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Effect of Altitude on the Antimicrobial and Cytotoxic Activities of Two *Pinus* species L Cultivated in AL-Jabel AL-Akhdar, Libya

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Abstract

Pinus halepensis and *Pinus pinea* Family Pinaceae; are plants indigenous to Al-Jabal Akhdar, Libya. Two different localities for each species were chosen: Sidi Alhamry (Ph-S) [high land, 830 meters above the sea level] and Alaslab (Ph-A) [low land, 75 meters above the sea level] for *P. halepensis*, while Werdama (Pp-W) [high land, 625 meters above the sea level] and Al Mansura (Pp-M) [low land,408 meters above the sea level] for *P. pinea*. The susceptibility of clinically isolated organisms to standard antimicrobial agents was compared to the results of the 4 crude extracts. The results revealed that Ph-S and Pp-M exhibited the strongest inhibition diameter against gram +ve bacteria specially *Micrococcus lutea*, *Mycobacterium phli* and *Bacillus subtilis* exceeding most reference standard antibiotics. Gram negative bacteria were mostly sensitive against Ph-A especially *Escherichia coli*. The fungus *Candida albicans* was sensitive to Ph-S and Pp-W. Three fractions from each locality were evaluated for their antimicrobial activity where they exhibited weaker activity when compared to the total extracts suggesting a synergistic effect of the active constituents when combined. The cytotoxic activities against breast and colon cell lines were evaluated where the Pp-W locality showed the strongest cytotoxic activity against both cell lines.

Keywords: Pinus; Altitude; Antimicrobial activity; Cytotoxicity.

1. Introduction

Pine trees (Pinaceae) are endogenous to Libyan flora [1] however, their cultivation has spread east and west and in most parts of the world, especially in the cold and temperate regions, and includes different types with more than 250 species [2, 3]. Pine trees are strong-growing, sustainable trees with a height of 50 meters or more and are considered ornamental, oleo-resin trees with valuable timbers, yet, others are important for medicinal uses [4] [5]. Different Altitudes have distinctive impacts on plants affecting their morphological and microscopical contents [6]. Altitude affects physical, mechanical, chemical, and biological properties of Pinus as described by [7-9]. Topical application of the plant oil treats eczema and possesses a rubefacient effect. The essential oil distilled from the fresh needle leaves is used as an inhalant to treat chronic laryngitis and catarrhal diseases of the respiratory tract. Blumenthal et al also prepared an aqueous infusion of pine shoots for oral ingestion for the same indications as the oil

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[10]. Chemical investigation of the essential oils and terpenes obtained from the entitled plants, as well as their antimicrobial and anti-parasitic activities have been previously reported by Ansari, M, Šarac, Z, et al and Ghaffari, T, et al [11-14]. The antimicrobial activity of the plant different species was investigated before by M Sadeghi et al, Llewellyn et al, [15], [16]. However, few reports were published regarding the antimicrobial and the cytotoxicity activities of both *P. halepensis* and *P. pinea* found at different altitudes of Al-Jabal Alakhdar. This provoked this study with the aim to evaluate the impact of the different Altitudes and to justify the potential usage of these plants in herbal medicine.

2. Experimental

2.1. Plant Material

Samples of aerial parts were obtained from two different localities (random locations in each locality), during spring 2017 and 2018 from plants in Al-Jabal Al-Akhdar in Libya. The plant is not endangered so no permissions were required. The locations are; Sidi Alhamry [high land, 2.5908° N, 21.7168° E at 830 meters above the sea level] and Alaslab [low land, 32.8830° N, 22.1803° E, at 75m above sea level] for *P.halepensis*, while Werdama [high land, 32.7564° N, 21.7376° E, at 625meters above the sea level] and Al Mansura [low land, 32.5908° N, 21.7168° E, at 408 meters above the sea level] for *P. pinea* as seen in figure 1. The plant materials (aerial parts) were collected and dried by placing them in the shade for 4 weeks in large, open, and clean places and uniqueness in a thin layer in an upright position with its green top up with continuous flipping till complete dryness.The plants were identified by Mr. Mossa Al-seayti, and according to the Libyan Flora as seen in figure 2 [17-19].

