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CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF *PINUS HALEPENSIS* MILL. OIL

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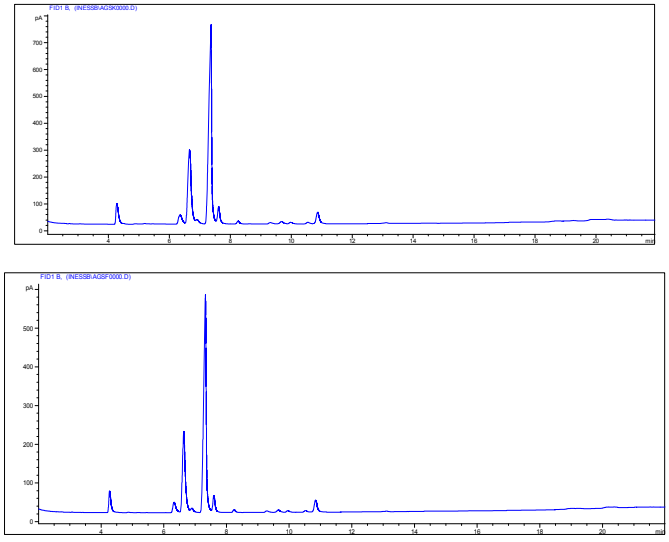
A discriminate analysis was applied taking as dependent variables the oil yield, phenolic content and fatty acids profile according to the growing location of *pinus halepensis* Mill. oil. The antioxydant activity of the oils collected from Kasserine center and Foussana country side has also been studied. *pinus halepensis* Mill.oil collected from Foussena had the highest value of oleic acid (62.45%) while oil collected from Kasserine was characterized by a high percentage of palmitic acid (5.73%) which makes this oil freeze at a low temperature. On the other hand, phenolic contents showed significant correlations with antioxydant activity. In addition, the anti-metastatic of fatty acids from *pinus halepensis* Mill. oil were investigated in U87-MG cell lines. The fatty acid extract showed significant inhibitory activity on cell proliferation and cell cycle progression in U-87 MG cell lines. These results give credence to the therapeutic potential of this plant against cancer. In consideration of potential utilization, detailed knowledge on the composition of *pinus halepensis* Mill. oil is of major importance. The diversity of applications to which *pinus halepensis* Mill. oil can be put gives this plant great industrial importance.



INTRODUCTION

Pinus halepensis Mill., a species belonging to the *Pineaceae* family, is naturally distributed in the Mediterranean region where it covers more than 25.000 km² and dominates forest formations in semi-arid and arid regions. Its continental range extends from Northern Africa (Morocco, Algeria, Tunisia and Libya) and Middle East (Syria, Lebanon, Jordan, Palestina and Turkey), up to Southern Mediterranean Europe (Eastern Greece, Croatia, Northern Italy, Eastern France and Eastern Spain).[1] Most bibliographical studies of chemical, biological, antimicrobial activities and genetic side

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of *p. halepensis* have been reported.[2] These studies report that diversity in *p. halepensis* did not follow a regular pattern and was a complex mixture of natural migration and human interventions. [3] As part of an extensive photochemical analysis of *pinus halepensis* Mill. oil, there is no comparative work has been published on chemical composition and biological activities of *pinus halepensis* Mill. oil with respect to the impact of geographic variation and environmental conditions. Therefore, we have projected for the first time to study the impact of geographic variation and environmental conditions on chemical composition and antioxidant activities as well as the antitumor activity of two of *pinus halepensis* Mill. oils provenances, distributed under different ecological conditions in Tunisia.

MATERIALS AND METHODS

Plant material

The sample of *pinus halepensis* seeds was collected from two different locations: Kassrine center (PHK 2017) (a city in the North of Tunisia, Governorate of Kasserine, 35° 10’ North, 8°50’ East in the sub-himid zone) and Foussana (PHF2017) (a city in the North of Kasserine, Governorate of Kasserine, 35° 20’58" North, 8°37’40" East in the sub-himid zone) on February 2017. The plant samples were identified by Dr. Maher Boukhris from Department of sciences of life, Sfax University (Tunisia) the voucher specimens of *pinus halepensis* needs are preserved (PHK 2017 and PHF 2017) at the Sciences of Faculty of Sfax, Sfax University, Tunisia.

