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Phytochemical Screening and Antibacterial Activity of *Pistacia atlantica* and *Pinus canariensis* Leaf Extracts

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Abstract: The qualitative detection of phytochemical compounds of extracts was carried out using color reagents, total content of phenols and flavonoids was specified using Folin-Ciocalteu and aluminum chloride method, respectively, and antioxidant activity was determined through its ability to free radicals scavenging using DPPH radical. The efficacy of the plant extracts against pathogenic bacteria was studied by agar well diffusion method with different concentrations, and microdilution method was used to measure minimum inhibitory concentration (MIC) of all plant extracts. The results showed presence of tannins, phenols, and flavonoids in all extracts of both plants, while saponins were found in aqueous extracts only, cardiac glycosides and coumarins were absent in all plant extracts. Ethanolic extract of *Pistacia atlantica* recorded the highest content of phenols and flavonoids as 263.76 ± 0.53 (mg GAE/g Dw) and 46.83 ± 0.55 (mg RE/g Dw), respectively. While aqueous extract of *Pinus canariensis* recorded the lowest content of of phenols and flavonoids 30.11 ± 0.37 (mg GAE/g Dw) and 5.43 ± 0.38 (mg RE/g Dw), respectively. Both plants have been shown to have good antioxidant activity, as ethanolic extract of P. atlantica recorded the best ability to free radicals scavenging 90.27% ±1.51, ethanolic extracts of both plants were the most effective in inhibiting bacteria especially at high concentrations (500 mg/mL); the inhibition zone diameter of *P. atlantica* extract reached 33.56 mm against *Shigella boydii*, while aqueous extract of *P. canariensis* was the most effective against *Pseudomonas aeruginosa*; the inhibition zone diameter was 21 mm. MIC ranged between 5.468 and 43.75 mg/mL depending on plant extract and bacterial species. This confirms the importance of plant extracts as a natural source of antibacterial to confront problems of increasing bacterial resistance to antibiotics that threaten public health.

Keywords: Total phenolic, flavonoid, DPPH, antibacterial activity, MIC value, Pistacia atlantica, Pinus canariensis.

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INTRODUCTION

Plants have shown an important role in treating and preventing many diseases in pharmacology since past time, and plant extracts were used to treat various diseases such as diarrhea, sleep disorders and cough, infections, cancer, cardiovascular, and diabetes, due to their wide spread and diversity, and they contain many compounds with therapeutic characteristics. In addition, medicinal plants have been shown to possess the advantage of having low side effects compared to antibiotics (1, 2). About 80% of the world's population depends on traditional medicine according to WHO estimates. As a result, the demands of plant extracts for medicinal purposes in many countries had been increased (3). It was focused on that secondary metabolites in medicinal plants are characterized with different medicinal properties. On the other hand, detection of genes of Staphylococci maintained increasing antibiotic resistance as well as: amoxicillin/ clavulanic acid 65%, ampicillin 70%. Percentage of presence of MRSA strains was 15% and MRCNS was 6.66% (4). Occurrence of bacterial multidrug resistance feature side effects of medicine use induced WHO to maintaining importance of plants therapy (5). The random use of antibiotics used to treat diseases sometimes led to negative side effects on the host as immune response, allergic reactions,

and hypersensitivity, which necessitated to develop alternative drugs from different sources such as plants (6). A positive effect of *Myrtus communis* extracts occurred against pathogenic bacteria (7), and the effect of Lamiaceae plants extracts as Mentha and Ocimum; which induce to isolate and study phytochemicals for explaining its effects against microorganisms (8).

Pistacia atlantica (Anacardiaceae) is a tree with a length of 2-5 m, its branches are grayish-white and have leaves composed of 9 to 11 leaflets (pinnate compound leaves). Oleoresin is secreted by the trunk featuring a yellowish-green color and a mild smell (9), and it contains many chemical compounds in various parts of the plant: α-pinene, limonene, β-pinene, terpinolene, camphene, bornyl acetate, sabinene, p-mentha-1 (7),8 diene, Δ^3-carene, spathulenol, masticadienonic acid, morolic acid, gallic acid, oleanolic acid, tetragalloylquinic acid, quinic acid, quercetin-3-glucoside, 3-O-acetyl-3-epiisomasticadienolic acid, 3-methoxycarpachromene, β-myrcene, (9, 10, 11). P. atlantica has antibacterial activity, as a research has indicated that it has widespread inhibitory effects against number of Gram (-) bacteria (*E. coli, Proteus mirabilis, Pseudomonas aeruginosa, Enterobacter cloacea, Salmonella typhi, Acinetobacter baumannii*) and Gram (+) (*Staphylococcus aureus, Listeria monocytogenes*) (12, 13), and antifungal properties against some microorganisms (*Aspergillus fumigates, Aspergillus flavus, Aspergillus niger, Candida sp*) (14), and anti-adenovirus agent (15), and anti-inflammatory activity (16).

Pinus canariensis (Pinaceae) is an evergreen tree reaches more than 30 m high, resin canals in wood, bark, leaves and often cones, Dwarf shoots (fascicles) hold three long (20– 30 cm) needles (17). Analysis of the essential oil showed 116 compounds; more than 100 substances belonging to terpenoids: (sesquiterpenes, monoterpenes, and diterpenes) by 52.1%, 42.7%, and 4.8% respectively, the most important substances of monoterpenes are (α-pinene 23.1%, β- pinene 1.6%, myrcene 5.8%, limonene 10.1%) (18). Another study showed the presence of monoterpenes 30.7%, the most important of which were (α- pinene 14.6%, β- pinene 1.2%, myrcene 6.4%, and limonene 7.9%), and sesquiterpenes 66.6%, (as germacrene D formed the main compound 44%, then β-caryophyllene 8.7%), and diterpenes 2.4% (19). Pinus in traditional medicine are used for respiratory system as antiseptic and expectorant, also for gastrointestinal disorders, urinary system diseases and for the treatment of skin diseases. Pine needles extracts showed effect against a range of bacteria as (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus cereus*) (20) pine needles showed exhibit strong antioxidant, antimutagenic and also antitumor effects in vivo and point to their potential usefulness in cancer prevention (21).