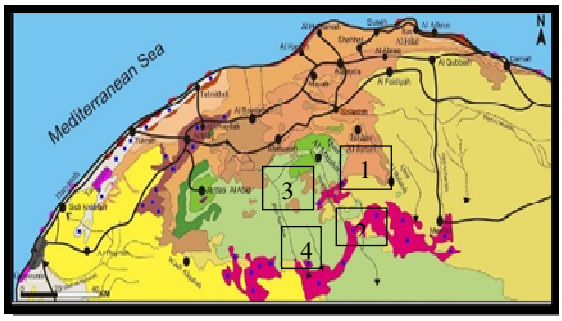

Figure 1: The different sites in Jabal Al Akhdar from which the plant samples were collected under study. Source: The Committee for the Assessment of Vegetation Cover in Libya1: Sidi Alhamry, 2: Alaslab, 3: Werdama, 4: Al Mansura

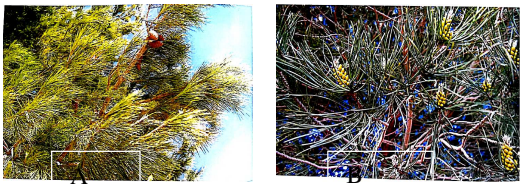

Figure 2: Morphological differences of A f A) *Pinus halepensis* and B) *Pinus pinea* X=0.2

2.2. Preparation of the ethanolic extracts

500 gm of the 4 plant powders under study were weighed and placed in the extraction unit consisting of 3 chambers of the Soxhlet assembly apparatus then ethanol 95% was added till complete exhaustion. The resulting ethanolic extract was evaporated in vacou using rotavapor to yield brown residue; the extracts were then stored in glass umber container till usage.

2.3. Fractionation of the extracts

Powdered plants of each locality (500 g) were fractionated by continuous hot extraction [20] as seen in figure 3**.** Three fractions were obtained as follows: Hexane (A), chloroform (B), and ethanol (C) respectively. All the prepared extracts were stored in amber glass containers for further usage.

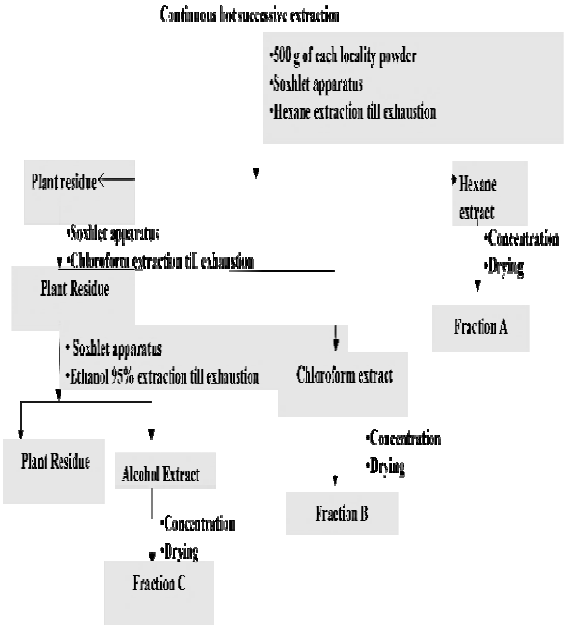

Figure 3: Fractionation scheme (done for each locality) using continuous extraction

2.4. Antibiotic susceptibility for different bacterial strains

Antibiotic susceptibility for different bacterial strains

Different strains of bacteria and fungi were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Omar Al-Mukhtar University. All the strains had been isolated from clinical cases from Al-Thawra Hospital, Al-Bayda city, and were classified into their different types as follows:

Gram positive: *Bacillus subtilis*, *Micrococcus lutea*, *Staphylcoccus aureus.* Gram negative: *E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumonia.* Acid fast bacteria: *Mycobacterium phli* and Fungal strain: *Candida albican.* Antibiotic susceptibility for the isolated bacteria was carried out according to [21, 22] and as described by the national committee for clinical laboratory standards (NCCLS 1997). The standard antibiotics used for the experiment are illustrated in table S1. The pure bacterial suspensions are transferred from a surface of nutrient agar plates to 5 ml of Muller Hinton broth tubes. The tubes were incubated at 37 ° C for 24 hours, then the suspensions were diluted by transferring 50 µl to 5 ml of a sterile physiological salt solution. The incubated microorganisms were Inoculated with a on Muller Hinton plates with cotton swab 3 times until complete and even distribution of the microorganisms over the surface of the plates. The dishes were left to dry for 5 minutes. Antibiotic discs are placed on the surface of the agar plates using sterile forceps and at equal distances from each other. The discs were placed in the incubator for a period of 24 hours at 37 ° C, after which the results are taken by measuring the areas of inhibition formed around the antibiotic discs using a ruler to determine the sensitivity for antibiotics.

2.5. In vitro antimicrobial activity:

The prepared extracts were tested for antimicrobial activity using the paper disc diffusion method [23-25]. Several concentrations were prepared according to the following formula and as seen in table S2: Required concentration = weight of extract / volume of solvent.The preparation of the discs saturated with different concentrations of the extract was done according to a method described by [26-28]. The bacterial agar media was prepared and sterilized by an autoclave at 121 ° C for 15 minutes, left to cool, and poured into the sterilized petri dishes then left to harden and placed in the refrigerator until use. Using a sterile inoculation needle, a small portion of the pure colonies were incubated in the nutrient broth tubes for 12 hours at 35 ° C to stimulate them and increase their growth. Finally, the bacterial suspensions were spread over the nutrient broth by cotton swabs followed by the equal distribution of extracts discs. The plates were incubated for 24 hours and poured into the sterilized petri dishes then left to harden and placed in the refrigerator until use. Using a sterile inoculation needle, a small portion of the pure colonies were incubated in the nutrient broth tubes for 12 hours at 35 ° C to stimulate them and increase their growth. Finally, the bacterial suspensions were spread over the nutrient broth by cotton swabs followed by the equal distribution of extracts discs. The plates were incubated for 24 hours at 37 ° C. the inhibition zones were measured by a ruler and recorded as according to [26].

2.6. In vitro Cytotoxic activity of the plant extracts

Potential cytotoxic activity of each ethanol extract was carried out in National Cancer Institute, Cairo University Egypt, using two tumor cell lines: breast cancer cell line [MCF-7] and colon cancer cell line [HCT-116]. Measurement of cytotoxicity was carried out using Sulfo-rhodamine-B assay method [29-31]. Cells were plated in 96 multi-well plate (10 cells/ well) for 24 hours before treatment with the different extract's concentrations (0,1, 2.5, 5 and 10 mg/mL) that were added to the cell monolayer, triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the tested extracts for *24* hours at 37 ° C and in an atmosphere of 5% CO_2. After incubation, cells were fixed, washed, and stained with Sulfo-Rhodamine-B-stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader.

3. Results and discussion

3.1. Sensitivity test

The antibiotic susceptibility tests were done to compare the results with that of the different Pinus plant localities. The pattern of sensitivity was illustrated in Table 1 and Figure 4. Our findings are consistent with previous research [32-37] as they all proved that while Gram-positive bacteria are usually labile to Sulfa, aminoglycosides, and fluoroquinolones antibiotics even the MRSA resistant *S. aureus;* Gram-negative bacteria including *Ps. Aeruginosa* bacteria are mainly susceptible to fluoroquinolones and aminoglycosides only. Those results triggered a debate about how plant extracts affect different types of bacteria.