Pinus Halepensis oil extraction

After leaf-removal and *pinus halepensis* seeds were washing. For laboratory scale simulation of industrial process, a hammer crusher (a stainless steel hammer mill operating at 3000 rpm provided with a 5 mm sieve), a malaxer and a centrifuge (Centrifuge MF 20, Awel Industries, France) were used. *pinus halepensis* oils were filled in dark colored glass bottles without headspace filled under nitrogen flux. The samples were kept at +4 ^{o}C in refrigerator. Experiments were performed in triplicate.

The oil extraction yield (%w/w) was calculated as follows:

$$\text{Oil extraction yield, \%} = \frac{\text{Mass extracted oil(g)}}{\text{Mass of olive paste(g)}} \times 100$$

Extraction of phenolic fraction from Pinus Halepensis Mill. Oil

The phenolic extracts were obtained following the procedure of Rigane *et al.* [4] with some modification. Briefly, the oil sample (4 g) was added to 2 mL of *n*-hexane and 4 mL of a methanol/water (60:40, v/v) solution in a 10 mL centrifuge tube. After vigorous mixing, they were centrifuged for 180 s. The hydroalcoholic phase was collected, and the hexanic phase was re-extracted twice with 4 mL of methanol/water (60:40, v/v) solution each time. Finally, the hydroalcoholic fractions were combined, washed with 4 mL of *n*-hexane to remove the residual oil, then concentrated and dried by evaporative centrifuge in vacuum at 35 °C.

F-C Test for measurement of total phenol concentration

The total phenol content of *pinus halepensis* Mill. oils was determined using the phenol reagent.[5] Briefly, 250 µL aliquot of different extracts was assayed with 1.5 mL of Folin–Ciocalteau reagent and 500 µL of aqueous sodium carbonate (7 %, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 min at room temperature, the absorbance was measured at 765 nm. The total phenols were expressed as gallic acid equivalents (mg gallic acid equivalent/g of oil), using a calibration curve of a freshly prepared gallic acid solution. For the gallic acid, the curve absorbance versus concentration is described by the equation $y = 5.844x$ ($r^2 = 0.9184$).

Total flavonoids determination

The flavonoids content in the extracts was determined by spectrophotometric procedure.[4] Briefly, 1 mL of methanolic-aqueous extract were added to 1 mL of 2 % $AlCl_3$ was added. After that, the obtained mixture was incubated at room temperature for 30 min. Rutin was used as a reference standard and results were expressed as mg of the rutin equivalent per g of oil. All determinations were performed in triplicates. For the rutin, the curve absorbance versus

concentration is described by the equation $y = 48.42x-0.045$ ($r^2 = 0.975$).

Fatty acid determination (GC)

The fatty acid composition of the oils was determined by gas chromatography (GC) as fatty acid methyl esters (FAMEs). FAMEs were prepared by saponification/methylation with sodium methylate according to the EEC 2568/1991.[6] A chromatographic analysis was performed in a Hewlett Packard 6890 gas chromatography using a capillary column (stabilwax, Restek, length 50 m, internal diameter 0.32 mm and film thickness 0.25 μm). The column temperature was isothermal at 180 °C and the injector 230 °C and detector temperatures were 250 °C. Results were expressed as relative percent of total area. Fatty acids were identified by comparing retention times with standard compounds. Seven fatty acids were considered in this study. These were tridecylic (13:0), myristic (14:0), palmitic (16:0), hypogeic (16:1n-9) + palmitoleic (16:1n-7), stearic (18:0), oleic (18:1n-9) + Z-vaccenic (18:1n-7), linoleic (18:2), linolenic (18:3), arachidic (20:0), gadoleic (20:1), behenic (22:0) and Erucic (22:1) acids expressed as percentages of fatty acid methyl esters.

Iodine values (IV) were calculated from the fatty acid percentages [7] by using the following formula:

IV = (% palmitoleic x 1.001) + (% oleic x 0.899) + (% linoleic x 1.814) + (% linolenic x 2.737)

Antioxidant activity evaluation

DPPH Assay. The method is based on the reduction of alcoholic DPPH (2,2-diphenyl-1-picrylhydrazyl) solutions in the presence of a hydrogen donating antioxidant. DPPH solutions show a strong absorption band at 517 nm with a deep violet color. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant. The DPPH radical scavenging capacity was measured according to Rigane *et al.* [2] with modifications. For each concentration, one milliliter was added to 250 μL of ethanolic solution of DPPH. The mixture was stirred vigorously and then incubated at room temperature for 30 min in the dark. Each experiment was analyzed in triplicate.

