 5, 7-trihydroxy-6-methoxy-flavone) and Skrofulin (4', 7-dihydroxy-6, 7'-dimethoxy-flavone) were isolated from this plant (Shen et al., 1994). In this study, Polyphenolic compounds (flavonoids and phenolic acids) of *A. herba alba* leaves were extracted, quantified and analyzed by HPLC. The antioxidant and free radical scavenging activities of the extracted materials were tested together with their antibacterial effects against selected bacterial strains. The antioxidant and antibacterial activities of some pure phenolic compounds were also examined.

MATERIAL AND METHODS

Plant material

A. herba alba was brought from a local medicinal herbs vendor in Setif (Algeria), and identified at the laboratory of Phytosociology, University Ferhat Abbas, Setif. A voucher specimen was kept at the laboratory. The leaves were dried in shadow and ground to a fine powder.

Extraction procedure

Powdered dried leaves were extracted with 3 volumes of 80% methanol 3 times and with 3 volumes of 50% methanol 3 times. The extracts were combined and kept at 4°C for 24 h. The resulting solution was then filtered and reduced to the aqueous phase under reduced pressure at 35°C. The resulting aqueous solution was kept at 4°C for 24 h to allow the precipitation of chlorophyll. The solution was filtered again and extracted with n-hexane 3 times. The n-hexane extract containing lipids and chlorophyll was discarded. The remaining aqueous solution was extracted 3 times with an equal volume of chloroform and 5 times with an equal volume of ethyl acetate. Extracts of the same phase were combined. The resulting phases (chloroform, ethyl acetate and the aqueous phase) were evaporated under reduced pressure at 35°C and freeze dried.

Determination of total polyphenols

Total polyphenols in lyophilized extracts were determined using the Purssian blue assay (Price and Butler, 1977; Graham, 1992) using gallic acid (25 - 500 mg/l) as a standard. Results were expressed as mg equivalent gallic acid per gram dry weight.

Determination of total flavonoids contents

The flavonoids contents in the lyophilized extracts were estimated by the Aluminum chloride solution according to the method described by (Bahorun et al., 1996). Briefly, 1 ml of the methanol solution of each extract was added to 1 ml of 2% $AlCl_3$ in methanol. After 10 min, the absorbance was determined at 430 nm. Quercetin (0 - 25 ug/ml) was used as a standard. Results were expressed as mg equivalent Quercetin per gram dry weight.

HPLC analysis of phenolic compounds

Phenolic compounds in the chloroform, the ethyl acetate and the aqueous phase of the methanol extract of *A. herba alba* leaves were identified using HPLC with diode array detection Fernandez de Sinas et al. (1993). A sample of 10 mg of the lyophilized material was dissolved in 1 ml 50% methanol for the aqueous phase and in 1ml methanol for the chloroform and the ethyl acetate phase. The solutions were filtered through a 0.22 μ m filter and a 10 aliquots of the filtrates were injected onto the HPLC system. This system was composed of RP Nova Pack C18 column (300 x 3.9 mm) packed with 4 μ m particles and a pre-column containing the same packing material. The apparatus composed of a Waters pump 600^E (Milford, MA. USA) and a U6K Universal injector and auto-injector (Waters 717 Auto-sampler). The UV-Vis spectra of the separated compounds were recorded at 280 nm by a Waters 996 Photodiode array detector. The columns were eluted in gradient with two solvent systems: Solvent A: water/ acetic acid (98:2, v/v) and solvent B: water/ acetonitrile/acetic acid (78:20:2, v/v/v). The flow rate ranges from 1 to 1.2 ml/min and the temperature was set at 23°C and the running time was for 90 min. The gradient elution was from 0 to 70 min by decreasing solvent A to 10% and increasing solvent B to 90%. Polyphenolic compounds were identified by comparison of retention times and spectra of each peak with those of known standards analyzed in the same conditions.

