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Research Article

Phytochemical analysis and antiproliferative properties of *Pistacia atlantica* leaves

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Abstract

The medicinal plants may serve as potential sources for the development of new drugs and more effective anticancer agents for future therapy. *Pistacia atlantica* (Vahl.) Masters (Anacardiaceae) is an important plant used in traditional medicine practice in Algeria, and North Africa countries. The present study has the objective to estimate the in vitro antiproliferative (on the RD and Hep2 human tumor cell lines using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay) properties of the aqueous extracts of *P. atlantica* leaves, offering a phytochemical characterization of its aqueous extracts, by spectrophotometry methods. Revealed by HPLC, phenolic compounds present among the five different flavonoids identified such as: ((epi)catechin, myricetin, quercetin and kaempferol glycoside derivatives). The MTT assay revealed that the tested extract had a good activity against Hep2 and RD cell lines with median inhibitory concentration (GI50) > 1000 µg/mL against Hep2 and (IC50) = 825,079 µg/mL against RD, (GI 50). The results showed a great bioactive potential for this species with a significant contribution of phenolic compounds, specially the flavonoids which makes it an interesting matrix in the development of novel pharmaceutical formulations. Planned future studies will involve the identification of different extract other than aqueous extract, determination of the mechanisms of action and the bioactive molecule of plant extracts.

Keywords: *Pistacia atlantica*; aqueous extract; antiproliferative; MTT, HPLC.

1. INTRODUCTION

For centuries, herbal medicines have been used by humans to treat their illnesses. Today, they are still the main source of therapeutic substances in developing countries¹. One of the most abundant problems in the biological and medical world is oxidative stress, it comes from an imbalance in redox homeostasis and results in the excessive formation or insufficient suppression of free radicals; this imbalance leads to an overproduction of reactive oxygen species (ROS) capable of causing damage to vital cellular components (lipids, proteins, DNA) leading to many diseases, such as cancer^{2,3}. Cancer is defined as a hyperproliferative disorder that involves damage and inflammation of cells in a specific tissue⁴, to protect themselves from it, the body puts in place a prominent antioxidant defense system⁵. However, it can develop deficiencies in endogenous antioxidants which require supplementation to maintain optimal bodily function^{6,7}. Algeria by its geographical location in the center of the Mediterranean, shelters a rich and diversified vegetation, a large number of plants grow there spontaneously among which *Pistacia atlantica* or "Betoum" of the family of Anacardiaceae, widely used in various fields

in particular in medicines traditional and in food⁸. Recently new approaches have demonstrated that plants are capable of having direct impacts on public health and the economy⁹, similarly traditional knowledge and scientific reports show that they are rich sources of biologically active compounds among these phenolic compounds considered to be natural antioxidants have attracted particular interest due to their implications in the prevention and treatment of several diseases^{10,11}. New sources of natural compounds with antioxidant activity are actively sought because synthetic antioxidants present potential toxicological risks^{12,13}. It is in this perspective we became interested in *Pistacia atlantica* to assess its antioxidant potential and its ability to inhibit the proliferation of cancer cells, in order to provide a scientific basis for its traditional use by in vitro study. From this goal we noticed that there has been very little phytochemical study on *Pistacia atlantica* in Oran (Algeria). In order to quantitatively characterize the extracts prepared from the leaves of *Pistacia atlantica*, an assay of total polyphenols and flavonoids was carried out. The choice of these substances lies in the fact that they play key roles in many biological activities^{14,15}. For the quantitative evaluation of our aqueous

extract of *P. atlantica*, an assay of polyphenols and flavonoids was performed in our study.

2. MATERIALS AND METHODS

2.1. Chemicals

All of the chemicals and reagents used in the experiments were of analytical grade. Thiazolyl blue tetrazolium bromide (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT), Gibco EMEM, dimethyl sulfoxide (DMSO), trypan blue (0.4%), phosphate buffered saline (PBS), penicillin / streptomycin (100 ×), trypsin-EDTA (1 ×) and fetal bovine serum mycoplex (FBS), Inverse phase microscope, filter of 0.20Um, tips at different filter, micropipette, incubator, flasks of culture; bell and candles.