Measurement of radical scavenging activity by ABTS. The radical scavenging activity of the *pinus halepensis* needles oils growing in Tunisia were also measured using the method of Rigane *et al.*[8] with some modifications. ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonate]) radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 24 h before use. Afterwards, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. After addition of 990 μL of diluted $ABTS^+$ solution to10 μL of sample, the absorbance reading was taken at 30 °C exactly 6 min after initial mixing, at 734 nm. Tests were carried out in triplicate.

Anti-tumorale Activity

U78 Cell lines and chemicals. The U78 cell lines were purchased from American type culture Collection Company. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma), contained 10% of fetal bovine serum (FBS; Sigma). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Before the Cell Proliferation Assays, U87 cell lines were trypsinized and seeded in 96-well round-bottomed tissue culture plates. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), penicillin and streptomycin mixture, l-glutamine (200 mM) and phosphate buffer saline (PBS), were from GIBCO-BCL. All plastic wares for cell culture were obtained from Techno Plastic Products AG, Tras Anti-proliferative activities.

Cell viability assay. The viability assay was performed according to the manufacturer's instructions (Promega, Madison, WI, USA). After starvation, cells were harvested and treated with the indicated agents for 15 min at room temperature. The treated cells were subsequently seeded onto 96-well plate for 72 h. Following wash with PBS, cells were incubated with 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) for 4 h. Formazan crystals resulting from MTT reduction were dissolved by adding stop solution and gently agitated for 30 min. The absorbance of the supernatant was then measured spectrophotometrically at 560 nm.

Cell proliferation assay

U87 cells were plated (3×10^3/well) in 96-well plates in their complete medium and were incubated for 24 h before addition of *pinus halepensis* Mill. oil. After the incubation, the normal medium was replaced for an extract containing medium at the concentrations of 10 μg/mL and treated for 48, 72, 96, and 120 h. The control cells were maintained in normal

medium. After washing, attached cells were fixed, stained by 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 590 nm.

Cell adhesion assays

Adhesion assays were performed as previously described elsewhere.[9] Briefly, cells in single cell suspension were added to wells coated with purified extracellular matrix (ECM) proteins and allowed to adhere to the substrata for 2 h at 37 °C. After washing, attached cells were fixed, stained by 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 590 nm.

Statistical Analysis

Results of the analytical determinations were expressed as mean ± standard deviation (SD) of 3 measurements. Statistical differences were calculated using a one-way analysis of variance (ANOVA), employing the Student's t-test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Oil yield, phenolic and flavonoid content

The oil content of *pinus halepensis* Mill. seed was not in itself a criterion for determining the quality of the oil, but it is above all a criterion to be considered during a varietal selection. The oil yields obtained from the seeds of two provenances were shown in Table 1. The obtained results showed that there was a significant difference in the extraction rate of the oil. In general, *pinus halepensis* Mill. seeds from the Foussena provenance have the highest oil extraction rate (22%) followed by the Kasserine provenance (17.6%). This value is close to that found by Chaaben *et al.*[10] (21.6% ± 0.10) for the same plant but harvested from other areas. However our value seems low compared to the result of Cheikh-Rouhou *et al.*[11] who found in his study an oil content equal to 43.3%. This may be due to the difference in oil extraction method and provenance. It is well known that phenolic substances contribute directly to the antioxidant activity of plant extracts. Therefore, it was quite important to evaluate the total phenolic content (TPC) and flavonoid content (FC) of *pinus halepensis* Mill. oils. The total content of phenolic and flavonoid compounds in *pinus halepensis* Mill. oils needles was significantly affected by geographic variation and environmental conditions. As shown in Table 1, the highest levels of TPC and FC were found in the *pinus halepensis* Mill. oil from Foussena (2 mg GAE.g^{-1} of oil and 33 RE.g^{-1} of oil respectively). Our oils were much richer than that found in organic extract from the same *pinus* species cultivated in Tunisia which contain 0.23–0.26 mg/g, according to the results obtained by Nasri & Triki.[12] Compared to other species such as Algerian pine[1], high levels of total phenolic compounds were detected in methanolic extracts of *pinus halepensis* Mill., *pinus pinea* L., *pinus pinaster* and *pinus canariensis*. Their phenolic content varied ranged from 3.71 and 9.67 mg/g of extracts. This variation depends on several factors including the interaction with the type of climate and soil.