The increase in the infectious diseases and the development of bacterial resistance to antibiotics, and their side effects necessitate search for new compounds that are effective against pathogenic bacteria. This research aims qualitative (alkaloids, cardiac glycosides, resins, tannins, flavonoids, saponins, phenols, and coumarins) and quantitative screening (total phenolic and flavonoid contents), and study of antioxidant activity of *Pistacia atlantica* and *Pinus canariensis* leaf extracts, and testing their bioactivity against pathogenic bacteria.

MATERIALS AND METHODS

Collection of Samples

Samples of *Pistacia atlantica* and *Pinus canariensis* leaves were collected in Sweida area (Syria) in September of 2019, and washed with distilled water to remove impurities, and dried in shadow for 14 days, and ground into a dry soft powder, and powders were stored in refrigerator at 4 °C until use (13).

Preparation of Extracts

Aqueous and ethanolic extracts were prepared using Soxhlet method; 50 grams of powdered leaves were separately extracted in 500 mL of water and ethanol at a ratio 1:10 (w/v) for 7 h, and filtered using Whatman filter paper №1. Filtrates were evaporated using a rotary evaporator under vacuum at 40 °C and kept in refrigerator at 4 °C until they were used (22). All extracts were sterilized before use by filtration through membrane filters 0.45 µm. Determination of percentage yield (%) was calculated using the formula (23):

yield % = (weight of final dried extract / weight of initial dried plant sample) x 100

Phytochemical Qualitative Screening Test

Test for Alkaloids

a-Dragendorff's reagent test: A few drops of Dragendorff's reagent were added to (1 mL) of each extracts and mixed, then diluted hydrochloric acid (2 mL) (HCl) were added. formation of precipitate of reddish-yellow color indicates appearance of alkaloids.

b- Mayer's test: To each 1 mL of studied extracts a few drops of Meyer's reagent were added. Formation of a creamy white precipitate indicates appearance of alkaloids (24).

Test of Cardiac glycosides

Keller Killiani Test: 1 mL of anhydrous acetic acid added to each extract of plant and shaken, then a few drops of ferric chloride were added, and 2-3 drops of sulfuric acid (concentrated) were added carefully to the test tube, appearance of a reddish-brown-colored ring at the junction of two layers, which confirms the positive test (24, 25).

Test for Resins
Turbidity test: 10 mL of distilled water was added to each plant extract, to which a few drops of 4% HCl were added. Appearance of turbidity in solution indicates presence of resins (25).

Test of Tannins
Lead acetate Test: A few drops of lead acetate were added to 1 mL of plant extract. Formation of a large white-brown precipitate indicates presence of tannins (24).

Tests for Flavonoids
a- Shinoda Test: 0.5 g of magnesium powder was added to each plant extract, then a few drops of concentrated hydrochloric acid were added. Appearance of a red color indicates presence of flavonoids.
b- Alkaline Test: Sodium hydroxide solution was added to 1-2 mL of each plant extract. A yellow to red color formed in test tube confirms presence of flavonoids (24, 25, 26).

Test for Saponins
One mL of plant extract was added to 20 mL of distilled water, and shaken vigorously for 5-10 minutes. Formation of a froth column that does not disappear by adding HCl indicates presence of saponins (25, 26, 27).

Test for Phenols
To each plant extract was added 1 mL of $FeCl_3$ (5%). Formation of bluish-black color indicates presence of phenols (25).

Test for Coumarins
One mL of each extract were taken in separate tubes, and covered with a filter paper moistened with 1N NaOH solution, and heated for a few minutes. When these tubes yield a yellow fluorescence under UV light, this indicates the presence of coumarins (24).

Phytochemical Quantitative Screening Test

Total phenolic content (TPC)
TPC in all plant extracts were measured by the Folin–Ciocalteu method, 1000 µL of each sample of concentration of 0.011 g/mL was added to 4.8 mL distilled water, 4 mL sodium carbonate 2% (Na_2CO_3) and 200 µL of Folin- Ciocalteu reagent and mixed fully, the absorbance was recorded at 760 nm by a spectrophotometer after 60 min of incubation, distilled water was used as a blank. A calibration curve of gallic acid solutions were prepared in ethanol at different concentrations 0 to 300 ppm (Figure 1), and the results were estimated as gallic acid equivalent for each gram of dry plant extract (mg GAE/g Dw). Total phenolic contents of samples were determined in triplicate (28, 29).

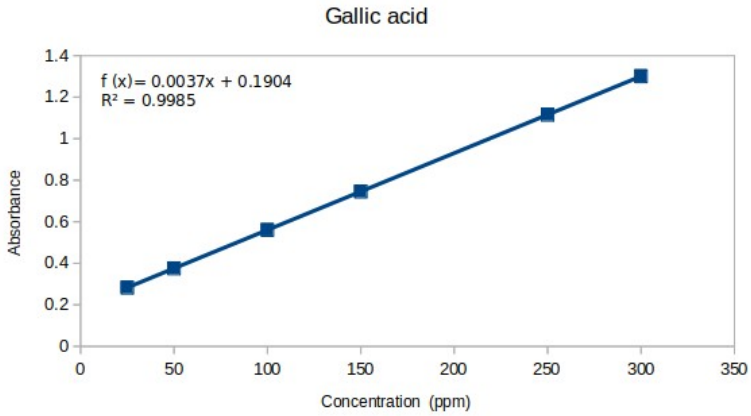


Figure 1. Calibration curve of gallic acid.

Total Flavonoid content (TFC)
TFC was measured using aluminum chloride method $AlCl_3$,$6H_2O$ in the plant extracts, each plant extract (0.5 mL) was added to distilled water (2 mL) and 150 µL of sodium nitrite $NaNO_2$ (5% w/v). After 5 minutes, 10% of aluminum chloride solution (150 µL) was added to mixture, then incubated in the dark for 6 min. Finally, 4% of NaOH (2 mL) was added and mixed well, after 15 minutes of incubation in the dark the solutions turned to pink. Distilled water was used as a blank, the absorbance was recorded at 510 nm by a spectrophotometer, a calibration curve of rutin solutions were prepared at different concentrations 0 to 150 ppm (Figure 2), and the results were estimated as rutin equivalent per gram of dry plant extract (mg RE/g Dw). Total flavonoid of samples were measured in triplicate (30).