Analysis of flavonoids by HPLC

Flavonoids in the lyophilized extracts were identified by HPLC-DAD system, using the conditions suitable for flavonoids separation described by Fernandez de Sinas et al. (1993). The HPLC system composed of a model pump 600^E, a U6K injector and a 991 Waters Photodiode Array detector. The column was a Nova Pack C18 (3.9 x 150 mm, WATO 36975) and the pre-column was of the same packing. The elution was isocratic with a solvent consisted of Water/methanol/acetic acid (57.5/187.5/25, v/v/v) and the flow rate was 1 ml/min, the oven temperature was set at 23°C and the running time was 40 min. The chromatograms were recorded at 350 nm. Flavonoids were identified by comparison of retention times and spectra of each peak with those of known standards analyzed in the same conditions.

Free radical scavenging activity

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure phenolic compounds were measured from the bleaching of the purple-colored methanol

solution of 2, 2'-O-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits and Bucar, 2000; Sanchez-Moreno et al., 1998). 50 µl of various concentrations of the extracts in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of the free radical DPPH (I%) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration. Tests were carried out in triplicate.

Antioxidant activity

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). A stock solution of β -carotene-linoleic acid mixture was prepared as follows: 0.5 mg β -carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water saturated with oxygen (30 min 100 ml/min) were added with vigorous shaking. 2500 µl of this reaction mixture were dispensed into test tubes and 350 µl portions of the extracts or phenolic compounds solutions, prepared at 2 g/l concentrations were added and the emulsion system was incubated for 48 h at room temperature. The same procedure was repeated with synthetic antioxidant, BHT, as positive control, and with a blank as negative control. After this incubation period, absorbance of the mixtures was measured at 490 nm. Antioxidative capacities of the extracts were compared with those of BHT and the blank.

Determination of the effect of extracts on lipid peroxidation

The level of TBARS (Thiobarbuteric Acid Reactive Substances) in rabbit brain homogenate was measured according to the method of Ohkawa et al. (1979). Rabbits weighing between 1.5 - 2 kg were used. Animals were anaesthetized with 25% urethane (1.2 g/ kg i.p.) and the blood was withdrawn from the animal by perfusing a sufficient volume of cold saline via the jugular vein. The brain was immediately removed and homogenized in ice cold 1.15% KCl. To induce autoxidation a 7% brain homogenate was incubated for 1 h at 37°C in a shaking water bath, in the presence of test solutions at a concentration of 10, 25 and 50 µg/ml or in the presence of an identical volume of 5% CMC for the total peroxidation tubes. The incubated solutions were then centrifuged for 10 min at 4000rpm and an aliquot of 2 ml was added to 0.5 ml of 8.1% SDS and 4 ml 0.8% TBA dissolved in 20% acetic acid. Tubes were then shaken and heated, in a water bath at 100°C for 1 h. After cooling with tap water; the tubes were centrifuged for 10 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm against a blank solution incubated without TBA. Peroxidation results were expressed as percentage of the inhibition in lipid peroxidation.

Bacterial strains

Three referenced bacterial strains were used in this study:

Pseudomonas aeruginosa ATCC 27853, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Six other pathogenic bacteria were isolated and identified in the laboratory of clinical bacteriology and parasitology, University Hospital of Sétif, Algeria. These bacteria were: *Bacillus* sp, *Klebsiella pneumoniae*, *Salmonella thyphi*, *Enterobacter agglomerans*, *Serratia marcescens* and *Citrobacter freundii*.

Antimicrobial screening test

All bacterial strains were grown in nutritive agar in 37°C for 6 h except for *Bacillus* sp which was grown in 30°C. The sensitivity of selected bacteria to plant extracts and phenolic compounds was evaluated by the cup-plate agar diffusion method (Cushnie and Andrew, 2005). 25 ml of Miller-Hinton were discarded in 9 cm Petri dishes and 5 ml of diluted cultures of the test bacteria. The Petri-dishes were rotated slowly to ensure a uniform distribution of microorganisms. They were then left to solidify in the dish. Discs of 6.0 mm diameter were put on the surface of the Petri dish. Different concentrations of the test extracts and phenolic compounds were inoculated into the discs using a micropipette. The dishes were allowed to stand for about 30 min at room temperature to allow for proper diffusion of the extracts to take place. The plates were then incubated at 37°C for 24 h, except for *Bacillus* sp which was incubated in 30°C. This procedure was repeated for each extract concentration three times and the mean diameter of zone of inhibitions were measured and recorded. The antibiotic Gentamicin was used for comparison. The minimum inhibitory concentration (MIC) was determined by comparing the different concentrations of a particular extract having different zones of inhibition and the selected lowest concentration for each extract.