2.2. Plant Material and Preparation of Aqueous Extract

Leaves of *P. atlantica* Desf used in this study were collected from Oran (Algeria) in October 2018. The plant material was authenticated in the botanic laboratory, University of Oran1. After the leaves were cleaned and air-dried, they ground to a fine powder and extracted with distilled water (1: 10, w/v) under the heat conditions (60 °C) during 60 min. The mixture was filtered. The obtained decoction was frozen and then lyophilized (freeze-dryer christalp 2-4 lsc d 37520, Germany).

2.2.1. High-performance liquid chromatography HPLC UV

HPLC detection was carried out at the Center for Research and Techniques for Physical and Chemical Analysis CRAPC, Algeria. High-performance liquid chromatography analysis Phenolic compounds were separated on a C18 (150 mm, 4.6mm, and 5µm) column at 25°C. The mobile phase contains water, acetic acid, and methanol in a gradient mode with a rate of flow of 1 ml/min (YLClarity-Céhroatography SW). The obtained chromatograms at 254 nm were compared with those obtained with standards of some well-known phenolic compounds.

2.2.2. Phytochemical analysis of plant.

Flavonoids: 1 ml of 10% lead acetate (H₂O) is added to 1 ml of extract. Their presence is indicated by a yellowish green color¹⁶.

Tannins: 2.5 ml of extract are added with 0.5 ml of 2% iron perchloride, MeOH (FeCl₃), a precipitate develops: greenish brown for the catechetal tannins or blue black for the gallic tannins¹⁶

Coumarins: 2 ml of extract are added with 3 ml of 10% sodium hydroxide, H₂O with stirring, a yellow color appears¹⁶.

Terpenoids: 2.5 ml of the sample are added to 1 ml of chloroform and 1.5 ml of concentrated sulfuric acid. The formation of a red or purple ring indicates their presence¹⁷.

Steroids: 2.5 ml of acetic anhydride and 500 µl of concentrated sulfuric acid are added to 2.5 ml of extract, then the mixture is left to react for 20 minutes. The blue-green appearance indicates the presence of steroids¹⁷.

Saponins: 3 ml of distilled water are added with 1 ml of extract then stirred vigorously for 2 minutes. Then the mixture is left to stand for 20 minutes. The formation of persistent foam indicates the presence of saponosides¹⁸

Free quinones: A few drops of sodium hydroxide (10%, H₂O) with 5 drops of HCl are mixed with 1 ml of the extract.

The appearance of a yellow, red or purple color explains their presence¹⁹.

Essential oils: 2 ml of extract are added to 100 µl of NaOH (10%) and 100 µl HCl (10%), the formation of a white precipitation confirms the presence of essential oils²⁰

2.3. Determining the antiproliferative effect

2.3.1. Cell lines and cell culture preparation

The cell lines used in this study were HEp-2 (laryngeal cancer cell line) and RD (from Atalnta USA laboratory) HEp-2 and RD cells were cultured in 89% Gibco EMEM with 10% FBS and 1% penicillin / streptomycin. All cells were cultured at 37 °C to 95% moisture and 5% CO₂ for 3 days to 80% - 90% confluences. Then, the used medium was replaced with fresh medium and incubated again for 24 h. The cell cultures were then washed with PBS 1-2 times and suspended using trypsin-EDTA. Fresh medium was added to the cells.

