Chemical composition and activity of bark and leaf extracts of *Pinus halepensis* and *Olea europaea* grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens

**Abstract**

The objective of this study was to identify the chemical composition and antimicrobial activity of acetone extracts of *Pinus halepensis* Mill (needles and bark) and *Olea europaea* L. (leaves and bark) obtained from three different altitudes in AL-Jabel AL-Akhdar, Libya. The chemical compositions were analyzed by GC/MS. The analyses of the extracts from needles of *P. halepensis* led to the identification of 38, 15 and 18 different components, representing 93.34, 100 and 97.00% of the total extract at three different altitudes (125, 391 and 851 respectively). However, the extracts from bark contained 6, 4 and 8 compounds, representing 100% of the total extract at three different altitudes, respectively. For the leaf extracts from *O. europaea*, 5, 8 and 10 compounds were identified, representing 100% of the total extracts at three different altitudes (125, 391 and 851 respectively). However, the crude extracts from bark led to the identification of 13, 16 and 15 compounds, representing 97.55, 99.70 and 98.05% of the total extract at three different altitudes, respectively. The chemical classes of the detected compounds confirmed that these extracts contained a complex mixture consisting of sugars, monoterpenes, hydrocarbons, sesquiterpenes, diterpenes, diterpenoids, terpenophenolics, triterpenes, tetraterpenoids, phenylethanoids, steroids, resins and phthalates. Four pathogenic bacteria and one fungal strain were used to determine the antimicrobial activity. The extracts exhibited antibacterial potency with varying degrees of inhibition with MIC values ranging from 480 to 1300mg/L and the best MICs values observed were 525, 530, 410 and 645mg/L against the growth of *Botrytis cinerea*, *Aspergillus niger*, *Corynebacterium fascians* and *Pseudomonas solanacearum*, respectively for the extract from *O. europaea* at altitude I. The extracts all extracts exhibited a significant antifungal potency against *Botrytis cinerea* with varying degrees of inhibition of growth with EC$_{50}$ values ranging from 71.10 to 154.71mg/L.

**Keywords:** *Pinus halepensis*, *Olea europaea*, acetone extracts, antibacterial activity, antifungal activity, GC/MS analysis

**Abbreviations:** GC, gas chromatography; MS, mass spectrometry; FID, flame ionization detector; EI, electron impact ionization; MICs, minimum inhibitory concentrations; NB, nutrient broth; DMSO, dimethyl sulfoxide; PDA, potato dextrose agar

**Introduction**

Al-Jabel Al-Akhdar region (Libya) has highest species diversity and having distinct environmental characteristics associated with evergreen forest along with the Mediterranean from the Atlas Mountains to the Levant, and it has an environment similar to other regions in Southern Europe such as Italy, the Greek islands and Turkey. 1 The number of plant species reach up to 1100 species from the total of plant species in Libya (2000 species) with about 75 species of plants that grow only in Al-Jabel Al-Akhdar and have been served as basis of traditional medicinal systems for thousands of years.2–5 In addition, such plants produce a remarkable diverse array of over 5,000,000 low and high molecular natural products which are known as secondary metabolites, which can be used as an alternative form of health care as well as screening for active compounds that have significant effects against human and plant pathogens.2,6–8

*Pinus halepensis* Miller is a steno-mediterranean species distributed along the coasts and in the islands, it prefers temperate regions include Al-Jabel Al-Akhdar region, Libya and have significant effects against human and plant pathogens.2,6–8

Historically, olive leaves and fruits of *Olea europaea* were used for the treatment of malaria and associated fever.14 The different parts and fruits of this tree are rich in phenolic substances including hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, vanillic acid, vanillin, oleanuropein, oleanolic acid, rutin, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside.15,16 These valuable products possess important antioxidant and antibacterial effects14,17–20 and have an ancient history of nutritional, medicinal and traditional usages especially as an important part of the Mediterranean.21–23 Phytochemical screening of aqueous extract of *O. europaea* leaves revealed the presence of flavonoids, saponins and steroid, which demonstrated the best inhibition against some bacterial strains.14 *Pinus halepensis* Miller is a steno-mediterranean species distributed along the coasts and in the islands, it prefers

**Volume 3 Issue 3 - 2017**

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**Received:** March 04, 2017 | **Published:** June 14, 2017
warmer calcareous areas like Libya, Italy, Algeria, Greece, Morocco, and Turkey, whereas it also succeeds in colonizing the less hospitable rocks because of its springiness.\textsuperscript{25–27} The genus \textit{Pinus} (Pinaceae) comprises 250 species and is widespread in the northern hemisphere, especially in the Mediterranean region, Caribbean area, Asia, Europe, North and Central America.\textsuperscript{25,28,29} The extracts of \textit{P. halepensis} are rich in secondary metabolites like terpenoids, essential oils, terpenes, turpentine and phenolic compounds.\textsuperscript{28,30–38} Recently, an increase interest in natural substances extracted from such plants has been observed in literatures due to their significant impact from an environmental point of view, as well as to find effective alternatives to the industrially synthesized chemicals.\textsuperscript{3,39–42} Therefore, the objective of this study was to analyze the chemical composition of acetone extracts of \textit{Pinus halepensis} Mill (needles and bark) and \textit{Olea europaea} L. (leaves and barks), collected from three levels of altitude in AL-Jabal AL-Akhdh, Libya, using gas chromatography/mass spectrometry (GC/MS). The GC/MS is an analytical instrument that a hyphenated system of GC and MS and it is a very compatible technique and the most commonly used technique for the identification and quantification of different substances within a test sample.\textsuperscript{43} The unknown organic compounds in a complex mixture can be determined by interpretation and also by matching the spectra with reference spectra.\textsuperscript{44} Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification. The percentages of leaf and bark components of selected trees in the present study were estimated from the obtained GC/MS spectra. The influence of altitude on the quantity and quality of the extracts was discussed in details. In addition, the identification of the most effective compounds and the antimicrobial activity against four pathogenic bacteria \textit{Agrobacterium tumefaciens}, \textit{Erwinia carotovora}, \textit{Corynebacterium fascians} and \textit{Pseudomonas solanacearum} and the fungus \textit{Botrytis cinerea} have been investigated.