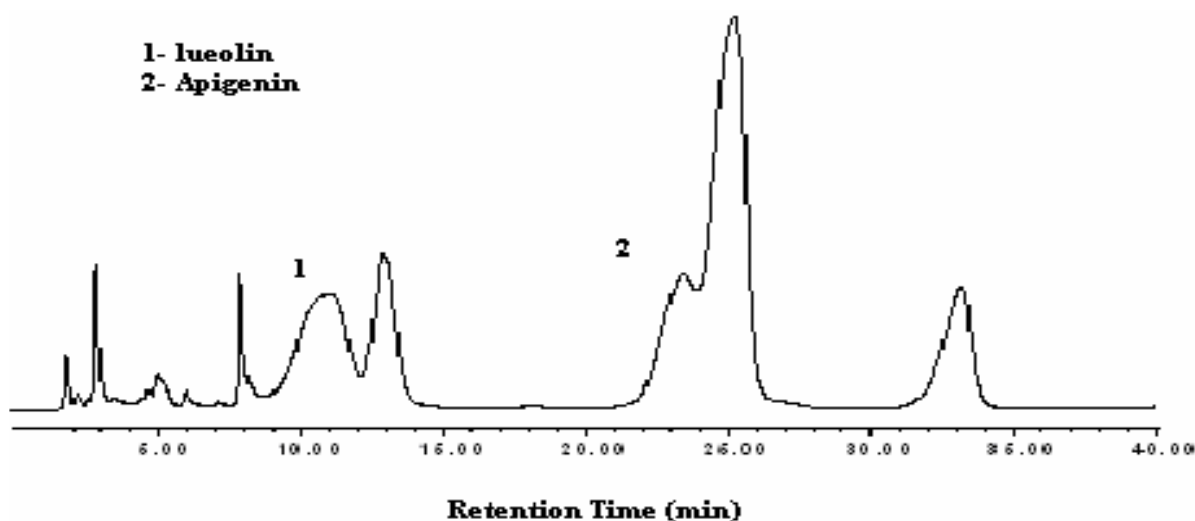
RESULTS AND DISCUSSION

Phenolic compounds and flavonoids in plant extracts

Total polyphenols were estimated in three different extracts, the ethyl acetate phase contains the largest amounts of polyphenols mainly flavonoids and phenolic acids. Total polyphenols and flavonoids in *A. herba alba* leaves extracts varied according to the nature of the extract. High levels of total polyphenols and of flavonoids were noticed in the ethyl acetate phase (Table 1). HPLC analysis of these extracts showed the presence of flavonoids (apigenin and luteolin) (Figure 1) and phenolic acids (protocatechic acid, caffeic acid, and ferulic acid) (Figure 2) in the ethyl acetate phase. The chloroform phase consists mainly of flavonoids aglycons (apigenin and luteolin) and phenolic acids (protocatechic acid, vanillic acid, caffeic acid and ferulic acid). It was previously reported that *A. herba alba* contains phenolic acids. Chlorogenic acid was reported in this plant (Mouhajir et al., 2001). This plant has been also reported to contain Quercetin-3'-glucoside, Quercetin-3-O-Rutinoside (Duke, 1992). Luteolin 7-O-rutinoside was also isolated from *Artemisia montana* and was reported to have a strong scavenging activity towards DPPH (Kim et al., 2000).

Table 1. Total polyphenols (mg gallic acid/g dry weight), flavonoids (mg quercetin/g dry weight) in *Artemisia herba alba* leaves extracts and their anti-lipid peroxidation activity.