Fatty acids composition

The main fatty acids profiles of studied *pinus halepensis* Mill. oils are shown in Table 2. The monounsaturated fatty acids have great importance because of their nutritional implication and effect on oxidative stability of oils. Oleic (18:1n-9) + Z-vaccenic (18:1n-7) was the predominant fatty acids in *pinus halepensis* Mill. oil growing in Foussana (62.45 %). In addition, *pinus halepensis* oil growing in Kasserine presented a higher palmitic acid level (5.73 %) which makes this oil freeze at low temperature. Hypogeic (16:1n-9) + palmitoleic (16:1n-7), arachidic (20:0), gadoleic (20:1), behenic (22:0), and erucic (22:1) acids were affected by the *pinus halepensis* oil origin. The percentages of saturated fatty acids (SFAs), MUFAs and PUFAs of the studied oils were also calculated (Table 2). The *pinus halepensis* oil growing in Kasserine had the lowest total SFAs (11 %) essentially due to the lower myrestic and stearic acids content (Table 2). Regarding the total monounsaturated fatty acids (MUFAs), *pinus halepensis* oil from Foussena contained the highest percentage (67 %) due to its high content in oleic acid. However, *pinus halepensis* Mill. oil growing in Kasserine was rich in total polyunsaturated fatty acids (PUFAs) (60.91 %) because of its high contents in linolenic acid representing the major fatty acid of this fraction, it also showed the highest iodine value (IV) (191.17). These results are different from those found by Cheikh-Rouhou *et al.*[11] who reported that the Tunisian *pinus halepensis* Mill. oil was rich in linoleic (48.8%) and oleic (48.8%) acids and those of Nasri *et al.*[12] and Nergiz and Domnez[13] who

reported that linoleic acid is the main unsaturated fatty acid in Tunisian and Turkish *p. pinea* L. seed oil with 47.28% and 47.6%, respectively. On the other hand, only *p. halepensis* Mill. oil from Kasserine is in agreement with those given for main saturated fatty acids in Tunisian or in Turkish *p. pinea* L. seed oils[12,13] with palmitic acid as the main saturated fatty acid (8.75%, and 6.49%). In addition, Kadri *et al.*[14] studied the lipid profile of four *pinus* (*pinus halepensis* Mill., *pinus pinea* L., *pinus pinaster* and *pinus canariensis*) seeds from North Algeria. They found that the major unsaturated fatty acids for the four species were linoleic acid (30–59%) and oleic acid (17.4–34.6%), while the main saturated fatty acid was palmitic acid (5–29%) and iodine values (93.3–160.4). From these results, our research team could be concluded, for the first time, that the fatty acid composition of *pinus halepensis* Mill. oil depending on geographic variation and environmental conditions.

Antioxidant activity

Herein, two different assays based on electron transfer were used for the *in vitro* evaluation of the antioxidant properties of *pinus halepensis* oil used in Tunisia. The results of scavenging activity on DPPH radicals and ABTS assay are shown in Table 3. All the studied oils showed a propensity to quench the free radicals. According to the *in vitro* assays, the lowest IC_{50} value was observed in *pinus halepensis* oil collected from Foussena (10 ± 0.12 and 231.23 ± 0.5 $\mu g.mL^{-1}$, respectively, for DPPH and ABTS assays) and thus presenting the highest radical scavenging activity. The difference of antioxidant the studied oils between two *pinus halepensis* origin may be related to the genotype and geographic origin. According to Othman *et al.*,[15] polyphenols have received considerable attention because of their physiological function, including antioxidant, antimutagenic and antitumour activities. These compounds are widely distributed in plants as it was mentioned by Li *et al.*,[16] who have focused their studies to the antioxidant activities and free radical-scavenging abilities of phenolic compounds, due to the beneficial implications for human health.[17] It is worth noting that the antioxidant activity of the phenolic compounds were attributed to its redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and have also metal chelating properties. Thus, it has been reported that free radical-scavenging activity is greatly influenced by the phenolic composition of the sample.[5,18]