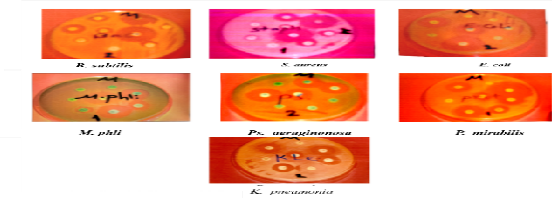

Figure 4 antibiotic susceptibility results of bacteria for different classes of antimicrobial agents

3.2. Antimicrobial activity of different pine extracts:
3.2.1. The total ethanolic extract

As seen in figure 5, The 4 tested ethanolic extracts revealed results that differed according to the geographical origin. Among the tested localities, AL Mansoura exhibited the strongest, the widest spectrum and the most significant ($P < 0.05$) antimicrobial activity against (*M. lutea, S. aureus, P. mirabilis, K. pneumonia,* and *M. phli*) with an inhibition zone diameter of (36, 25, 25, 29 and 33 mm) respectively which even exceeded most of the standard tested antibiotics. However, *E. coli* showed resistance to this extract. Alaslab locality was the strongest against gram negative bacteria *E. coli* with inhibition diameter of (14 mm) but it had weaker activity compared to the standard Chloramphenicol (23 mm), Alaslab also showed moderate to low activity against the rest of the tested bacterial. Sidi Alhamry was the most active against gram positive bacteria *B. subtilis* with inhibition zone of (28 mm) resembling the standards Erythromycin and Tetracycline (26 mm and 27 mm) respectively; the extract even showed significant ($P < 0.05$) antimicrobial activity when compared to clavulanic acid derivatives (Amoxiclav 11 mm), Cephalosporins (Cephotaxime and Cephalexin 10 mm) and aminoglycosides (Chloramphenicol 15 mm). Werdama extract revealed moderate to low antimicrobial activity to all the tested microorganisms with *M. lutea* being the most susceptible to it (28 mm). As seen in figure 6, *Candida albicans* was resistant to Sidi Alhamry and AL Mansoura extracts but revealed moderate sensitivity to Werdama and Alaslab extracts giving inhibition diameters (10 mm and 9 mm) respectively approaching that of Amphotericin B (>15 mm).

3.2.2. Hexane fraction:
Further fractionation of the crude ethanolic extract, gave three different fractions with different polarities, the first of which was the hexane fractions that gave weak to moderate activities against most of the tested microorganisms. Sidi Elhamry and Werdama revealed weak antimicrobial activities against *B. subtilis, E. coli and M. lutea* with no activity against the rest of the tested bacteria. Alaslab hexane extract did not reveal any antibacterial activity except a moderate activity against *B. subtilis* with inhibition diameter (19 mm) that approached the standard antibiotic gentamicin and even exceeded that of chloramphenicol and Amoxiclav (15 mm and 11 mm) respectively and a weak activity against *M. lutea* with inhibition diameter of (9 mm). Mansoura extract was weakly active against *B. subtilis, P. mirabilis* and *K. pneumonia. St. aureus, M. phil* and *Ps. aeurigonsa* were resistant to the four hexane extracts. The tested yeast *C. albicans* was found susceptible to Werdama and Alaslab as they both showed nhibition diamters of (9 mm and 10 mm) respectively.

3.2.3. Chloroform fraction:
The chloroform fractions revealed only moderate to weak antibacterial activities against all tested bacterial strains with *B. subtilis* being the most susceptible to the four extracts. Alaslab had the most prominent activity against *B. subtilis* with inhibition diameter (19 mm) exceeding the standard antibiotics chloramphenicol and Amoxiclav (15 and 11 mm respectively and approaching the inhibition diameter of standard antibiotic Gentamicin. *E. coli* was slightly susceptible to Alaslab extract (10 mm) while *M. lutea*, *Ps. aeruginosa, P. miribilis, K. pneumonia*, and *M. phil* were only susceptible to Werdama and Mansoura extracts with inhibition diameters ranging from (1 to 16 mm). *St. aureus* was found to be resistant to the 4 tested extracts. It was recorded that the four *Pinus* extracts did not exhibit any antifungal activity against *C. albicans*.