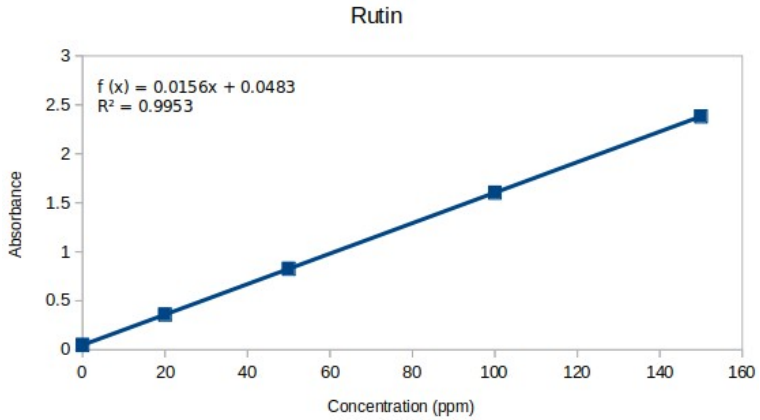


Figure 2. Calibration curve of rutin.

Antioxidant Activity

DPPH Radical Scavenging Activity Assay

Each plant extract (300 µL) of concentration of 0.001 g/mL was added in test tubes separately, and 3 mL of DPPH in ethanol (45 µg/mL) was added to each tube and mixed vigorously, after 30 min of incubation without light the absorbance was recorded at 515 nm by a spectrophotometer. The results were presented compared to ascorbic acid which was prepared as standard with different concentrations from 0 to 0.5 mM/L (Figure 3). The results were calculated as a percentage (%) using the following formula (31):

$$\text{Scavenging DPPH (\%)} = A_{control} - A_{sample} / A_{control} \times 100$$

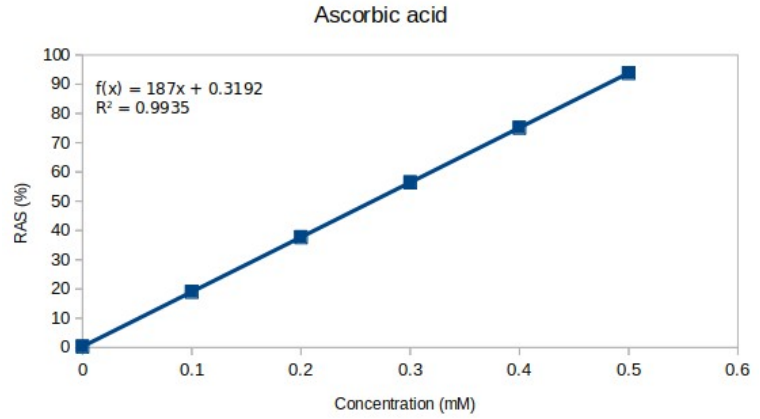


Figure 3. Calibration curve of ascorbic acid.

Antibacterial Susceptibility Test

Bacterial Isolates

The antibacterial test was carried out using: *Staphylococcus aureus, Klebsiella pneumoniae, E. coli, Proteus mirabilis, Pseudomonas aeruginosa, Shigella boydii,* and *Enterobacter cloacae*, were obtained from the microbiology Laboratory - Department of Plant Biology, Faculty of Science at the university of Damascus (Syria).

Antibacterial Activity

The bacterial susceptibility tests for plant extracts were performed by agar well diffusion method on Mueller-Hinton agar. The bacterial isolates were activated for 24 h at 37 °C on nutrient agar, then a bacterial suspension was prepared in a saline solution [sterile NaCl 0.85% (w/v)]. Turbidity of prepared bacterial suspension was 0.5 McFarland (10^8 CFU/mL). The suspension was used to inoculate 9-cm-diameter Petri dishes with a sterile cotton swab, after that 5-6 wells (4 mm diameter) were punched in the agar plate, and 50 µL of plant extracts were added in each well. All plates were placed in the refrigerator (4 °C) for 2 h in order to allow diffusion of plant extracts into the medium. Then the plates were incubated for 24 h at 37 °C. After incubation, inhibition zone diameters were measured to determine the effectiveness of extracts against tested bacteria. Tests were performed in triplicates per experiment and the average of the results was taken, the plant extracts were dissolved in dimethyl sulfoxide solution (DMSO) to obtain the concentrations 500, 350, 250, 150, 50 mg/mL. DMSO solution was used as negative control, and the antibiotics Moxifloxacin 5 mcg and Gentamicin 10 mcg as positive control (30, 32).

Determination of MIC

MIC was measured by nutrient broth microdilution in microtitration plates containing 96 well (33, 34)

with some modifications. The turbidity of prepared bacterial suspension was 0.5 McFarland (10^8 CFU/mL). First, the dry extracts were dissolved in DMSO to obtain a concentration of (350 mg/mL) to be tested, then serial two-fold dilution was performed in a concentration range of 0.683 to 350 mg/mL. For each test batch, two control wells were prepared; negative control: nutrient broth medium (50 µL) and bacterial suspension (50 µL) in first well, positive control: plant extract (50 µL) and bacterial suspension (50 µL) in second well, all wells except the second were filled with the nutrient broth (50 µL). Then, 50 µL of plant extract at the highest concentration (350 mg/mL) were added to the third well and mixed, 50 µL of mixture was taken for fourth well and so until serial decreasing concentrations were obtained in the rest of the wells. Then the wells were inoculated with bacterial suspension 50 µL, and the plates were incubated for 24 h at 37 °C. Each well contained 100 µL (final volume). After incubation, a solution (20 µL) of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in water (0.01%, w/v) was added to each well and plates were incubated for an additional 2 h. Results were estimated visually by observing the color change from yellow to red, which is an indication of bacterial growth and determined MIC as lowest concentration in which it appeared medium in yellow (no red color) (33).