Extract	Total polyphenols	Flavonoids	% inhibition of lipid peroxidation		
			Extract concentration (mg/ml)		
			10	25	50
Chloroform	160.47	41	0	0	3
Ethyl acetate	320.43	76.55	41.5 ± 4.9	46.4 ± 5.1	45.7 ± 5.6
Aqueous	133.43	17.80	23 ± 1.8	39 ± 5.3	43 ± 5.12

**Figure 1.** HPLC profile of flavonoids in the ethyl acetate phase of the methanol extract of *Artemisia herba alba* leaves.

Antioxidant and free radical scavenging activity of phenolic compounds and *Artemisia* extracts

The capacity of the extracts to inhibit lipid peroxidation is shown in Table 1. The ethyl acetate phase inhibited lipid peroxidation by 45%. This extract showed a strong anti-lipoperoxidation activity and a high scavenging capacity compared to the other extracts. Phenolic compounds and all plant extracts showed high ability in scavenging DPPH radical (Table 2). The IC_{50} for these extracts was 32.9 ± 0.036 and 154 ± 0.014 $\mu\text{g/ml}$ for ethyl acetate extract and aqueous extract, respectively. The synthetic antioxidant butylated hydroxyl toluene (BHT) had a value of $IC_{50} = 17.8 \pm 0.022$ $\mu\text{g/ml}$. Pure phenolic compounds were more effective than BHT, the IC_{50} for gallic acid and tannic acid reached 2.1 ± 0.05 and 2.3 ± 0.009 $\mu\text{g/ml}$ respectively (Table 2). Anti-radical power of phenolic compounds is very known (Hanasaki et al., 1994). Plant extracts and polyphenolic compounds used in this study strongly inhibited the oxidation of linolenic acid, the percentage inhibition varied between 86 and 98% (Figures 3 and 4). Phenolic compounds were reported to be very strong antioxidants (Pietta, 2000). Kim et al. (2003) reported anti-oxidant activities of the extracts from

the herbs of *Artemisia apiacea*. The antioxidant activity of phenolic compounds and plant extracts was closer to that of the synthetic antioxidant BHT. The antioxidant and free radical scavenging activities of plant extracts is correlated with total polyphenols levels.

Antibacterial activity of phenolic compounds and *Artemisia* extracts

As shown in (Table 3), the antibacterial activity of both extracts of the leaves of the plant was very poor. Both *Artemisia herba-alba* aqueous and ethyl acetate extracts had a weak or no antibacterial activity against the selected bacterial strains. The antibacterial activity of both extracts of the leaves was very poor. The weak activity was noticed against *Staphylococcus aureus*. Phenolic compounds especially phenolic acids (gallic acid, caffeic acid and tannic acid) showed high antibacterial activity against *Staphylococcus aureus* but not against other bacteria (Figure 5). Antibacterial activities of flavonoids were described previously (Middleton et al., 2000). It was reported that *Artemisia herba-alba* has a weaker antibacterial activity than the