3.2.4. Ethanol fraction:
The last fractions which were extracted with ethanol revealed moderate to slightly potent antimicrobial activities. Among the four tested localities, AL-aslab showed the widest spectrum of antibacterial activities affecting Gram positive bacteria (*B. subtilis, M. lutea, St. aureus* and *M. phil)* with inhibition diameters (16 mm, 21 mm and 11 mm and 18 mm) respectively, exceeding or approaching the inhibition diameters of the Amoxiclav standard antibiotic .Gram-negative bacteria were also susceptible to ALaslab extract (*E. coli, Ps. aeruginosa, P. mirabilis and K. pneumonia*) with inhibition diameters of (10 mm, 14 mm, 14 mm and 11mm). Werdama exhibited antimicrobial pattern that resembled ALaslab except that it did not affect *M. phil* nor *P. mirabilis*.). However, Sidi EL Hamry was mainly against Gram-positive bacteria especially *B. subtilis*, *M. lutea* and *St. aureus* with inhibition diameters of (11mm, 10 mm and 8 mm) respectively. Gram negative bacteria were resistant to this locality extract except *E. coli* with inhibition diameter < 8mm. Mansoura extract was active against *B. subtilis*, *M. lutea, Ps. aeruginosa, P. mirabilis,* and *K. pneumonia,* but it was inactive against *M. phil, St. aureus and E. coli.*

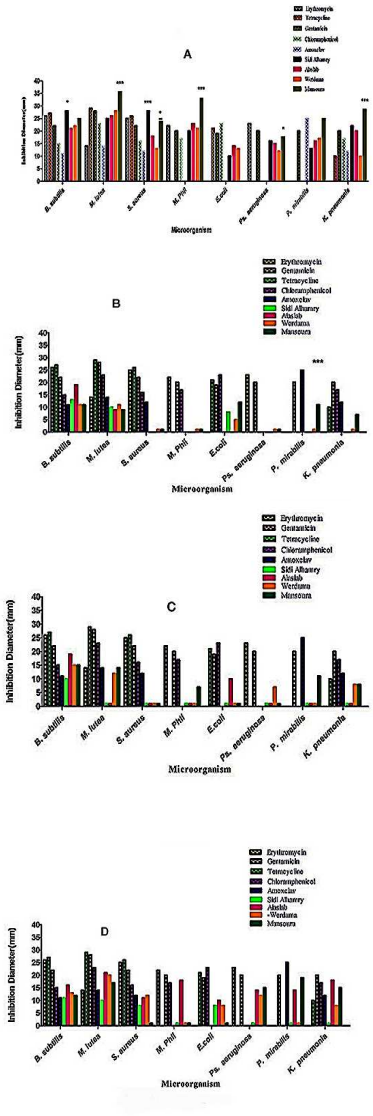

Figure 5: A comparison between the antibacterial activity (inhibition diameter) the four Pinus different extracts A) Total ethanol, B) Hexane fraction C) Chloroform fraction and D) Ethanol fraction under investigation and the different classes of standard antibiotics.: Macrolides (Erythromycin), Tetracycline, Aminoglycosides (Chloramphenicol), and clavulanic acid derivative (Amoxiclav).

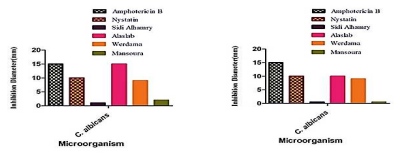

Figure 6: A comparison between the antifungal activity (inhibition diameter) the four Pinus different extracts A) Total ethanol, B) Hexane fraction under investigation and the Nystatin and Amphotericin B
N.B Chloroform and ethanol fractions did not reveal any antifungal activity.

3.3. An in vitro cytotoxic activity of the total crude ethanol extracts against breast cell lines [MCF-7] and colon cell lines [HCT-116]:

Since the 4 crude ethanolic extracts of sites exhibited potent antimicrobial effects sometimes exceeding standard agents, it was considered necessary to study their cytotoxic potential against different cell line. The total ethanolic extract obtained from *P. pinea* (Werdama) showed the most potent effects (IC_{50} = 0.81 µg/ml and 1.60 µg/ml) for the breast and colon cell lines, respectively, while (AlMansura) had a moderate effect on the two cell lines [IC_{50} = 2.95 µg/ml and 4.5 µg/ml] suggesting that the high altitude increased the cytotoxic potential of this species to enhance the activity of the standard doxorubicin (IC_{50} = 0.42 µg/ml and 0.72 µg/ml) for breast and colon cancer, respectively. Both extracts of *P. halepensis* showed moderate cytotoxic activities, (Sidi Alhamry) was more active against breast cancer [IC_{50} = 2.62 µg/ml] while the locality Alaslab showed greater activity against colon cancer [2.80 µg/ml]. It is important to mention that these results comply with what was carried out by [38, 39] against different cell lines. In 2008 Yu et al [40] reported the cytotoxic effect of the bark extract obtained from *P. massoniana* against human breast cell lines (MCF-7) and lung cell lines (HELF).Moreover, Simard et al 2008 [41] proved the cytotoxic effect of the wood alcohol extract prepared from *P. resinosa* against colon and lung cancer cell lines.

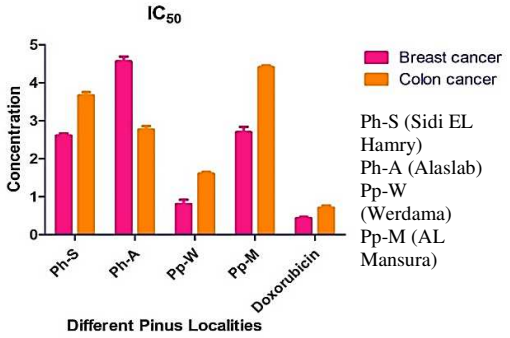

Figure 7: Cytotoxic activity of the total crude ethanol extracts obtained from both studied species

4. Discussion

As previously reported by one of our authors, Mariam gonaid et al [8], the volatile oil profile of the investigated *Pinus* species was highly affected when examined by GC_MS due to the variation in the

altitude**.** In 2014, , Fekih and Nadia reported the antibacterial activity of *Pinus halepensis* against bacteria [42] and Demirci reported the antibacterial activity of *Pinus pinea* [43] while in 2020, El Omari and Venditti reported the cytotoxic activities of *Pinus halepensis* and *Pinus pinea* respectively [44] [45]. This gave us a clue to design a study to find the optimum Altitude responsible for the best antimicrobial and cytotoxic activity among both plant species in addition to confirm the previously reported activity of *Pinus* plant. Both alcoholic extracts showed broad spectrum antibacterial and cytotoxic activities. There are evidences [6, 46] that altitude changes and topography affect plants as higher altitudes, leads to lower temperature range. Elevation plays a great role in the health and growth of plants [47, 48]. The type and amount of sunlight, water and nutrients availability which plants absorb differ at different altitudes causing significant effects on the distribution of the plant species and eventually on the active metabolites present in the plants [49]. This may rationalize that Werdama and Sidi El Hamri localities, growing at high lands for both species exhibited different patterns of antimicrobial and cytotoxic activities than ALaslab and ALmansura localities. In *Pinus pinea* species it was observed that the Inhibition diameters of the ALmansura locality was much higher than that of Werdama locality against all susceptible strains, confirming that the antimicrobial activity of this species was the best at low land altitude. However, the cytotoxic activity was optimum among the Werdama locality growing at high land with (IC_{50}=0.81µg/ml and 1.60µg/ml) approaching the standard drug doxorubicin (IC_{50}=0.42µg/ml and 0.72 µg/ml) for MCF-7 and HCT-116, respectively. As for *P. halepensis* species Alaslab locality was the strongest against gram negative bacteria *E. coli* while Sidi Alhamry was the most active against gram positive bacteria *B. subtilis* with inhibition zone of (28 mm) resembling the standards Erythromycin and Tetracycline (26 mm and 27 mm) respectively. As for the cytotoxicity it was noted that Sidi Alhamry was more potent against MCF-7 while ALAslab was more potent against (HCT-116). In this study, fractionation (hexane, chloroform, and ethanol) was done on the two species to explore the most active fraction. Interestingly when testing the three fractions for antimicrobial activities, they showed weaker activities when compared to crude extracts either in inhibiting the bacteria or the fungus suggesting that the active constituents may act better when combined in the total extract due to synergistic effect. Bacterial resistance in their free-living forms is a main concern for the health system, the problem is even aggravated nowadays due to the miss use of antibiotics. National institutes of health announced that more than 75% of microbial infections that occur in the human body are corroborated by the development and firmness of biofilms. A survey conducted in 2012, included around 2000 healthcare facilities and 300,000 patients revealed that most nosocomial infections are highly associated with strong *E. coli, Staphylococcus sp., P. aeruginosa, and K. pneumonia [50]*. In this study, *Pinus* species were significantly effective in inhibiting microorganisms. The inhibition diameters of the extracts approached many classes of standard antimicrobial agents as seen in figure (6). This finding is very promising in searching for a broad-spectrum antimicrobial agent with potent effect that may be used alone or in combination with antibiotics. Interestingly in the present study the inhibition diameter of AL-Mansura locality against gram positive bacteria was ranging from (36-29 mm) exceeding many standard antibiotics like (amoxicillin, amoxiclav and Penicillin). *C. albicans* is the fourth most frequent cause of bloodstream infections and are the major fungal species segregated from medical device infections [51]. Mechanical heart valves, pacemakers, urinary and central venous catheters, contact lenses, joint prostheses, and dentures are all susceptible to *C. albicans* infections. Since fungal infections are generally harder to eliminate, high antifungal dosages with elimination of the colonized medical tools are usually obligatory to cure infections with the risk of higher doses toxicity or removal of serious devices such as artificial heart valves and joints [51]. Accordingly, finding the active locality like Sidi EL hamri with potential to inhibit the *C. albicans* in this study may be of a great importance.