Statistical Analysis

SPSS software (version 22) was used by one-way ANOVA to analyze the data statistically. Pearson's correlation was used to determine the correlation between TPC, TFC and antioxidant activity. Data was considered statistically significant at minimum level of $P < 0.05$.

RESULTS AND DISCUSSION

Phytochemical qualitative screening of active chemical compounds

Pistacia atlantica extracts were distinguished by their high (very rich) of phenolic, tannin, and flavonoid contents and they were higher than and Pinus canariensis extracts (Table 1).

Saponins were found with a high content in P. canariensis aqueous extract, as a column of foam was formed more than 3 cm high and foam did not disappear after adding HCl.

A high content of resin were found in ethanolic extract of P. canariensis, while it was absent in the aqueous extract of Pistacia atlantica, while alkaloids were absent in aqueous and ethanolic extracts of P. atlantica, and found only in a small amount in aqueous extract of Pinus canariensis. Coumarins and cardiac glycosides were not found in all extracts of both plants. For detection of active chemical compounds in P. atlantica leaves extracts; two studies in Libya and Armenia indicated that extracts contained phenols, flavonoids, and tannins. However, the study in Armenia showed the presence of coumarins, and this difference with our research may be due to the difference in the genetic combination of the plant, climatic conditions, and geographical location, in addition to the extraction methods and the quality of the solvents used (35, 36). No references were found concerned with phytochemical screening of Pinus canariensis extracts. Our results were however similar to many studies that indicated presence of phenols, saponins, and tannins in other species of the pine genus (37).

Table 1. Phytochemical screening of *Pistacia atlantica* and *Pinus canariensis* leaves extracts.

Chemical components	***P. atlantica***		***P. canariensis***	
	Aqueous extract	**Ethanolic extract**	**Aqueous extract**	**Ethanolic extract**
Alkaloids	-	-	+	-
Cardiac glycosides	-	-	-	-
Resins	-	+	+	++
Tannins	+++	+++	+++	++
Phenols	+++	+++	++	++
Flavonoids	++	++	+	+
Saponins	+	-	+++	-
Coumarins	-	-	-	-

−: absence , +: presence in small quantities, ++: presence in high quantities, +++: presence in very high quantities.

Determination of yields, TPC, and TFC of P. atlantica and P. canariensis leaves extracts

Yields of plant extracts differed according to extraction solvent used and plant species. The yields of ethanolic and aqueous extracts of Pistacia atlantica were 30.12% ± 0.14 and 24.20% ± 0.08, respectively, significantly higher than the yields of ethanolic and aqueous extracts of Pinus canariensis which amounted to 20.53% ± 0.09 and 15.77% ± 0.04, respectively (Table 2).

Results showed that TPC and TFC varies between both plants; P. atlantica extracts contained higher concentrations of phenols and flavonoids compared to P. canariensis extracts, phenolic contents of ethanolic extracts of P. atlantica and P. canariensis were 263.76 ± 0.53, and 40.52 ± 0.58 mg GAE/g

Dw, respectively, while aqueous extracts reached 241.64 ± 0.16 and 30.11 ± 0.37 mg GAE/g Dw for the studied species, respectively. While the flavonoid contents of ethanolic extract of P. atlantica and P. canariensis reached 46.83 ± 0.55 and 9.80 ± 0.12 mg RE/g Dw, respectively, and the aqueous extract of P. atlantica and P. canariensis reached 31.81 ± 0.26 and 5.43 ± 0.38, respectively (Table 2).

Results of our study are consistent with many studies that prove that P. atlantica leaves extracts contain a good content of phenols and flavonoids at varying proportions, as one of the studies conducted in Tunisia showed TPC and TFC in ethanolic extract is higher than in aqueous, as TPC of ethanolic and aqueous extracts reached 68.23 ± 0.8 and 20.07 ± 0.2 mg GAE/g Dw, respectively, while TFC reached 44 ± 0.8, and 15 ± 0.2 mg RE/g Dw for ethanolic and aqueous extracts, respectively (38).

TPC in aqueous, ethyl acetate, and n-butanol extracts of P. atlantica leaves in Algeria were 421.01 ± 8.92, 514.81 ± 2.10, and 376.34 ± 3.43 mg GAE/g Dw, respectively, while TFC were 44.51 ± 0.29, 126.43 ± 1.31, and 103.77 ± 1.07 mg CE/g DW for previous extracts, respectively (39). One study was concerned in studying the effect of growing area, harvest time, and gender on phenolic and flavonoids compounds of P. atlantica leaves extracts. Results showed that phenols ranged between 79.00 ± 13 and 259 ± 8 mg GAE/g Dw, while flavonoids ranged between 0.65 ± 0.10 and 2.81 ± 0.88 mg QE/g DW depending on the study period, phenolic contents of leaves is shown to decrease from spring to autumn; the content was affected by harvest time and growing region more than plant gender (male or female) (40).

No references were found concerning with TPC and TFC of P. canariensis extracts. Therefore, these results were compared with the results of research conducted on other species of pine genus, one of these studies determined TPC and TFC in aqueous, ethanol, and n-butanol extracts of Pinus roxburghii and Pinus wallichiana; phenolic contents of different solvents ranged 3.94 ± 0.03, 10.08 ± 0.06, and 8.55 ± 0.28 mg GAE/g Dw respectively for Pinus roxburghii, while ranged 4.09 ± 0.43 and 4.06 ± 1.12 mg GAE/g Dw for ethanolic and butanol extracts respectively for Pinus wallichiana, while the phenolic contents were absent in aqueous extract for Pinus wallichiana (41). Results of the study conducted in Tunisia to determine content of phenols and flavonoids in ethanol extracts of leaves of 19 subspecies of Pinus nigra showed that total phenols ranged from 15.67 ± 1.95 and 47.53 ± 1.32 mg GAE/g Dw, and amount of flavonoids varies from 1.69 ± 0.32 and 3.97 0.17 mg RE/g Dw (42), while the study conducted in Romania showed a good content of phenols 78.22 ± 0.44 mg GAE/g Dw and flavonoids 19.84 ± 0.57 mg CE/g DW for Pinus cembra needle extract (aqueous methanol extract 80%) (20).