**Materials and methods**

**Plant materials**

\textit{Pinus halepensis} Mill (needles and bark), and \textit{Olea europaea} L. (leaves and bark), were collected from three altitudes in AL-Jabal AL-Akhdh, Libya during August 2014, where the trees were selected within three classes of different levels of altitudes. The species were identified and were chosen from three levels of altitude. First level (125m) represents the area Alwista considered as littoral zone near to the Mediterranean Sea. Second level (391m) of Wadi-alkuf area which includes the area between 200 height to 450m height above the sea level and third level (851m) of Sidi-Alhemre which includes sites that begin height of about greater than 450meters above the sea level. Collected fresh plant materials were examined and the old, pest-infected leaves were removed. The fresh bark and leaves of each species of tree were washed with clean water and thoroughly dried at room temperature and then ground into powders by an electric mill. The leaves and powdered bark of each tree were stored at room temperature in dark places until they were subjected to the extraction process.

**Sample extraction**

The dry extracts (100g) of each sample were extracted into acetone (500mL) by immersing the powder in the solvent for 48h at room temperature in the dark. Acetone was selected as it is more of a non-polar solvent than others are and most organic molecules present in plant samples are more soluble in it. The contents were then filtered with Whatman No. 1 filter paper. The resulting filtrates were then concentrated with a rotary evaporator at 60°C to give a dark colored solid. The extracts were stored in dark bottles and refrigerated at 4°C before use.\textsuperscript{45}

**GC/MS analysis of the extracts**

Acetone extracts were analyzed for their chemical composition by gas chromatography/mass spectrometry (GC/MS)\textsuperscript{46,47} with the following specifications: A Trace GC Ultra/Mass Spectrophotometer ISQ (Thermo scientific, Austin, TX, USA) instrument equipped with flame ionization detectors (FID) and a direct capillary column TG-5MS (30m 9 0.25mm 9 0.25m film thickness) apparatus at Central Laboratory, Faculty of Agriculture, Alexandria University; Alexandria, Egypt. Helium (average velocity 39cm s\textsuperscript{-1}) was used as the carrier gas (flow rate of 1mL/min), and the temperature program was 120°C/min raised at 6°C/min to 320°C, injector temperature was 260°C and detector temperature was 320°C with post run (off) at 320°C. Sample (1µL) was injected at 250°C, with split/split-less injector (50:1 split ratio) in the split less mode flow with 10 mL/min. All mass spectra were recorded in the electron impact ionization (EI) at 70 electron volts. The mass spectrometer was scanned from 50-500m/z at five scans per second. Scan time: 1.5s; mass range: 40 to 300mu. The ion source and transfer line temperatures were set at 200 and 250°C, respectively. Peak area percent was used for obtaining quantitative data with the Xcalibur\textsuperscript{TM} Software (Xcalibur 4.0 with Foundation 3.1 SP1, Thermo Scientific Technologies) without using the use of response factor correction. The compounds were identified by comparing their mass spectra with MS library (NIST and Wiley) data.\textsuperscript{48,49} Quantification of the percentage of each component was obtained by integrating the peak area of the chromatogram obtained by FID.

**In-vitro antibacterial assay**

Four plant pathogenic bacteria namely, \textit{Agrobacterium tumefaciens} (Family: Rhizobiaceae), \textit{Erwinia carotovora} (Family: Enterobacteriaceae), \textit{Corynebacterium fascians} (Family: Nocardiaceae) and \textit{Pseudomonas solanacearum} (Family: Pseudomonadaceae) were provided by Department of Pesticide Chemistry and Technology, Faculty of Agriculture, Alexandria University, Egypt. The bacterial strains were maintained on nutrient agar (NA: Peptone 5g, Beef extract 3g, NaCl 8g), medium at 37°C. The \textit{in vitro} antibacterial activities as minimum inhibitory concentrations (MICs) of the extracts were determined by micro dilution broth assay method using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) as a chromogenic marker.\textsuperscript{50,51} Nutrient broth (NB) medium was used to grow the bacterial strains to a final inoculum size of 5×10\textsuperscript{6}CFU/mL. The extracts were dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled water to obtain a final stock solution of 2000mg/L. For the broth microdilution test, 20µL of each bacterial suspension in NB medium was added to the wells of a sterile 96-well microtiter plate already containing 40µL of serially diluted compounds and 140µL NB medium. The final volume in each well was 200µL. Control wells were prepared with culture medium, bacterial suspension, and solvent. The contents of each well were mixed on a micro plate shaker at 200rpm prior to incubation for 24h at 37±2°C. To indicate respiratory activity, the presence of color was determined after adding 10µL/well of TTC dissolved in water (0.01%, w/v) and incubated under appropriate cultivation condition for 30 min in dark. The absorbance was measured at 492nm in an Ultra Micro plate Reader (Robonik, PVT.LTD). Positive controls were wells with
a bacterial suspension. Negative controls were wells with growth medium and the tested compounds. All measurements of MIC values were repeated in triplicate. The MIC was the lowest concentration, where no viability was observed after 24h on the basis of metabolic activity.

**In-vitro antifungal activity**

A culture of *Botrytis cinerea* (Pers.) (Moniliaceae; Class: Deuteromyces), was provided by Department of Plant Science and Technology, Faculty of Agriculture, Alexandria University; Alexandria, Egypt. *B. cinerea* was obtained by growing the fungus in potato dextrose agar medium (PDA) at 26°C. The antifungal activity on the mycelial growth of *B. cinerea* was tested using mycelia radial growth technique according to Badawy et al. The extracts were dissolved in DMSO with different concentrations ranging from 50 to 2000mg/L and then added to sterilize the PDA medium immediately before pouring into sterilized Petri dishes. Each concentration was tested in triplicate. Parallel controls were maintained with DMSO mixed with PDA medium. Mycelial discs (0.5 cm in diameter) of fungus, from an 8-day culture on PDA plates, were transferred aseptically to the center of the Petri dishes. Plates were incubated in the dark at 26°C. Colony growth diameter was measured when fungal growth in the control had completely covered the Petri dishes. The percent inhibition of mycelial growth was calculated as follows:

\[
\text{Mycelial growth inhibition} \% = \left( \frac{\text{DC} - \text{DT}}{\text{DC}} \right) \times 100
\]

Where the DC and DT are average diameters of fungal colonies of control and treatment respectively.