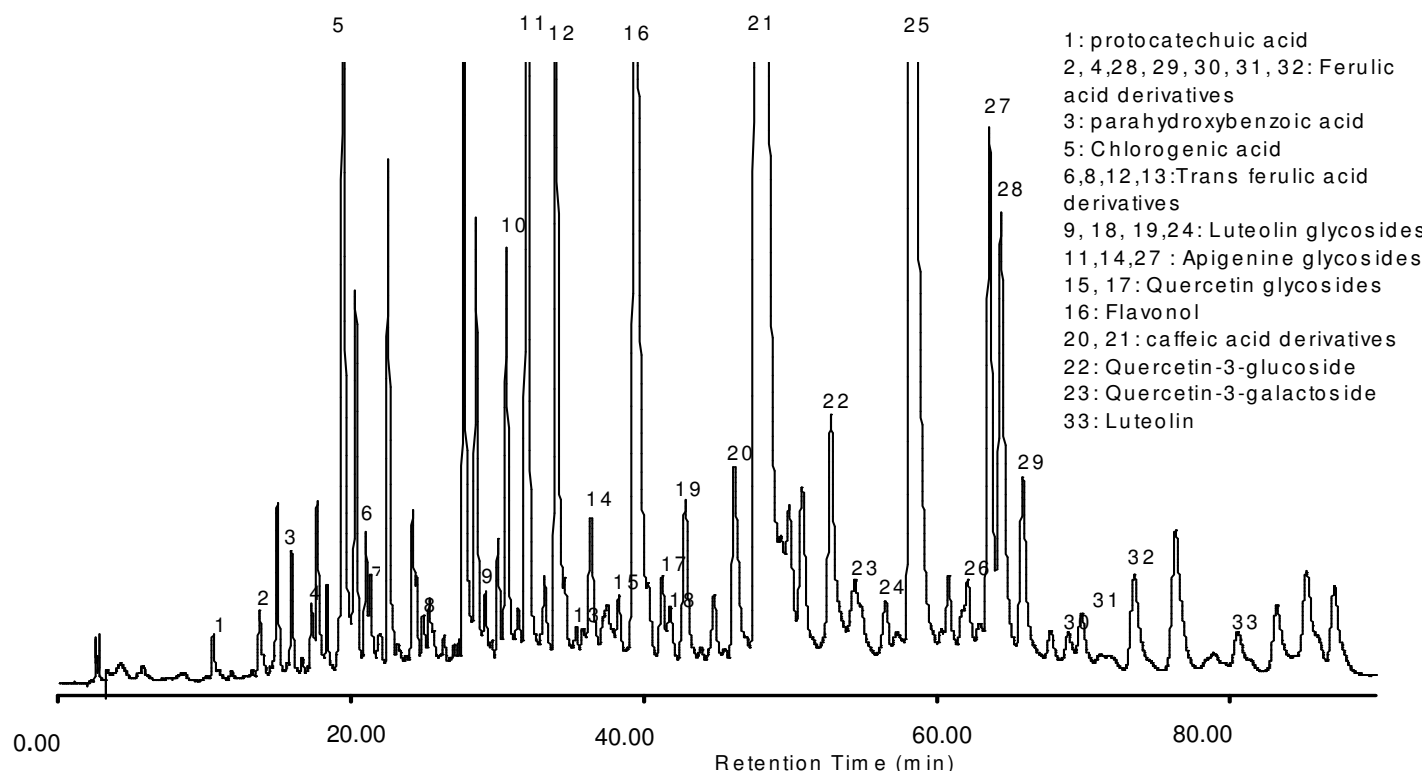


Figure 2. HPLC Profile of the ethyl acetate phase of the methanol extract of *Artemisia herba alba* leaves.

Table 2. Scavenging activity of DPPH radicals by phenolic compounds and *Artemisia* extracts. Results represent the IC₅₀ values (μg/ml) of each extract and phenolic compound.

Extracts and compound	IC ₅₀ ug/ ml
<i>Artemisia herba alba</i> (EA)	32.9 ± 0.036
<i>Artemisia herba alba</i> (PA)	154.6 ± 0.014
Quercetin	5.4 ± 0.022
Rutin	8.6 ± 0.019
Gallic acid	2.1 ± 0.05
Tannic acid	2.3 ± 0.009
Caffeic acid	4.8 ± 0.005
Catechin	4.3 ± 0.014
BHT	17.8 ± 0.022

related species of *Artemisia* (Yashphe et al., 1979; Kaur and Shinna, 1992). Marri et al. (1995) reported that the extract of this plant possesses relatively weak antibacterial activity and little or no inhibitory activity against the yeast *Saccharomyces cerevisiae*. The essential oil of *Artemisia annua* aerial parts remarkably inhibited the growth of *Enterococcus hirae* (Juteau et al., 2002).

It is concluded that organic and aqueous extracts from *Artemisia herba alba*, exert strong free radical scavenging and antioxidant activities which are related to

their polyphenol contents. The ethyl acetate extract of this plant contains flavonoids like apigenin and luteolin and phenolic acids like caffeic acid, ferulic acid and protocatechuic acid. This extract had antilipoperoxidant activity, antioxidant and free radical scavenging capacities. These activities are due, at least in part, to flavonoids and phenolic acids present in this plant, since other phenolic compounds used in this study showed high antioxidant activity. A weak or no antibacterial activity of this plant extracts was noticed against selected bacteria.