The antiproliferative activity of extracts against human cancer cell lines was tested using the microtitration colorimetric method of MTT reduction with a minor modification. MTT is used to determine cell viability in cell proliferation and cytotoxicity assays. Exponential phase cells that were 80% to 90% confluent were harvested from maintenance cultures and counted using a hemocytometer with a trypan blue solution. Cell suspensions (100 µl) were distributed in 96 well culture plates at optimized concentrations of 1.0 x 10⁴ cells / ml per cancer cell line. After 24 h incubation at 37 °C, 100 µL of culture medium were removed from the wells and 100 µL of fresh medium containing the extracts (1000, 100, 10, 1, 0.1, 0.01, 0.001 µg / mL) were added to each well and incubated for an additional 48 hours. Wells containing EMEM were used as negative controls. At the end of the treatment period, the medium in each well was aspirated and replaced with 20 µL of MTT 5 mg working solution (MTT stock solution mixed with media to reach a final concentration of 0.5 mg / mL). Briefly, the MTT powder was dissolved in PBS to form a MTT stock solution (5 mg / mL). The stock solution was sterilized by filtration through a 0.22 µm filter and stored at -20 °C until use. The cells were incubated at 37 °C for 4 h, then the medium was aspirated and replaced with 100 µl of DMSO to dissolve formed formazan crystals. The culture plates were shaken for 5 minutes and the absorbance (OD) of each well was read using an enzyme immuno-assay reader at 570 nm (reference wavelength = 630 nm).

The percentage of antiproliferative activity compared to untreated cells was determined as follows:

$$\text{Cell viability (\%)} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\% \quad (1)$$

$$\text{So Cell Death} = 100\% - \text{Cellular Viability} \quad (2)$$

Where OD means Optical Density and it signifies absorbance. each experiment was performed four times. The relative viability of the treated cells compared to that of the control cells is expressed as cell viability.

3. RESULTS AND DISCUSSION

Composition of *P. atlantica* in phenolic compounds

The results confirm the presence of constituents which are known to exhibit medicinal as well as physiological activity (table1), important phytochemicals, such as terpenes, flavonoids, tannins, steroids, fatty acids, essential oils, and other compounds are present in the fruit, leaf, stem-bark, root bark and mastic of *P. atlantica* subspecies. Today, over

150 compounds have been isolated and identified from the *P. atlantica* subspecies.²¹

The phenolic profile of the crude aqueous extract of *P. atlantica* leaves was recorded at 280, Fig. 1 shows a representative chromatographic profile obtained for the Aqueous extract. The compounds characteristics, identities and quantification are presented in Table 1. The chromatographic separation allowed the elucidation of 17 phenoanic compounds, a (Table 1). Compounds 2 and 3 were positively identified as (+)gallique acide and Rutin according to their retention time, characteristics by comparison with commercial standards. Both these compounds were previously described in extracts of *P. atlantica*²¹

Antiproliferative activity

The antioxidant activities of natural compounds are frequently accompanied by an antiproliferative property^{22,23}. To determine whether *P. atlantica* extract affects cell viability, the MTT test was used to evaluate mitochondrial activity and indirectly, the cell viability of treated cells. A rather remarkable increase in cell viability was observed at higher treatment concentrations in both cell lines. Conversely, viability appears to be lower with lower treatment concentrations of the extracts. These results suggest interactions between the cell viability test reagents and the extract²⁴. Indeed, our results (expressed as a percentage of viability compared to untreated control cells) show that the cell viability of human cancer cell lines (Hep2 and RD) was significantly reduced after treatment with an aqueous extract of *P. atlantica*. According to the table 2. *P. atlantica* has a remarkable antiproliferative effect on both cell lines (RD and Hep2) but the IC50s differed from one line to the other. Our extract exerted an interesting inhibitory effect on the proliferation of Hep2 cancer cells (IC50=324.91 µg/mL) however, the effect is less on the RD cancer cell line with an IC50 of 434.912 µg/mL, the fact that the effect is different from one line to the other could testify to the specificity of the antiproliferative activity of this extract on the one hand and the resistance of Hep2 cells on the other

hand. This result is consistent with that of Hashemi et al., (2017)²⁵ who showed that *P. atlantica* was more active on AGS cells than on HeLa with respective IC50s of 382.3 µg/mL and 332.3 µg/mL. The antiproliferative activity of *P. atlantica* can be attributed to its high polyphenolic content²⁶. Although the MTT test is a rapid and specific test²⁷, a high concentration of NADH can lead to inaccurate MTT test results²⁸, non-targeted effects of the molecules studied can also lead to an under- or overestimation of cell viability²⁴.

Table 1: Result of phytochemical tests.