**Statistical analysis**

The experimental data are presented as mean±standard error or standard deviation and the ANOVA was performed and the property values were separated (P<0.05) with the Student-Newman-Keuls (SNK) by the SPSS program (Statistical Package for Social Sciences, version 21.0, United States). The concentration which inhibited 50% of the fungal mycelial growth (EC50) and its corresponding 95% confidence limits were calculated by probit analysis.

**Results and discussion**

**The yield of crude acetone extracts**

The data in Table 1 showed that the yield percentage of crude extracts (weight/dry weight of plant) was varied significantly between tree species and the source of extract that obtained from bark or leaves. The data showed that the highest percentages of the yield were found in crude extracts of *O. europaea* leaf with 9.00%, 11.7% and 14.99% at levels I, II, and III, respectively, while lowest percentages were found in *P. halepensis* needle extract with 3.75%, 3.70% and 3.74% at levels I, II, and III respectively. On the other hand, the yield of crude extracts of bark showed highest relatively percentages found in *P. halepensis* with 6.43%, 6.61% and 3.74%, at levels I, II, and III respectively. The data in Table 1 showed that the yield percentage of crude extracts yield (%) isolated from *P. halepensis* (bark and needles) and *O. europaea* (bark and leaves) at different levels of altitudes summarized in Table 3, were 6.61±3.94, 5.20±3.70, 4.88±1.23, and 3.75±1.71, 4.80±0.93, 3.70±1.33, 3.74±1.06, 11.70±1.21, 9.00±3.99, respectively, respectively.

**Chemical composition of crude extracts of *P. halepensis***

GC/MS analyses of the crude extracts from needles of *P. halepensis* led to the identification of 38, 15 and 18 different components, representing 93.34, 100, and 97.00% of the total extract at three different altitudes (125, 391 and 851, respectively) (Table 2). However, the crude extracts from bark led to the identification of 6, 4, and 8 compounds, representing 100% of the total extract at three different altitudes (Table 3). The identified compounds are listed in Tables 2 & 3 according to their elution order on a TG-5MS capillary column. The results show some differences between extracts of three different altitudes in chemical constituents and their amount in each extract. The major components detected in needle extracts (Table 2) at three altitudes were caryophyllene (3.76%, 5.04% and 8.72%), α-caryophyllene (0.99%, 1.05% and 1.95%), β-cubebene (1.37, 2.44, and 5.49%), carophyllene oxide (2.22%, 3.11% and 1.11%), cembrene (1.56, 5.80, and 5.29%), dibutyl phthalate (2.54, 1.00, and 0.92%), 3,7,11-trimethyl-14-(1-methyl-hylyl)-[S-(E,Z,Z,E)-1,3,6,10-cyclooctadecatetraene (23.0, 14.29, and 15.84%), thunbergol (4.64, 30.71, and 19.46%), saleroil (3.78, 9.76, and 6.21%), antipodal acid (3.04, 19.20, and 11.79%), retinoic acid methyl ester (2.04, 3.00, and 9.65%), methyl retinoate (0.87, 1.00, and 1.39%), and mono (2-ethylhexyl) phthalate (4.07, 2.07, and 0.85%). Compounds of isopulegyl acetate, L-glucose, D-fructose, 1,3,6-trideoxy-3,6-epithio-, sucrose, l-sorbose, gamaerane D, 8-cadinene, α-cadinol, 17-hydroxy-17-methyl-1(17α)-androsta-4,14-dien-3-one, aromadendrene oxide-(2), l(+)-ascorbic acid 2,6-dihexadecanoate, phytol, linolenic acid, di-n-octyl phthalate, dehydroabietal, 1-heptatriacotanol, α-pimaric acid, androsterone, androstadienedione, 1-Heptatriacotanol, 17-Pentatriacontene, and α-sitosterol were only detected in needle extract from altitude I with percentages ranged from 0.77 to 9.28%. However, androstenediol, toretal and retinol were only detected in needle extract from altitude III with percentages ranged 1.51, 3.10, 1.80% respectively. The chemical classes of the detected compounds of needle extracts confirmed that these extracts contain a complex mixture consisting of sugars, monoterpen hydrocarbons, sesquiterpenes, diterpenes, diterpenoids, terpenophenolic, steroids, resins, and phthalates (Table 2).

The main components found in three altitudes of crude extracts from the bark of *P. halepensis* summarized in Table 3, were D-glucose, 4,6-o-ethylidene (40.19, 50.40, and 17.68%), dibutyl phthalate (10.88, 14.00, and 2.95%), di-n-octyl phthalate (13.04, 13.10, and 24.56%), and β-sitosterol (18.57, 22.50, and 32.08%). L-3,4-dihydroxyphenylalanine was detected only in extract of altitude I with 12.33% of the total weight and disappeared from extracts of altitudes II and III. However, l(+)-ascorbic acid 2,6-dihexadecanoate,
2,2’-dimethyl-6,6’-dinitro-1,1’-biphenyl, 2,2’-methylenebis[3,4,6-trichloroanisole], and lycopluyl were only detected in extract of altitude III with 14.52, 2.12, 2.87, and 3.22% of the total weight (Table 3). The data in this table showed that the D-glucose, 4,6-o-ethylidene and β-sitostanol were the greatest abundance in three extracts followed by di-n-octyl phthalate and butylhexyl phthalate. In addition, the chemical classes of the detected compounds of the bark extracts confirmed that these extracts contain a complex mixture of essential phytochemicals of amino acids, sugars, diterpenes, phthalates, retinol, carotenoids and sterols. Generally, the samples showed a variable composition, which are linked to different sites and their characteristics. In line with our results, several studies have demonstrated the variability of secondary metabolite production due to climatic and soil conditions.\textsuperscript{35,36} \textit{P. halepensis} is a pioneer and expansionist species that colonizes abandoned agricultural lands characterized by high biodiversity and by the richness of secondary metabolites such as terpenoids and/or phenolic compounds through several processes.\textsuperscript{35} Several phytochemical analyses of \textit{P. halepensis} have been published on terpenes, turpentine and phenolic compounds.\textsuperscript{36,37,40} The literature reports some works on the chemical composition of \textit{P. halepensis} essential oil and extracts from Italy, Turkey, Algeria, Nigeria, Greece, Morocco, Tunisia.\textsuperscript{28,33,36,38,62}