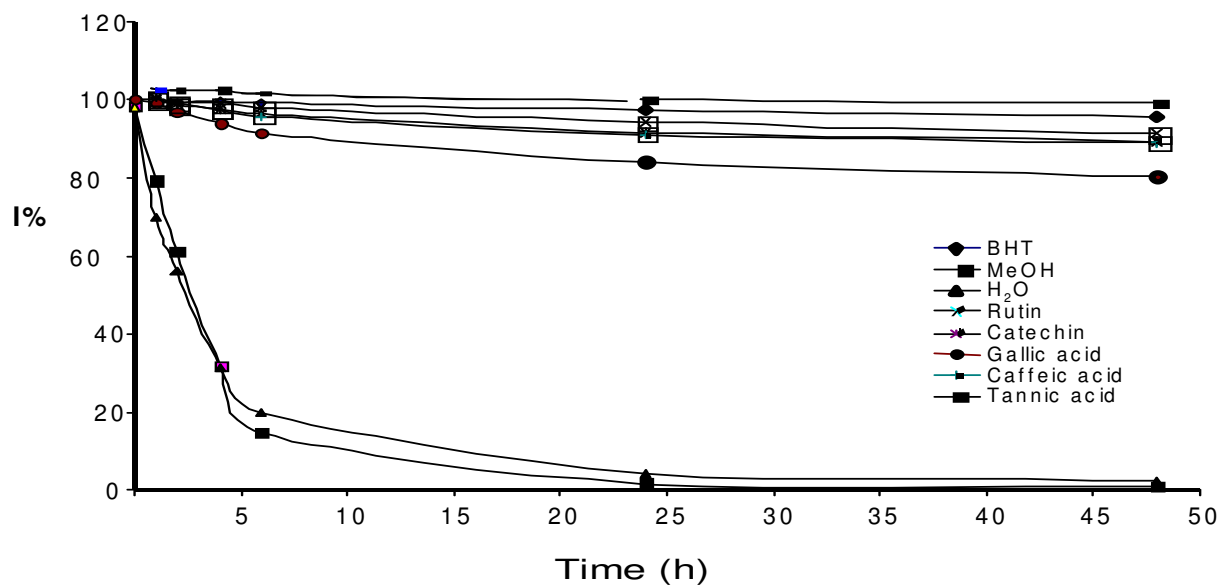


Figure 3. Effects of phenolic compounds on the oxidation of β -carotene-linoleic acid. Antioxidative capacities (I%: Percentage of inhibition) of phenolic compounds were compared with those of BHT and blank. Tannic acid showed a strong antioxidant activity more than BHT.