Table 1

Total Phenols and Flavonoids in *pinus halepensis* Mill. Oils

	Kasserine	***Foussena***
Yield oil	17.6 ±0.1[a]	22±0.2[b]
Total phenol (mg GAE.g^{-1} of oil)	1.2 ± 0.22[a]	2 ± 0.32[b]
Flavonoid content (µg RE.g^{-1} of oil)	32± 0.00[a]	33 ± 0.09[b]

Results are expressed as mean ± standard deviation of 3 determinations.[a,b] Different letters for the same parameter indicate significant differences among varieties (p <0.05).

Table 2

Fatty acid composition of *pinus halepensis* Mill. oil from Kasserine center and Foussana country side

	Kasserine	**Foussena**
Tridecylic acid (C 13:0)	0.02±0.00[a]	0.05±0.00[a]
Myristic acid (C 14:0)	0.05±0.00[a]	5.25±0.10[b]
Palmitic acid (C 16:0)	5.73±0.28[a]	0.13±0.00[b]
Hypogeic (C 16:1n-9) + palmitoleic (C 16:1n-7) acids	0.19±0.02[a]	3.6±0.18[b]
Stearic acid (C 18:0)	3.53±0.25[a]	25.99±0.19[b]
Z-vaccenic (C 18:1n-7) + Oleic (C 18:1n-9) acids	27±0.30[a]	62.45±0.45[b]
Linolenic acid (C18:3)	60.91±0.30[a]	0.76±0.02[b]
Arachidic acid (C20:0)	0.76±0.03[a]	0.54±0.02[b]
Gadoleic acid (C20:1)	0.55±0.10[a]	0.91±0.14[b]
Behenic acid (C22:0)	0.91±0.05[a]	0.29±0.00[b]
Erucic acid (C22:1)	0.34±0.00[a]	0.04±0.00[b]
∑ SFAs	11±0.61[a]	32.25±0.31[b]
∑ MUFAs	28.08±0.42[a]	67±0.77[b]
∑ PUFAs	60.91±0.33[a]	0.76±0.02[b]

Table 2 (continued)

∑ UFAs	88.99±0.75[a]	67.76±0.79[b]
∑ MUFAs / ∑ PUFAs	0.46±0.10[a]	88.15±0.23[b]
IV	191.17±1.37[a]	61.82±0.63[b]

SFAs, saturated fatty acids; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; UFAs, unsaturated fatty acids. Results are expressed as mean± standard deviation of 3 determinations.[a, b] Different letters for the same parameter indicate significant differences among varieties (p <0.05).

Table 3

Antioxidant activity of *pinus halepensis* oils assessed using DPPH and ABTS assays.

	Kasserine	*Foussena*
DPPH assay (IC_{50} expressed as µg.mL^{-1})	14 ± 0.22[a]	10 ± 0.12[b]
ABTS assay (IC_{50} expressed as µg.mL^{-1})	254.23± 0.3[a]	231.1 ± 0.5[b]

Results are expressed as mean ± standard deviation of 3 determinations.[a,b] Different letters for the same parameter indicate significant differences among varieties (p <0.05).

Cytotoxicity and anti-proliferative activities

The main contribution of this work was the demonstration of a new biological activity of the *pinus halepensis* oil. However, their anti-proliferative activity against human glioma cells has not been reported. Indeed, the effect of *pinus halepensis* oil on cells viability was assessed by the MTT assay.[19] Cultured cells were exposed to increasing concentrations of extracts for 48 h. As shown in (Figure 2, A), *pinus halepensis* oil (from 25 to 400 µg/mL) was unable to affect viability of U87 cells. The proliferation assay was measured over 5 days in four separate independent experimental trials to determine if the administration of *pinus halepensis* oil (from 25 to 400 µg/mL) was sufficient to inhibit cellular proliferation. Cells did not receive fresh medium during these periods of incubation. Our results demonstrated that comparison of treated cells to untreated control cells revealed that the *pinus halepensis* oil has significant effect on cell proliferation when applied at 300 µg/ml and the inhibition of proliferation reached 75% at 400 µg/mL (Figure 2, B). These results may be due to different polyphenol compounds of *pinus halepensis* oil.