\textbf{Table 2} Chemical composition and main constituents of the crude extracts isolated from \textit{P. halepensis} needles at different altitudes in AL-Jabal AL-Akhdar region by GC/MS analysis:

\begin{table}[h]
\centering
\begin{tabular}{llllllll}
\hline
\textbf{Compound name} & \textbf{Molecular formula} & \textbf{Chemical class} & \textbf{Altitude (m)} & \textbf{I (125 m)} & \textbf{Area (\%)} & \textbf{II (391 m)} & \textbf{Area (\%)} & \textbf{III (851 m)} & \textbf{Area (\%)} \\
\hline
Isopulegyl acetate & C\textsubscript{15}H\textsubscript{15}O\textsubscript{3} & Monoterpane hydrocarbons & 3.89 & 2.25 & - & - & - & - \\
L-Glucose & C\textsubscript{12}H\textsubscript{22}O\textsubscript{8} & Sugars & 4.12 & 1.02 & - & - & - & - \\
D-Fructose,1,3,6-trideoxy-3,6-epithio- & C\textsubscript{6}H\textsubscript{10}O\textsubscript{5} & Sugars & 4.29 & 3.14 & - & - & - & - \\
Sucrose & C\textsubscript{12}H\textsubscript{22}O\textsubscript{11} & Sugars & 4.38 & 1.16 & - & - & - & - \\
D-Sorbose & C\textsubscript{12}H\textsubscript{22}O\textsubscript{11} & Sugars & 4.92 & 6.79 & - & - & - & - \\
Caryophyllene & C\textsubscript{15}H\textsubscript{20} & Sesquiterpenes & 5.62 & 3.76 & 5.62 & 5.04 & 5.56 & 8.72 \\
α-Caryophyllene & C\textsubscript{15}H\textsubscript{20} & Sesquiterpenes & 6.1 & 0.99 & 6.11 & 1.05 & 6.05 & 1.95 \\
Germacrene D & C\textsubscript{15}H\textsubscript{14} & Sesquiterpenes & 6.41 & 0.87 & - & - & - & - \\
β-Cubebeene & C\textsubscript{20}H\textsubscript{32} & Sesquiterpenes & 6.51 & 1.37 & 6.55 & 2.44 & 6.5 & 5.49 \\
α-Cadinene & C\textsubscript{17}H\textsubscript{26} & Sesquiterpenes & 7 & 0.79 & 7.01 & 0.8 & 7.06 & 0.94 \\
δ-Cadinene & C\textsubscript{17}H\textsubscript{26} & Sesquiterpenes & 7.13 & 1.52 & - & - & - & - \\
Caryophyllene oxide & C\textsubscript{15}H\textsubscript{20} & Sesquiterpenes & 8.15 & 2.22 & 8.15 & 3.11 & 8.12 & 1.11 \\
α-Cadinol & C\textsubscript{17}H\textsubscript{26} & Sesquiterpenes & 9.28 & 3.47 & - & - & - & - \\
17-hydroxy-17-methyl-\{17a\}-androsta-1,4-dien-3-one & C\textsubscript{18}H\textsubscript{24}O\textsubscript{2} & Diterpenes & 12.06 & 2.03 & - & - & - & - \\
Aromadendrene oxide-(2) & C\textsubscript{15}H\textsubscript{20} & Sesqueterpenes & 12.62 & 1.15 & - & - & - & - \\
Cembrone & C\textsubscript{29}H\textsubscript{20} & Diterpenes & 13.8 & 1.56 & 13.83 & 5.8 & 13.8 & 5.29 \\
l-\{\textbf{+}\}-Ascorbic acid & C\textsubscript{14}H\textsubscript{20}O\textsubscript{4} & Hydrocarbons & 14.17 & 3 & - & - & - & - \\
Dibutyl phthalate & C\textsubscript{15}H\textsubscript{20}O\textsubscript{2} & Phthalates & 14.22 & 2.54 & 14.23 & 1 & 14.27 & 0.92 \\
3,7,11-Trimethyl-14-(1-methyltetrahydro-
[S-\{E.Z,E,E\}]-1,3,6,10-cycloetradecatriene & C\textsubscript{18}H\textsubscript{20} & Hydrocarbons & 15.69 & 2.3 & 15.69 & 14.29 & 15.68 & 15.84 \\
Thunbergol & C\textsubscript{23}H\textsubscript{30} & Diterpenes & 15.88 & 4.64 & 15.88 & 30.71 & 15.87 & 19.46 \\
Phytol & C\textsubscript{20}H\textsubscript{38}O & Acyclic diterpene alcohol & 16.54 & 1.94 & - & - & - & - \\
Linolenic acid & C\textsubscript{18}H\textsubscript{28}O & Fatty acids & 16.89 & 2.95 & - & - & - & - \\
\hline
\end{tabular}
\end{table}

\textbf{Citation:} Mohareb ASO, Kherallah IEA, Badawy MEI, et al. Chemical composition and activity of bark and leaf extracts of \textit{pinus halepensis} and \textit{olea europaea} grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens. J Appl Biotechnol Bioeng. 2017;3(3):331–342. DOI: 10.15406/jabb.2017.03.00067
Table Continued...