5. Conclusion

Different extracts obtained from both *P. halepensis* and *P. pinea* exhibited various biological activities viz. cytotoxic, antibacterial, and antifungal effects. Since the altitude can be considered as a factor that affect the chemical and biological response of plants, it requires further ecological investigation.

6. Conflicts of interest

"There are no conflicts to declare".

7. Formatting of funding sources

This research received no funding.

Table1
Sensitivity tests of the bacteria to the conventional antibiotics

Micro-organism / Anti biotic	*B. subtilis*	*M. phli*	*K. pneumonia*	*P. mirabilis*	*Ps. aeruginosa*	*E. coli*	*S. aureus*	*M. lutea*
Cephotaxime	10	12	-	25	10	27	10	27
Erythromycin	26	22	-	-	23	-	25	14
Polymyxin-B	11	12	11	-	10	12	9	18
Cephalexin	10	-	-	-	-	-	-	21
Amoxicillin	-	-	-	15	-	-	-	-
Tetracycline	27	-	10	-	-	21	26	29
Streptomycin	20	15	15	15	17	-	15	27
Nalidixic Acid	20	-	17	20	-	-	-	17
Fusidic Acid	23	-	-	-	-	-	30	20
Amoxiclav	11	-	12	25	-	-	12	14
Penicillin-G	-	-	-	-	-	-	-	-
Carbenicillin	9	13	-	28	13	-	-	11
Gentamicin	22	20	20	20	20	19	22	28
Cloxacillin	-	-	-	-	-	-	-	-
Chloramphenicol	15	17	17	-	-	23	16	23
Amikacin	27	22	20	21	22	19	23	21
Ceftriaxone	12	20	16	20	19	27	20	20

Table 2
Measurement of the potential cytotoxic activity according to IC_{50} values [µg/ml] from both studied species

Localities / Cell lines	**Doxorubicin**	***P. halepensis***		***P. pinea***	
		Sidi El-hamry	**Al-Aslab**	**Werdama**	**Al-Mansura**
Breast (MCF-7)	**0.42**	**2.62µg/ml**	**4.56µg/ml**	**0.81µg/ml**	**2.95µg/ml**
Colon (HCT-116)	**0.72**	**3.69µg/ml**	**2.80µg/ml**	**1.60µg/ml**	**4.50µg/ml**

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