It should be noted that content of phenols and flavonoids in plant species in general is affected by different environmental factors characteristic of each geographical region, in addition to the difference in time of samples collection which in turn depended on growth rate, genetic diversity, different methods of storing and drying samples, and difference in extraction methods and solvent used in preparation of plant samples (39).

Table 2. Yields, TPC, and TFC in *Pistacia atlantica* and *Pinus canariensis* leaves extracts.

Plant species	Plant extract	Yields (%)	Contents	
			Total phenolic (TP) (mg GAE/g Dw)	Total Flavonoid (TF) (mg RE/g Dw)
P. atlantica	Aqueous	24.20 ± 0.08	241.64 ± 0.16	31.81 ± 0.26
	Ethanolic	30.12 ± 0.14	263.76 ± 0.53	46.83 ± 0.55
P. canariensis	Aqueous	15.77 ± 0.04	30.11 ± 0.37	5.43 ± 0.38
	Ethanolic	20.53 ± 0.09	40.52 ± 0.58	9.80 ± 0.12

Antioxidant activity

Antioxidant activity was determined by calculating percentage of ability to DPPH radical scavenging as shown in Table 3. Results showed a good efficacy of plant extracts in scavenged DPPH radical, as antioxidant efficacy was arranged as follows: ethanolic extract of *P. atlantica* 90.27%, aqueous extract of *P. atlantica* 81.77%, ethanolic extract of *P. canariensis* 52.40%, aqueous extract of *P. canariensis* 38.44 %, this arrangement corresponds to the order of total content of phenols and flavonoids in the studied plant extracts and confirms the role of these compounds as antioxidants. It was found that the concentration of ascorbic acid corresponding to the concentration of plant extracts and is able to record the same percentage of DPPH scavenging less than the concentration of the plant extracts by 11.42 - 19.04 double, as shown in Table 3. Therefore, both plants have good capacity in scavenging DPPH radical, and thus have antioxidant efficacy.

Phenolic compounds power as antioxidant is due to their ability to chelate metals, and their capacity as

donors of hydrogen and electron from hydroxyl group allowing scavenging free radicals (43, 44), and corresponds to many studies that have shown that there was a strong correlation between content of phenols and antioxidant activity, confirming their responsibility as antioxidants (30, 45).

Results of the statistical study using Pearson's correlation showed a strong positive correlation 0.976 between efficiency of extracts in DPPH radical scavenging and their total phenolic contents, and their total flavonoid contents 0.974, this confirms responsibility of these compounds for plant extracts efficiency as antioxidant.

Table 3. Antioxidant activity of *P. atlantica* and *Pinus canariensis* leaves extract.

Species	Plant extracts	Concentration of extract (g/mL)	DPPH (%)	Corresponding concentration of ascorbic acid (g/mL)	Comparison of extracts efficiency with ascorbic acid
Pistacia atlantica	Aqueous	0.001	81.77 ±1.32	7×10^{-5}	14.28
	Ethanolic	0.001	90.27 ±1.51	8.75×10^{-5}	11.42
Pinus canariensis	Aqueous	0.001	38.44 ± 0.33	3.5×10^{-5}	28.57
	Ethanolic	0.001	52.40 ± 0.47	5.25×10^{-5}	19.04

Antibacterial activity

Antibacterial activity of Pistacia atlantica

Table 4 and Figure 4 shows results of antibacterial activity of P. atlantica leaves extracts, ethanolic extract was found to be more effective and broad-spectrum inhibition of bacterial growth compared to aqueous extract.

The highest inhibition zone diameter average of ethanolic extract was 14.83, 19.33, 20.96, 23, 26, and 33.56 mm for *E. coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis, Staphylococcus aureus,* and *Shigella boydii*, respectively, at 500 mg/mL.

The lowest inhibition zone diameter average of ethanolic extract was 10.7, 10.9, 12.16, 18.5 and 26.0 mm for *Pseudomonas aeruginosa, Proteus mirabilis, Enterobacter cloacae, Staphylococcus aureus,* and *Shigella boydii*, respectively, at 50 mg/mL.

Ethanolic extract showed inhibitory activity of bacteria at all studied concentrations except for *E. coli* and *Klebsiella pneumoniae* at 50 mg/mL.

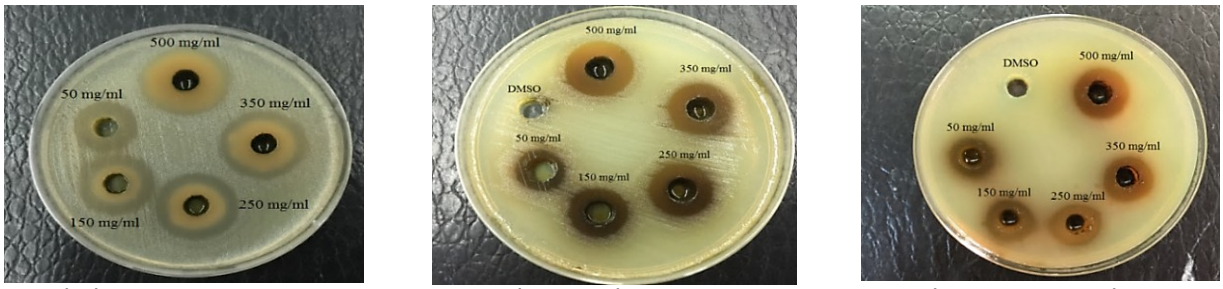
The highest inhibition zone diameter average of aqueous extract was 11.0, 12.0, 13.25, 18.25, 24.65 and 29.25 mm for *E. coli, Enterobacter cloacae, Proteus mirabilis, Staphylococcus aureus* and *Shigella boydii,* respectively at 500 mg/mL.

Aqueous extract didn't show any antibacterial activity at 50 mg/mL except of *E. coli, Pseudomonas aeruginosa,* and *Shigella boydii*, as inhibition zone diameter average was 5.5, 7.5 and 18.2 mm, respectively.