| Compound name                        | Molecular formula | Chemical class       | Altitude (m)                  | Rt (min) | Area (%) | Rt (min) | Area (%) | Rt (min) | Area (%) |
|--------------------------------------|-------------------|----------------------|-------------------------------|----------|----------|----------|----------|----------|----------|
|                                      |                   |                      | I (125 m)                     |          |          | II (391 m) |          |          | III (851 m) |          |          |
|                                      |                   |                      | (% of Total)                  |          |          | (% of Total) |          |          | (% of Total) |          |          |
| Sclareol                             | C_{15}H_{22}O_{3} | Di-terpenoids        | 18.37 | 3.78 | 18.37 | 9.76 | 18.36 | 6.21 |
| Androstenediol                       | C_{19}H_{30}O_{2} | Steroids             | - | - | - | - | 18.84 | 1.51 |
| Totarol                              | C_{20}H_{30}O_{2} | Terpenophenolics     | - | - | - | - | 19.38 | 3.1 |
| Anticopalic acid                     | C_{20}H_{32}O_{2} | Di-terpenes          | 19.88 | 3.04 | 19.89 | 19.2 | 19.94 | 11.79 |
| Mono(2-ethylhexyl) phthalate         | C_{19}H_{22}O_{2} | Phthalates           | 20.07 | 3.32 | - | - | - |
| Retinol                              | C_{20}H_{30}O     | Retinol              | 20.79 | 2.04 | 20.8 | 3 | 20.81 | 9.65 |
| Retinoic acid methyl ester           | C_{20}H_{32}O     | Di-terpenes          | 21.11 | 5.6 | - | - | - |
| Dehydroabietal                       | C_{20}H_{32}O     | Retinol              | 21.69 | 9.28 | - | - | - |
| I-Hepatriacetol                      | C_{20}H_{30}O     | Hydrocarbons         | 21.96 | 0.87 | 21.96 | 1 | 21.96 | 1.39 |
| Methyl retinoate                     | C_{20}H_{30}O     | Retinol              | 22.43 | 0.77 | - | - | - |
| α-Pimaric acid                       | C_{20}H_{32}O     | Retinol              | 22.52 | 4.07 | 22.52 | 2.07 | 22.52 | 0.85 |
| Di-n-octyl phthalate                 | C_{20}H_{32}O     | Phthalates           | 22.67 | 2.61 | - | - | - |
| Androsterone                         | C_{20}H_{32}O     | Steroids             | 22.79 | 0.7 | 22.79 | 0.73 | 22.8 | 0.98 |
| Obacunone                            | C_{20}H_{32}O     | Triterpenoids        | 23.15 | 1.59 | - | - | - |
| Androstadienedione                   | C_{20}H_{34}O_{2} | Steroids             | 23.29 | 1.64 | - | - | - |
| 1-Hepatriacetol                      | C_{20}H_{30}O     | Hydrocarbons         | 28.93 | 0.89 | - | - | - |
| 17-Pentatriacetone                   | C_{20}H_{30}O     | Hydrocarbons         | 31.15 | 1.68 | - | - | - |
| α-Sitosterol                         | C_{20}H_{30}O     | Sterols              | 93.34% | 100.00% | 97.00% |

Total peak area (%)

Table 3 Chemical composition and main constituents of the crud extracts isolated from bark of P. halepensis at different altitudes in AL-Jabal AL-Akhdar region by GC/MS analysis
Chemical composition and activity of bark and leaf extracts of *Pinus halepensis* and *Olea europaea* grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens

The data in Tables 4 and 5 represent the identified compounds with their retention times and peak area. In total, 5, 8 and 10 different components, representing 100% of the total extracts at three different altitudes (125, 391 and 851 m, respectively) were detected in *O. europaea* (Table 4). However, extracts of crude from the bark led to the identification of 13, 16 and 15 compounds, representing 97.55, 99.70 and 98.05% of the total extract at three different altitudes respectively (Table 5). The major components detected in leaf extracts of altitudes I, II and III (Table 4) were 3,4-dihydroxy-benzenecacetic acid (57.65, 18.26, and 5.31%), methyl elaidate (8.38, 9.20, and 18.67%), and di-n-octyl phthalate (12.73, 7.83, and 15.86%). It can be noted that the compound of 3,4-dihydroxy-benzenecacetic acid was gradually increase in their percentages with increase the level of altitude. Acetic acid, 1, 7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester was only detected in extract of tree at altitude I with high abundance of 18.68% of total weight however, tyrosol was only detected in extracts of tree at altitudes I and II with 2.38 and 1.45%, respectively. The compounds include betulin, astaxanthin, oleanolic acid and tararosterate were found in the two extracts obtained from the leaves at altitudes II and III with abundance ranging from 1.80 to 33.61% of the total weight. Other compounds, including oxiraneoctanoic acid, phytol and totarol, appeared only in altitude extract III with 4.99, 8.30 and 6.23%, respectively. The chemical classes of the detected compounds of leaf extracts confirmed that these extracts contain a complex mixture composed of phenylethanol, sesquiterpene hydrocarbons, diterpene hydrocarbons, diterpenoids, terpenophenols, phthalates, terpenes, and tetroperes (Table 4). The result of the chemical composition of the acetone extracts obtained from the *O. europaea* (Table 5) showed that the compounds of totarol, gibeloletic acid, bis-ethylhexyl hydroxydimethoxy benzylmalonate, betulin and β-sitosterol were found in leaf extracts of altitudes I, II and III and progressively increased in percentage with the increase in altitude level. However, tyrosol, 3,4-dihydroxy-benzenecacetic acid, methyl elaidate, dibutyl phthalate, oleic acid, and di-n-octyl phthalate, DNP-L-arginine were gradually decreased in leaf extracts of altitudes I, II and III. Compounds of (2-phenyl-1,3-dioxolan-4-yl)methyl (9E)-9-ocdecanoate, didigoxigenin, and preen-4-ene-3,20-dione,17,21-dihydroxy-bis(O-methyloxime) were found in both extracts obtained from leaves at altitudes II and III with abundance ranged from 1.80 to 33.61% of total weight and disappeared from altitude I. The chemical classes of the detected compounds of bark extracts confirmed that these extracts contained a complex mixture consisting of Phenylethanol, amino acids, fatty acids, diterpene hydrocarbons, sesquiterpenes, phthalates, terpenophenols, phthalates, hormones, and steroids. It was observed that ingested olive leaf extract was effectively to deliver oleuropein and hydroxytyrosol metabolites to plasma in humans.53 Seven phenolic compounds: caffeic acid, verbascoside, oleuropein, luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside and luteolin 4’-O-glucoside were found in *O. europaea* leaf aqueous extract.55 Tyrosol, similar to our findings were found both extract leaves and bark.64 Keskin et al.64 reported the GC/MS analysis revealed that the major constituents of the olive leaves extract were cyclotrisiloxane hexamethyl (36.98%), cyclotetrasiloxane octamethyl (15.18%) and cyclopentasiloxane decamethyl (14.59%). Tyrosol, hydroxy tyrosol and cinnamic acid derivatives were reported in olive leaves and may cause antimicrobial activity from.65 Recently, phenolic composition with tannin contents and their biological activities of fruit extracts from Italian and Algerian *Olea europaea* L. cultivars were studied.66 Fourteen different phenolic compounds including hydroxytyrosol, tyrosol, p-hydroxybenzoic acid, and verbascoside as major constituents were identified, and their profiles showed remarkable quantitative differences among analyzed extracts. The authors reported that there were no significant qualitative differences among samples, but it is possible to note important quantitative differences.