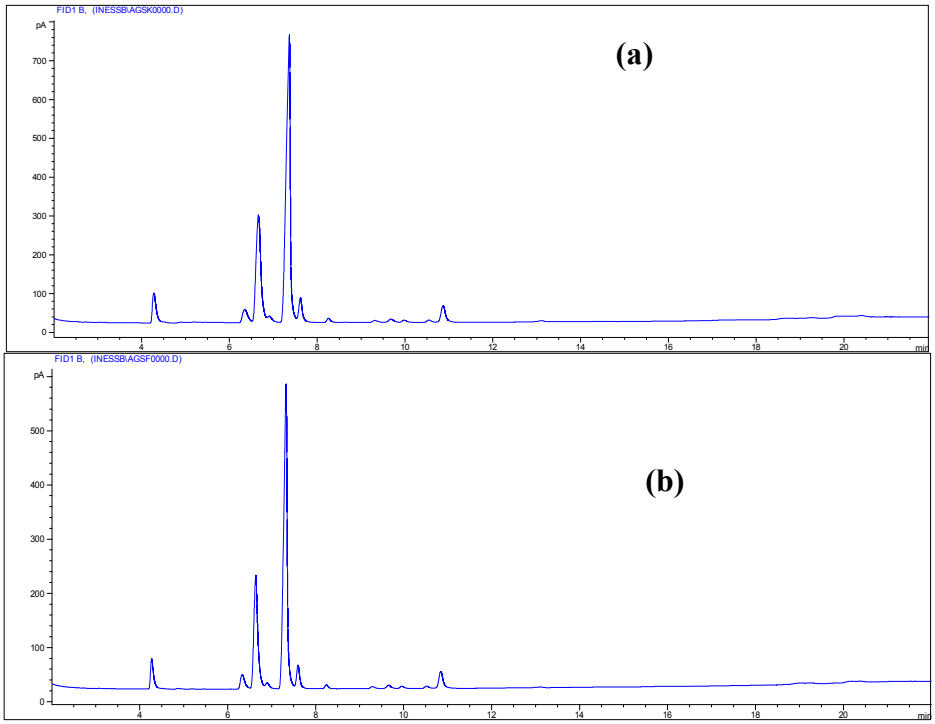

Fig. 1 – GC-FID chromatogram of fatty acid composition
of *pinus halepensis* oil from Kasserine center (a) and Foussana country side (b).