While *Klebsiella pneumoniae* and *Enterobacter cloacae* didn't show sensitivity against aqueous extract except at concentrations 350 and 500 mg/mL, and equal efficacy of ethanolic and aqueous extracts against *Pseudomonas aeruginosa* were observed at a concentration of 500 mg/mL, the inhibition zone diameter average were 16.83 and 16.74 mm, respectively. The higher of extracts concentration increases their efficiency in bacteria.

Table 4. Antibacterial activity (inhibition zone diameters average, mm) of *Pistacia atlantica* leaves extracts.

Bacterial Strains	Concentrations mg/ mL	Aqueous Extract	Ethanolic Extract	Moxifloxacin 5 mcg	Gentamicin 10 mcg	DMSO
E.coli	50	5.5 ± 0.5	0.0	0.0	16.5 ± 0.5	0
	150	6.75 ± 0.25	8.9 ± 0.1			
	250	8 ± 0.0	10.43 ± 0.40			
	350	8.5 ± 0.5	13.5 ± 0.5			
	500	12 ± 1	14.83 ± 0.28			
Klebsiella pneumoniae	50	0.0	0.0	31.5 ± 0.5	18.16 ± 0.28	0
	150	0.0	10.03 ± 0.55			
	250	0.0	13.33 ± 0.76			
	350	6.5 ± 0.5	15.96 ± 0.45			
	500	11 ± 1	19.33 ± 0.57			
Enterobacter cloacae	50	0.0	12.16 ± 0.76	29 ± 0.0	17.75 ± 0.25	0
	150	0.0	15.03 ± 0.25			
	250	0.0	16.7 ± 0.3			
	350	10.5 ± 0.5	18.5 ± 0.5			
	500	13.25 ± 0.75	20.96 ± 0.45			
Shigella boydii	50	18.2 ± 0.8	26 ± 1	30.25 ± 0.25	25.75 ± 0.75	0
	150	20.5 ± 0.5	28.5 ± 0.5			
	250	21.5 ± 0.5	30.4 ± 0.4			
	350	24.5 ± 0.5	31.25 ± 0.25			
	500	29.25 ± 0.75	33.56 ± 0.51			
Pseudomonas aeruginosa	50	7.5 ± 0.5	10.7 ± 0.46	22.75 ± 0.75	21.5 ± 0.5	0
	150	11.1 ± 0.1	12.5 ± 0.5			
	250	12 ± 0.0	13.8 ± 0.28			
	350	14.5 ± 0.9	14.9 ± 0.17			
	500	16.74 ± 0.8	16.83 ± 0.65			
Proteus mirabilis	50	0.0	10.9 ± 0.55	0.0	0.0	0
	150	8.85 ± 0.15	14.5 ± 0.5			
	250	12 ± 1	18.05 ± 0.25			
	350	14.9 ± 0.1	20.13 ± 0.40			
	500	18.25 ± 0.25	23 ± 0.2			
Staphylococcus aureus	50	0.0	18.5 ± 0.5	32 ± 1	10.25 ± 0.25	0
	150	14.25 ± 0.75	20.33 ± 1.15			
	250	17.4 ± 0.2	22 ± 1			
	350	21.5 ± 0.5	24 ± 0.0			
	500	24.65 ± 0.35	26 ± 0.2			

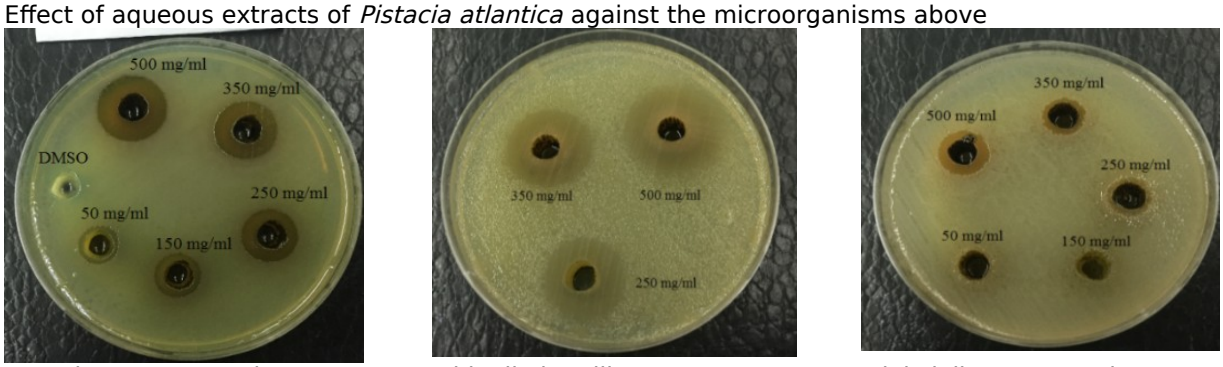


Staphylococcus aureus *Enterobacter cloacae* *Pseudomonas aeruginosa*

Effect of ethanolic extracts of *Pistacia atlantica* against the microorganisms above

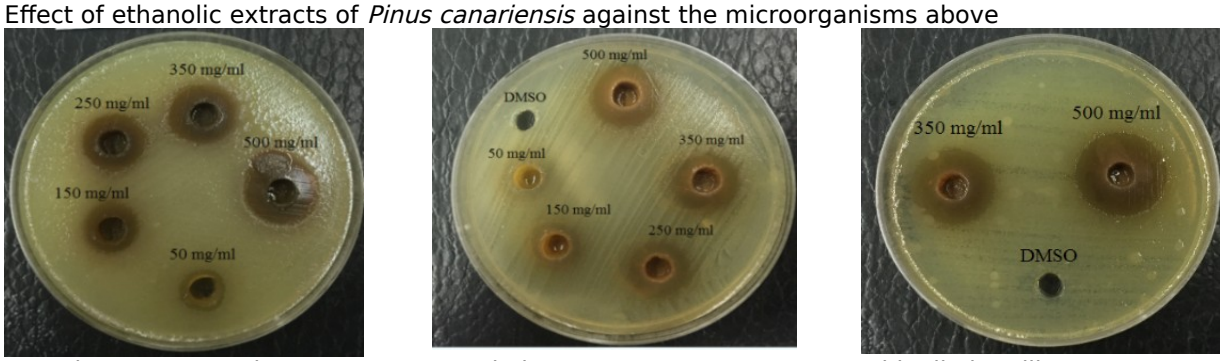
Pseudomonas aeruginosa *Staphylococcus aureus* *Proteus mirabilis*

Effect of aqueous extracts of *Pistacia atlantica* against the microorganisms above



Pseudomonas aeruginosa *Shigella boydii* *Klebsiella pneumoniae*

Effect of ethanolic extracts of *Pinus canariensis* against the microorganisms above



Pseudomonas aeruginosa *Staphylococcus aureus* *Shigella boydii*

Effect of aqueous extracts of *Pinus canariensis* against the microorganisms above

Figure 4. Effect of different extracts of plants against bacteria.