Table 4 Chemical composition and main constituents of the crude extracts isolated from *O. europaea* leaves at different altitudes in AL-Jabal AL-Akhdar region by GC/MS analysis

| Compound name | Molecular formula | Chemical class | Altitude (m) | Area (%) | Rt (min) |
|---------------|------------------|----------------|-------------|----------|----------|
| Tyrosol       | C_{8}H_{7}O      | Phenylethanol   | I (125 m)   | 5.38     | 2.38     |
| Oxiraneoctanoic acid, 3-octyl-cis- | C_{8}H_{15}O_{3} | Sesquiterpene hydrocarbons | II (391 m) | 9.03     | 57.65    |
| 3,4-Dihydroxy-benzenecacetic acid | C_{8}H_{16}O_{4} | Hydrocarbons | III (851 m) | 11.41    | 8.38     |
| Methyl elaidate | C_{9}H_{16}O_{2} | Diterpene hydrocarbons | I (125 m)   | 11.14    | 11.41    |
| Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester | C_{10}H_{15}O_{3} | Monoterpene hydrocarbons | II (391 m) | 12.16    | 11.41    |
| Phytol        | C_{20}H_{20}O_{2} | Acyclic diterpene alcohol | III (851 m) | 11.41    | 11.41    |
| Totarol       | C_{20}H_{35}O_{2} | Terpenophenolics | I (125 m)   | -        | -        |
| Di-n-octyl phthalate | C_{20}H_{35}O_{4} | Phthalates | II (391 m) | 22.51    | 22.51    |
| Betulin       | C_{20}H_{35}O_{2} | Triterpenes | III (851 m) | -        | -        |
| Astaxanthin   | C_{34}H_{82}O_{10} | Tetraterpenoids | I (125 m)   | -        | -        |

Citation: Mohareb ASO, Kherallah IEA, Badawy MEI, et al. Chemical composition and activity of bark and leaf extracts of *Pinus halepensis* and *Olea europaea* grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens. J Appl Biotechnol Bioeng. 2017;3(3):331–342. DOI: 10.15406/jabb.2017.03.00067
Table 5 Chemical composition and main constituents of the crude extracts isolated from bark of *O. europaea* at different altitudes in AL-Jabal AL-Akhdar region by GC/MS analysis

| Compound Name                  | Molecular formula | Chemical class     | Altitude (m)       |
|--------------------------------|-------------------|--------------------|--------------------|
|                                |                   |                    | I (125 m)          |
|                                |                   |                    | Rt (min) Area (%)  |
|                                |                   |                    | II (391 m) Area (%)|
|                                |                   |                    | III (851 m) Area (%)|
| Tyrosol                        | C_{10}H_{10}O      | Phenylethanoid     | 5.38 2.24          |
| 3,4-Dihydroxy-benzeneacetic   | C_{9}H_{10}O_{1}   | Hydrocarbons       | 9.04 34.44         |
| acid (2-Phenyl-1,3-dioxolan-4-yl) methyl (9E,9'-octadecenoate | C_{22}H_{34}O | Hydrocarbons | - - 9.55 1.97 |
| Methyl elaidate                | C_{10}H_{16}O      | Diterpene hydrocarbons | 11.41 5.88 |
| Widdrol hydroxyether           | C_{15}H_{22}O      | Sesquiterpenes     | 14.08 3.74         |
| Dibutyl phthalate              | C_{11}H_{14}O_{2}  | Phthalates         | 14.19 1.85         |
| Oleic Acid                     | C_{18}H_{34}O      | Fatty acids        | 16.85 1.96         |
| Totarol                        | C_{10}H_{18}O      | Terpenophenolics   | 19.37 3.03         |
| Di-n-octyl phthalate           | C_{12}H_{20}O      | Phthalates         | 22.5 2.55          |
| DNP-L-arginine                 | C_{10}H_{16}O      | Amino acids        | 25.93 8.37         |
| Gibberelic acid                | C_{9}H_{12}O       | Hormones           | 26.43 6.57         |
| bis-ethylhexyl hydroxydimethox  | C_{14}H_{16}O      | Diterpene hydrocarbons | 26.78 18.38 |
| benzyloxalane                  |                   |                   | 26.75 19.88        |
| Astaxanthin                    | C_{20}H_{32}O      | Tetraterpenoids    | 30.64 4.87         |
| Dihydroxigenin                 | C_{20}H_{32}O      | Steroids           | 30.77 6.18         |
| Pregn-4-ene-3,20-dione,17,21-   | C_{20}H_{32}O      | Diterpene hydrocarbons | - - 30.93 |
| dihydroxy-bis(O-methyloxime)   |                   |                   | 31.34 1.34         |
| β-Sitosterol                   | C_{20}H_{32}O      | Sterols            | 31.14 2.26         |