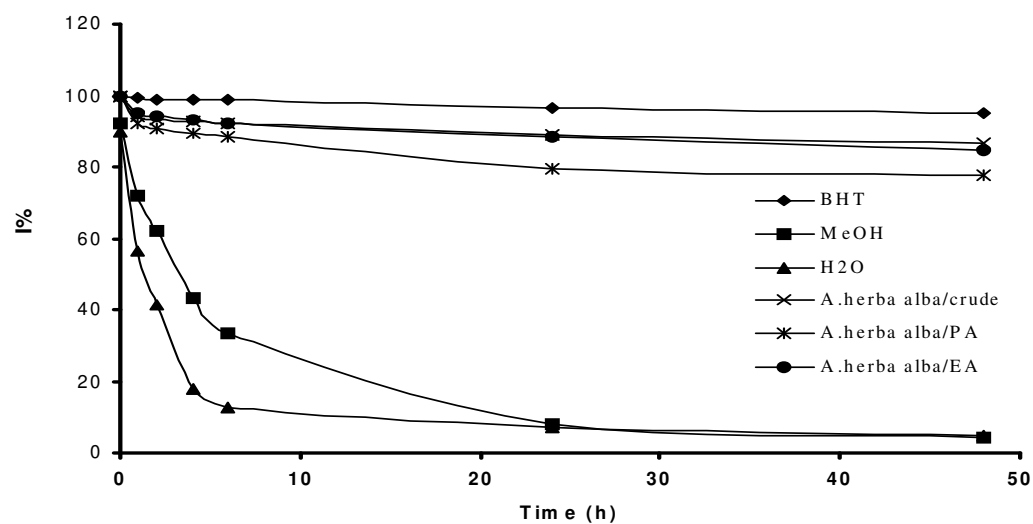


Figure 4. Effects of *Artemisia herba alba* extracts on the oxidation of β -carotene-linoleic acid. Antioxidative capacities (I%: Percentage of inhibition) of extracts were compared with those of BHT and blank. EA: ethyl acetate extract, PA: Aqueous extract, Crude: methanol extract.

Table 3. Effects of *Artemisia* extracts on the growth of some bacterial strains.1: *Serratia marcescens*, 2: *Citrobacter freundii*, 3: *Enterobacter agglomerans*, 4: *Salmonella typhi*, 5: *Klebsiella pneumoniae*, 6: *Bacillus* sp, 7: *Pseudomonas aeruginosa*, 8: *Escherichia coli*, 9: *Staphylococcus aureus*.

Bacteria extracts and compounds	1	2	3	4	5	6	7	8	9
<i>A. herba alba</i> (PA)	6	6	6	6	6	6	6	6	6
<i>A. herba alba</i> (EA)	6	7.5	6	6	6	6	6	6	8.5
Gentamicin	24	22	23	17	18	23	17	24	22

E A: ethyl acetate extract; PA: aqueous extract. Results are expressed as diameter of inhibition zone IZ (mm).

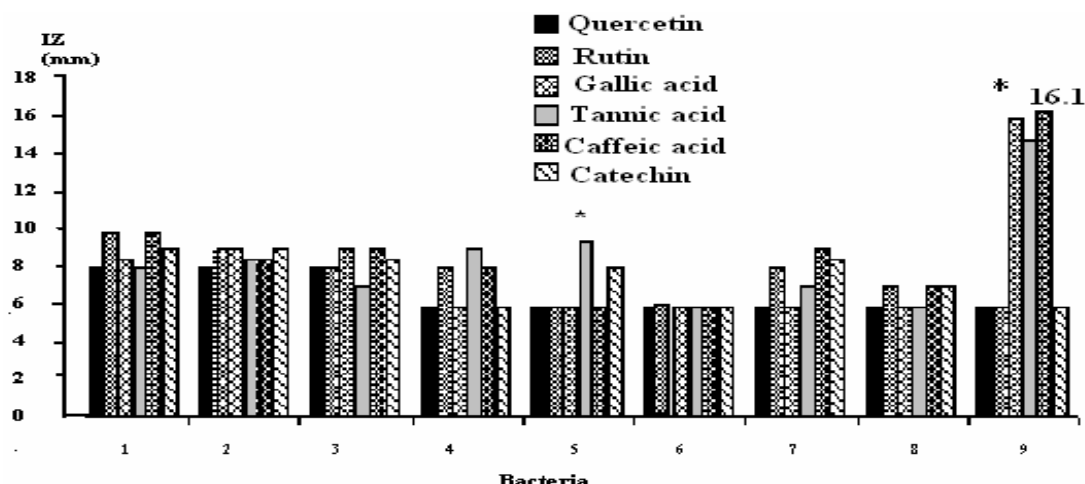


Figure 5. Effects of some phenolic compounds on the growth of some bacterial strains: 1: *Serratia marcescens*, 2: *Citrobacter freundii*, 3: *Enterobacter agglomerans*, 4: *Salmonella typhi*, 5: *Klebsiella pneumoniae*, 6: *Bacillus sp*, 7: *Pseudomonas aeruginosa*, 8: *Escherichia coli*, 9: *Staphylococcus aureus*. * Phenolic acids (gallic acid, caffeic acid and tannic acid) showed high antibacterial activity against *Staphylococcus aureus*. Results are expressed as diameter of inhibition zone (mm).

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