Results are in agreement with one of studies that indicated efficacy of ethanolic extract against *E. coli* and *Pseudomonas aeruginosa* reached 14 ± 0.9 and 16 ± 0.3 mm, respectively, while its results were less effective against *Staphylococcus aureus* which reached 14±1 mm (38). Also, efficacy of Pistacia atlantica extracts against *Klebsiella pneumoniae* was reported, which was recorded 13 ± 0.3 mm (46), and the results of this research did not agree with results (35); which did not show of *P. atlantica* leaves extract efficacy against Gram(-) bacteria *Proteus vulgaris*, and *E. coli*, but its efficacy against *Staphylococcus saprophyticus* was 8 mm, and boiled distilled water extract of *P. atlantica* leaves showed an inhibitory effect for *Streptococcus mutans* and *Streptococcus mitis* 19 and 25 mm, respectively, it was less effective against *Streptococcus salivarius* 5 mm (47).

Antibacterial activity of Pinus canariensis

Results of Table 5 and Figure 4 showed that aqueous leaves extract was more effective than ethanolic extract in inhibition of Pseudomonas aeruginosa growth at all concentrations; the highest inhibition zone diameter average was 21 mm at a concentration of 500 mg/mL. Ethanolic extract was the most effective in inhibition of *E. coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis, Staphylococcus aureus,* and *Shigella boydii*; the highest inhibition zone diameter average was 14.16, 14.25, 15.5, 15.75, 22.75 and 29.56 mm, respectively at concentration 500 mg/mL.

Effect of aqueous and ethanolic extract on bacteria at concentration 50 mg/mL was not observed with exception of *Pseudomonas aeruginosa* and *Shigella boydii*.

The highest inhibition zone diameter average of aqueous extract for *Staphylococcus aureus* and *Shigella boydii* 18.5 and 23.4 mm respectively, and the lowest inhibition zone diameter average for *Proteus mirabilis* and *E. coli* 11.5 and 11.16 mm respectively, at 500 mg/mL. Aqueous extract showed no effect in of *Proteus mirabilis, Enterobacter cloacae, Klebsiella pneumoniae,* and E. coli at concentrations of 50, 150 and 250 mg/mL, inhibition zone diameter average didn't exceed 13 mm in *Klebsiella pneumoniae* and *Enterobacter cloacae* at concentration of 500 mg/mL, the effect of concentrations of extracts in bacteria growth was observed, as with increasing concentration, inhibition zone diameter increased. Compared with another studies, Pinus leaves extracts (water, ethanol, chloroform, and petroleum ether) showed efficacy against E. coli, Klebsiella pneumoniae, Salmonella typhi and Enterobacter aerogene, and better efficacy was of petroleum ether, chloroform, water, and ethanol extracts, inhibition zone diameter average didn't exceed 10 mm in water and ethanol extracts (22). Results of the research are in agreement with the study conducted in Iran, where efficacy of ethanolic leaves extract reached 70% against clinical isolates Pseudomonas aeruginosa and E. coli and Proteus vulgaris 17, 15.66 and 15.50 mm, respectively, while it was less effective against Staphylococcus aureus, which was 16 mm (48).

Aqueous and ethanolic extract of Pinus leaves showed a lower effect against Pseudomonas aeruginosa with inhibition zone diameter average of 9.5 and 11 mm, respectively, while our results show a better effect against this Pseudomonas aeruginosa, effect of ethanolic extract was absent on Salmonella typhi, and inhibition zone diameter average of aqueous extract against E. coli was 11 mm, and this is in agreement with the results of our study (49) .

The difference in efficiency of these extracts compared to previous studies is due to various reasons, the most important of which are: difference of tested bacterial isolates, as a current study used multi-resistant bacteria, the difference in solvent and thus difference in quality of active compounds extracted, and the difference of extraction methods and concentration of used plant extract. Results showed that some plant extracts have better antibacterial activity than antibiotics Moxifloxacin 5 mcg and Gentamicin 10 mcg, and DMSO solution didn't show any effect in tested bacteria, this confirms that DMSO doesn't have any antimicrobial activity, where plant extracts recorded significant ($P<0.05$) antibacterial activity between all bacterial inhibition zone diameters averages.

The efficiency of plant extracts is due to they contain many chemical compounds (secondary metabolites) that have antibacterial activity with different mechanisms; phenolic compounds interaction with bacterial cell wall (either they bind to outer membrane or peptidoglycan), and interaction with membrane proteins (increasing membrane permeability). In addition to their ability to inhibition of biofilm formation, and to inhibition of bacterial enzymes, thus preventing bacterial growth. Found that Gram(-) bacteria are more resistant than Gram(+) bacteria to phenolic compounds actions; due to differences in cell wall structure, as outer membrane of Gram(-) bacteria is mainly composed of lipopolysaccharides (LPS) (50, 51, 52). It should be noted that this antimicrobial activity is not only related to quantities of phenolic compounds but also related to structure of these compounds (site(s) and number of hydroxyl groups on phenol group) (53). Nonspecific interactions of flavonoids can induce structural changes in properties of membrane and its can cause metabolic dysfunction and finally lead to bacterial death. Moreover, they are inhibit of synthesis of cell envelope, nucleic acid, and ATP, in addition to their ability to inhibition of bacterial toxins (54). Tannins may be related to their ability to inactivate microbial adhesins, have a role on inhibition of enzymes essential to metabolism process; such as proteolytic macerating enzymes, and their ability to inactivate cell envelope transport proteins, and their ability to complex with cell wall, while, saponins cause cell walls permeability disruption, and thus cause toxicity in cell (53).