Total peak area (%)                | 97.55%  99.70%  98.05% |

Antibacterial activity

The antibacterial activity (MIC values in mg/L) of acetone extracts from bark and leaves of *P. halepensis* and *O. europaea* trees against *A. tumefaciens*, *E. carotovora*, *C. fascians* and *P. solanacearum* is shown in Table 6. Generally, all extracts exhibited promising antibacterial potency with varying degrees of inhibition with MIC values ranging from 480 to 1300mg/L. Among all the extracts obtained from the three altitudes of the studied trees, the best MICs values observed were 525, 530, 410, and 645mg/L against the growth of *A. tumefaciens*, *E. carotovora*, *C. fascians* and *P. solanacearum*, respectively for the extract from *O. europaea* at altitude I followed by the leaf extract (MIC=545, 650, 480, and 700mg/L, respectively) for *O. europaea* at the same level of altitude (125m). It is noted that the extracts obtained from barks and leaves of *O. europaea* at three altitudes showed that antibacterial activity decreased with increasing altitude. The extract obtained from the *P. halepensis* needles showed that Level I extract was the most active of the three levels of altitude with MIC values of 600, 840, 585 and 900mg/L against the growth of *A. tumefaciens*, *E. carotovora*, *C. fascians* and *P. solanacearum*, respectively. However, the level II extract showed the lowest activity with MIC of 735, 1200, 850 and 1300mg/L against the same bacteria, respectively. On the other hand, the extract obtained from the bark indicated that the altitude extract III was the most active among the three altitude levels.
levels (MIC=555, 600, 610 and 835mg/L against A. tumefaciens, C. fascians and P. solanacearum, respectively). Several studies of extracts derived from the aerial parts of P. halepensis and O. europaea have shown that it has good antibacterial activity and that the activity of plant extracts is mainly attributed to the presence of phenolic compounds, alkaloids, flavonoids, terpenoids and other essential phytochemicals.\textsuperscript{22,26,66–72} The aqueous extract of O. europaea leaves showed good activity against Escherichia coli with MIC of 150μL/mL and inhibition zone of 15.3mm in diameter.\textsuperscript{24} Keskin et al.\textsuperscript{69} reported that aqueous extract of olive leaves show antimicrobial activity against opportunistic pathogens like met-oxa res S. aureus, P. aeruginosa, E. coli and K. pneumonia.\textsuperscript{66} Korukluoglu et al.\textsuperscript{73} reported that water extract of olive leaves did not show any inhibition effects on all the tested bacteria, but Gungumjee et al.\textsuperscript{69} showed that O. europaea leaf extract had higher activity against tested organisms than the stems. In addition, Aliabadi and others reported that O. europaea extracts did not present broad-spectrum antibacterial activity,\textsuperscript{72} but was found to be most active against Campylobacter jejuni, Helicobacter pylori and S. aureus with MICs as low as 0.31-0.78% (v/v).\textsuperscript{72}

Markin et al.\textsuperscript{74} reported that olive leaf extract with a concentration of 0.6% (w/v) killed E. coli, P. aeruginosa, S. aureus and K. pneumonia in 3h exposure, and on the other hand inhibited B. subtilis only when the concentration was increased to 20% (w/v) which may be due to the ability to form spores of this species.\textsuperscript{74} Olive leaf extracts showed good inhibitory effects on pathogenic bacteria and fungi and all the extracts were a higher activity showed good inhibitory effects toward E. coli and B. cereus, which could be related to a higher yield of flavonoids.\textsuperscript{74,75} Korukluoglu et al.\textsuperscript{72} studied the effect of the different solvent extraction on the bioactivity of olive leaf extract against some bacterial pathogens, and the results showed that the solvent type affected the distribution of phenolic and concentration in extracts. They reported that ethanol extract of O. europaea leaves showed the highest antimicrobial efficiency against E. coli and S. enteritidis, while, acetone extracts showed the activity against S. thypimurium.\textsuperscript{76} However, acetone leaf extract of O. europaea showed Inhibition concentration of 55μg/mL against S. aureus, 60μg/mL with Escherichia coli, 110μg/mL against S. typhimurium, and 170 μg/mL against S. enteritidis.\textsuperscript{20} Additionally, phenolic compounds within olive leaf extract have shown antimicrobial activities against several microorganisms including: E. coli, S. aureus, K. pneumoniae, B. cereus, S. typhi and V. parahaemolyticus.\textsuperscript{64} Oleuropein, the main glycoside present in olives, has been shown to have strong antimicrobial activity against both Gram-negative and Gram-positive bacteria.\textsuperscript{75} In addition, hydroxytyrosol and oleuropein have been proven to inhibit orderly the rate of growth of several human intestinal or respiratory tract pathogens.\textsuperscript{74}

**Antifungal activity**

The in vitro antifungal activity of the extracts obtained from bark and leaves of P. halepensis and O. europaea with different altitudes is presented in Table 7 as EC\textsubscript{50} values in mg/L with their statistical parameters. The results showed all extracts exhibited a significant antifungal potency with varying degrees of inhibition of growth with EC\textsubscript{50} values ranging from 71.10 to 154.71mg/L. Leaf extracts of O. europaea at altitude II showed the highest effect with EC\textsubscript{50} of 71.10mg/L followed by the bark extract from altitude III (EC\textsubscript{50}=89.55mg/L). As can be seen that the extracts from bark and leaves of O. europaea showed higher antifungal activity than extracts of bark and leaves of P. halepensis. On the other hand, the extracts obtained from the leaves of P. halepensis had higher activity (EC\textsubscript{50}=119.20, 134.20, and 115.42mg/L) than the bark extracts (127.30, 154.71, and 139.70mg/L) at altitudes I, II, and III, respectively. It can be noted that the bark extracts of altitude I were the most powerful followed by altitude III then the extract of altitude II. However, the leaf extract of altitude III was the most powerful followed by altitude, then the extract of altitude II. This result is explained by the chemical composition of the extracts, as shown in Table 2, which indicates that the GC/MS analyzes of the acetone extracts of the P. halepensis needles identified 38, 15 and 18 different components To three different altitudes (125, 391 and 851 m, respectively). The antimicrobial activity of plant extracts might not be due to the action of a single active compound, but the synergistic effect of several compounds that are in minor proportion in a plant.\textsuperscript{39,40} Although the individual phenolic compounds in olive extract may show strong in vitro activities, antioxidant and antimicrobial activities of combined phenolics showed similar or better effects than the individual phenolics.\textsuperscript{15,16,63,64,72}

Markin et al.\textsuperscript{74} confirmed that the extracts may be more beneficial than isolated constituents since a bioactive component can change its properties in the presence of other compounds present in the extract.\textsuperscript{73} They also reported the antifungal capacity order for several concentrations of olive leaf and olive extracts against Candida albicans and C. neoformans. The inhibitory activity of crude extracts isolated from P. halepensis and O. europaea against B. cinerea in this study was higher than reported by Abdelgalei et al.\textsuperscript{74} who observed the five sesquiterpenes compounds isolated from Ambrosia maritima were shown EC\textsubscript{50} ranged between 316.6–495.5mg/L on B. cinerea.\textsuperscript{84} Results of biological assays with crude extracts of leaf and bark extracts against B. cinerea could be related to the high percentage of chemical composition. These results are in agreement with those of other essential oils which showed the largest inhibition zone 18.3±0.6 in the presence of essential oil of fraction T6 from branches while none Effect of root essential oils against B. cinerea.\textsuperscript{85} In study by Tamokou et al.\textsuperscript{86} evaluated the antibacterial and antifungal activities of the crude extract and the isolated compounds.\textsuperscript{83} The results showed that the crude extract prevented the growth of all microorganisms tested. The crude methanolic extract as well as some isolated compounds showed antifungal and antibacterial activities. Although some compounds have only antibacterial activities (MIC=12.5-200mg/ml) and no antifungal activity.\textsuperscript{83}