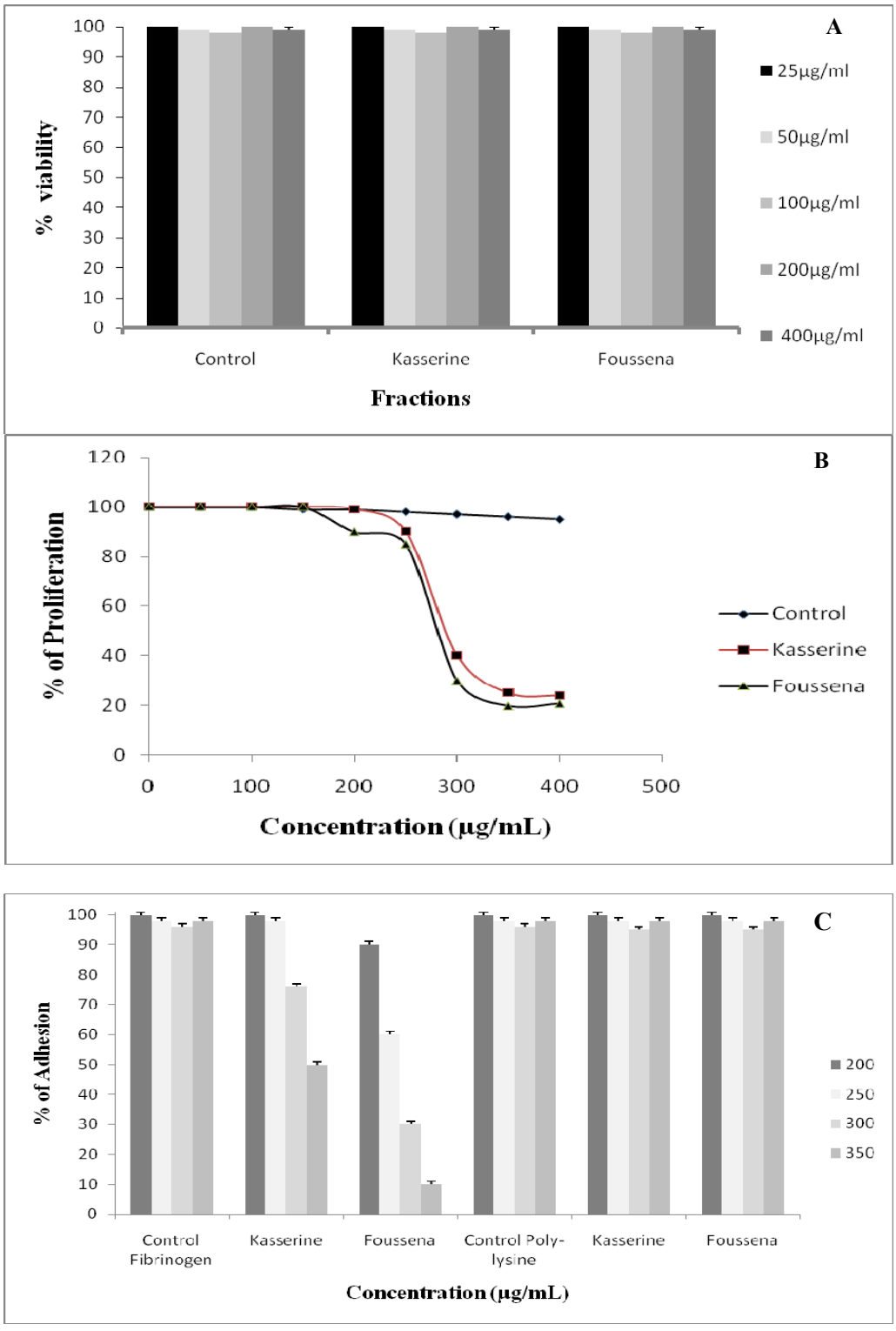

Fig. 2 – Effect of *pinus halepensis* oil on U87 cells: (A) Cells were treated with extract at the indicated concentrations for 24 h. Cell viability was determined using the MTT assay and expressed as percentages of control which was exposed to vehicle only. Control value was taken as 100%. (B) the *pinus halepensis* oil inhibited U87 cells proliferation. U87 cells were cultured in DMEM containing 10% FCS for the indicated periods of time in the absence (control) or in the presence of different concentration of *pinus halepensis* oil. U87 cells were quantified by staining with 0.1% crystal violet, solubilization with 1% SDS and measure of absorbance at 590 nm. All data represent the mean ± SEM of three separate experiments performed in triplicate. (C) Effect of *v* on U87 cells adhesion. Cells were preincubated with different concentrations of *pinus halepensis* or 30 min at room temperature. Cells were then added to 96-well microtiter plates coated with fibrinogen or poly-L-lysine and allowed to adhere for 2 h at 37 °C. After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 590 nm.

Adhesion assays

Because cell-ECM adhesion is a fundamental process through which cells interact and communicate with the environment in biology and pathology,[20] we first performed cell adhesion assays by using a fibrinogen ECM proteins. *pinus halepensis* oil, at 400 µM, notably blocked the adhesion of U87 to fibrinogen, while no effect could be observed either on the other matrix, or on the integrin-independent substratum, poly-L-lysine (Figure 2, C), suggesting that the effect of *pinus halepensis* may involve the integrin family of adhesion receptors. The inhibitory action of *pinus halepensis* on fibronogen was dose-dependent with IC_{50} values of 250 µM and 350 µM respectively from Kasserine and Foussena locality (Figure 2, C).

CONCLUSION

In summary, one of the main findings in this study was that the selected Tunisian *pinus halepensis* oil. medicinal herbs demonstrated good antioxidant activity, contained significantly good amounts of phenolics compounds probably with anti-proliferative properties. Based on the results described above, we can speculate that Tunisian *pinus halepensis* oil. Medicinal plants have high health prevention potential and could be considered in the formulation in new food products and dietary recommendations.

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