Determination of MIC for P. atlantica and P. canariensis extracts

MIC of aqueous extract of *P. atlantica* ranged from 10.937 mg/mL for *Proteus mirabilis* to 43.75 mg/mL for *Pseudomonas aeruginosa*, while ethanolic extract of *P. atlantica* ranged from 5.468 mg/mL for *E. coli, Enterobacter cloacae, Shigella boydii, Proteus mirabilis,* and *Staphylococcus aureus* to 10.937 mg/mL for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, the aqueous extract *of*

P. canariensis ranged from 21.875 mg/mL for *E. coli, Shigella boydii,* and *Staphylococcus aureus* to 43.75 mg/mL for *Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, and Proteus mirabilis,* while ethanolic extract of *P. canariensis* ranged from 5.468 mg/mL for *Staphylococcus aureus* to 21.875 mg/mL for *Klebsiella pneumoniae, Enterobacter cloacae,* and *Pseudomonas aeruginosa* as shown in Table 6. Compared with the Tunisian study, MIC of ethanolic extract of *P. atlantica* leaves reached 25 mg/mL for *E. coli*, 6.25 mg/mL for *Pseudomonas aeruginosa* and 12.5 for Staphylococcus aureus and Salmonella typhimurium, the values obtained in the current study are better for E. coli and Staphylococcus aureus (38). MIC of ethanolic extract of pinus leaves reached 7.29 mg/mL for Pseudomonas aeruginosa, 9.37 mg/mL for Staphylococcus aureus, 16.66 mg/mL for E.coli and Proteus vulgaris, results of MIC in the current study are better for E. coli and Staphylococcus aureus (48). Difference in these values can be explained by different sensitivity and resistance of tested bacterial isolates, and difference in environment and genetic combination of plant.

CONCLUSION

The current study showed of *P. atlantica* extracts gave a higher yield than of *P. canariensis* extracts. Phytochemical compounds (tannins, phenols, and flavonoids) were found in the extracts of both plants, while saponins were present in the aqueous extracts only. *P. atlantica* extracts contained a higher content of phenols and flavonoids compared to *P. canariensis* extracts, while all extracts had antioxidant activity which could be a suitable alternative to synthetic antioxidants, and all extracts showed antibacterial activity, but not all concentrations showed bioactivity against some of tested bacterial species, it was found that antibacterial activity increases with increasing concentration of plant extracts, and *Shigella boydii* was the most sensitive against of extracts of both plants, therefore, more studies are needed to isolate bioactive compounds from both plants extracts, which will help in development of medicinal and pharmaceutical products.

Table 5. Antibacterial activity (inhibition zone diameters average, mm) of *Pinus canariensis* leaves extracts.

Bacterial Strains	Concentrations mg/ mL	Aqueous Extract	Ethanolic Extract	Moxifloxacin (5 mcg)	Gentamicin (10 mcg)	DMSO
E. coli	50	0.0	0.0	0.0	0.5 ± 16.5	0
	150	0.0	0.0			
	250	0.0	9.75 ± 0.25			
	350	0.0	12.7 ± 0.3			
	500	11.16 ± 0.76	14.16 ± 0.76			
Klebsiella pneumoniae	50	0.0	0.0	31.5 ± 0.5	18.16 ± 0.28	0
	150	0.0	0.0			
	250	0.0	10.5 ± 0.5			
	350	11.75 ± 0.25	12.13 ± 0.23			
	500	12.75 ± 0.25	14.25 ± 0.25			
Enterobacter cloacae	50	0.0	0.0	29 ± 0.0	17.75 ± 0.25	0
	150	0.0	9.5 ± 0.5			
	250	0.0	12.03 ± 0.55			
	350	8.66 ± 0.76	13.25 ± 0.25			
	500	13 ± 1	15.5 ± 0.5			
Shigella boydii	50	14.1 ± 0.36	19.25 ± 0.25	30.25 ± 0.25	25.75 ± 0.75	0
	150	17.66 ± 0.57	21.3 ± 0.3			
	250	19.5 ± 0.5	23 ± 0.0			
	350	20.6 ± 0.4	24.66 ± 0.57			

	500	23.4 ± 0.36	29.56 ± 0.51			
Pseudomonas aeruginosa	50	10.5 ± 0.5	10.25 ± 0.25	22.75 ± 0.75	21.5 ± 0.5	0
	150	13.3 ± 0.2	12.25 ± 0.25			
	250	16.25 ± 0.25	14.5 ± 0.5			
	350	17.7 ± 0.3	17 ± 0.0			
	500	21 ± 1	18.5 ± 0.5			
Proteus mirabilis	50	0.0	0.0	0.0	0.0	0
	150	0.0	0.0			
	250	0.0	9.5 ± 0.5			
	350	9.25 ± 0.25	11.75 ± 0.75			
	500	11.5 ± 0.5	15.75 ± 0.25			
Staphylococcus aureus	50	0.0	13 ± 0.5	32 ± 1	10.25 ± 0.25	0
	150	8.4 ± 0.36	15.25 ± 0.25			
	250	13.53 ± 0.50	18.06 ± 0.11			
	350	15.75 ± 0.25	20 ± 0.0			
	500	18.5 ± 0.5	22.75 ± 0.75			

Table 6. MIC of *Pistacia atlantica* and *Pinus canariensis* leaves extracts.

Bacterial strains	*P. atlantica*		*P. canariensis*	
	Aqueous extract mg/mL	**Ethanolic extract mg/mL**	**Aqueous extract mg/mL**	**Ethanolic extract mg/mL**
E. coli	21.875	5.468	21.875	10.937
Klebsiella pneumoniae	21.875	10.937	43.75	21.875
Enterobacter cloacae	21.875	5.468	43.75	21.875
Shigella boydii	21.875	5.468	21.875	10.937
Pseudomonas aeruginosa	43.75	10.937	43.75	21.875
Proteus mirabilis	10.937	5.468	43.75	10.937
Staphylococcus aureus	21.875	5.468	21.875	5.468

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