**Table 6** Antibacterial activity of crude extracts isolated from bark and leaves of P. halepensis and O. europaea trees grown at the three levels of altitude in Al-Jabal Al-Akhdar (Libya) against A. tumefaciens, E. carotovora, C. fascians and P. solanacearum

| Extracts       | Altitude | MIC (mg/L) | A. tumefaciens | E. carotovora | C. fascians | P. solanacearum |
|---------------|----------|------------|----------------|---------------|-------------|-----------------|
| P. halepensis bark | I(125m) | 695        | 790            | 750           | 940         |
|               | II(39m) | 630        | 720            | 665           | 850         |
|               | III(851m) | 555       | 600            | 610           | 835         |

**Citation:** Mohareb ASO, Kherallah IEA, Badawy MEI, et al. Chemical composition and activity of bark and leaf extracts of pinus halepensis and olea europaea grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens. J Appl Biotechnol Bioeng. 2017;3(3):331–342. DOI: 10.15406/jabb.2017.03.00067
Table Continued...

| Extracts          | Altitude | MIC (mg/L) | A. tumefaciens | E. carotovora | C. fascians | P. solanacearum |
|-------------------|----------|------------|---------------|---------------|-------------|-----------------|
| P. halepensis leaf| I(125m)  | 600        | 840           | 585           | 900         |
|                   | II(39m)  | 735        | 1200          | 850           | 1300        |
|                   | III(851m)| 675        | 940           | 730           | 1050        |
|                   | I(125m)  | 525        | 530           | 410           | 645         |
| O. europaea bark   | II(391m) | 665        | 680           | 640           | 840         |
|                   | III(851m)| 810        | 830           | 670           | 930         |
|                   | I(125m)  | 545        | 650           | 480           | 700         |
| O. europaea leaf   | II(391m) | 775        | 780           | 580           | 720         |
|                   | III(851m)| 910        | 800           | 680           | 825         |

Table 7 Antifungal activity of crude extracts isolated from bark and leaves of *P. halepensis* and *O. europaea* trees grown at the three levels of altitude in Al-Jabal Al-Akhdar (Libya) against *B. cinerea*.

| Crud extracts     | Altitude | EC<sub>50</sub> (mg/L) | 95% confidence limits (mg/L) | Slope ± SE | Intercept ± SE (χ²)<sup>a</sup> |
|-------------------|----------|--------------------------|-------------------------------|------------|-------------------------------|
|                   |          |                          | Lower                        | Upper      |                               |
| P. halepensis bark| I(125m)  | 127.3                    | 66.82                         | 200.82     | 1.97±0.151                   | -4.16±0.347 | 18.481 |
|                   | II(391m) | 154.71                   | 67.126                        | 277.94     | 1.93±0.143                   | -4.24±0.337 | 28.750 |
|                   | III(851m)| 139.7                    | 54.982                        | 198.657    | 2.02±0.156                   | -4.20±0.355 | 22.932 |
| P. halepensis leaf| I(125m)  | 134.2                    | 86.291                        | 191.65     | 2.24±0.166                   | -4.78±0.378 | 13.374 |
|                   | II(391m) | 119.2                    | 63.679                        | 185.537    | 2.11±0.162                   | -4.39±0.366 | 18.583 |
|                   | III(851m)| 115.42                   | 59.643                        | 182.037    | 2.20±0.166                   | -4.54±0.380 | 20.478 |
| O. europaea bark   | I(125m)  | 109.9                    | 40.854                        | 184.91     | 2.32±0.183                   | -4.70±0.404 | 30.659 |
|                   | II(391m) | 101.16                   | 47.784                        | 179.381    | 2.34±0.187                   | -4.71±0.410 | 31.325 |
|                   | III(851m)| 89.55                    | 55.078                        | 153.879    | 2.27±0.181                   | -4.56±0.398 | 17.359 |
| O. europaea leaf   | I(125m)  | 117.14                   | 11.829                        | 139.282    | 1.49±0.173                   | -2.76±0.320 | 22.799 |
|                   | II(391m) | 99.6                     | 38.312                        | 169.993    | 1.83±0.150                   | -3.66±0.342 | 21.802 |

<sup>a</sup>The concentration causing 50% mycelial growth inhibition.

<sup>b</sup>Chi-Square.

**Conclusion**

Acetone extracts from *P. halepensis* Mill (needles and bark) and *O. europaea* L. (leaves and bark), obtained from three different altitudes in the Al-Jabel Al-Akhdar, Libya, were analyzed by GC/MS and considered as potential natural antimicrobial agents against certain pathogenic bacteria and plant fungi. The results revealed differences in the yield of extracts and the chemical composition of different altitudes (125, 391 and 851m of sea level). Although the main components of all extracts are common, their percentages are different based on the tree species and the altitude level. These differences are the results of an adaptive process to particular ecologic conditions, which are in direct relation with the altitudes.

*O. europaea* leaf and bark extracts showed good activity against the studied bacterial strains *A. tumefaciens*, *E. carotovora*, *C. fascians* and *P. solanacearum* and the fungus used *B. cinerea*. Additionally, the acetone extract from *P. halepensis* needles at level I showed good activity against *A. tumefaciens*. Based on these results, the acetone extracts from bark and leaves of *P. halepensis* and *O. europaea* could be suggested as alternative antimicrobials. However, as these tests have been done in vitro, the next step may be further investigations as in vivo on plants.

**Acknowledgements**

None.

**Conflict of interest**

The author declares no conflict of interest.

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**Citation:** Mohareb ASO, Kherallah IEA, Badawy MEI, et al. Chemical composition and activity of bark and leaf extracts of *pinus halepensis* and *olea europaea* grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens. J Appl Biotechnol Bioeng. 2017;3(3):331–342. DOI: 10.15406/jabb.2017.03.00067
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Citation: Mohareb ASO, Kherallah IEA, Badawy MEI, et al. Chemical composition and activity of bark and leaf extracts of Pinus halepensis and Olea europaea grown in Al-Jabel Al-Akhdar region, Libya against some plant phytopathogens. J Appl Biotechnol Biobeng. 2017;3(3):331–342. DOI: 10.15406/jabb.2017.03.00067

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