Chemical groups	Presence or absence
polyphenol	+++
Flavonoids	+++
Tannins	+++
Coumarins	+++
Terpenoids	+++
Steroids	++
Saponins	++
Free quinones	-
Essential oils	+++

(+++): Strongly present (++): Moderately present (+): Weakly present (-): Test negative

Table 2: Cytotoxic properties of *P. atlantica* leaves in human tumor cell lines and non-tumor liver primary cells (mean ± SD, n = 9).

Human tumor cell lines (GI50 values. µg/mL)

Hep2	RD
324.91±9.5	434.91±17

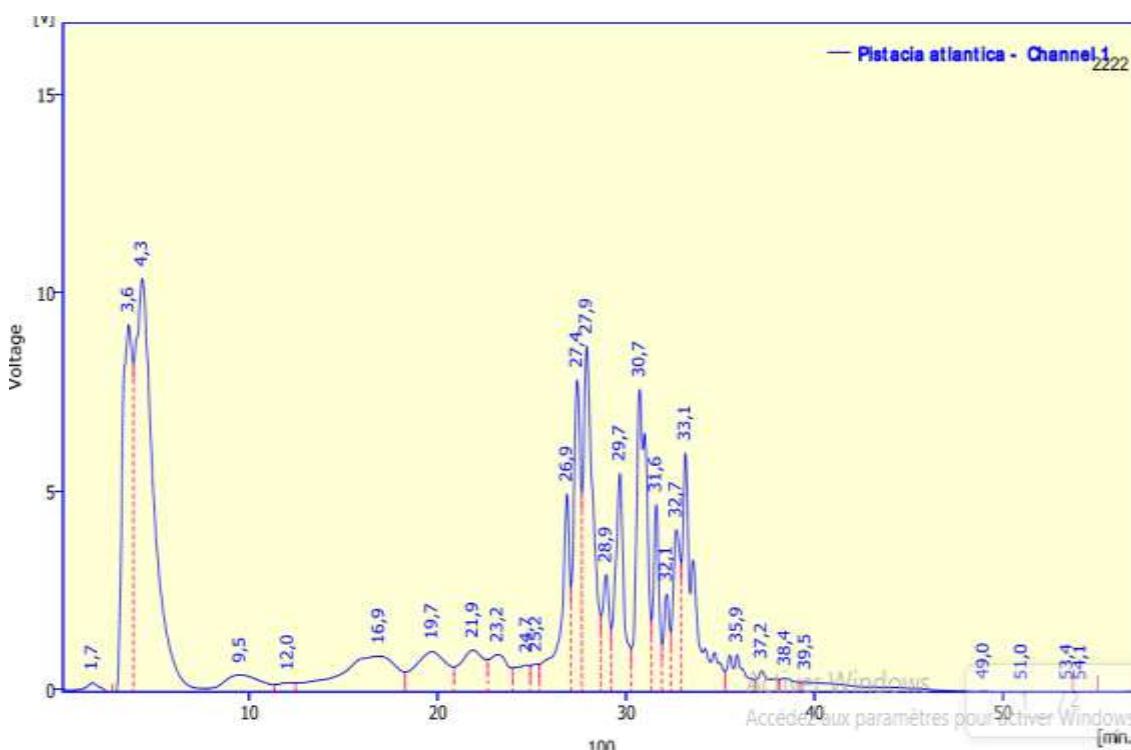


Figure 1: High-performance liquid chromatography analysis of *Pistacia atlantica* polyphenols

Table 3: Composition of polyphenols

Pic	Rt (min)	Tentative Identification	Formule
1	1,657	Quinic acid	C ₇ H ₁₁ O ₆
2	4,300	Gentisic acid	C ₇ H ₅ O ₄
3	9,503	Galloyl quinic acid (isomer)	C ₁₄ H ₁₆ O ₁₀
4	12,043	Rosmarinic acid	C ₁₈ H ₁₆ O ₈
5	16,887	Naringenin	C ₁₅ H ₁₂ O ₅
6	19,703	Digalloylquinic acid (isomer 1)	C ₂₁ H ₂₀ O ₁₄
7	21,9	Catecheine	C ₁₅ H ₁₄ O ₆
8	23,2	Epicatechin	C ₁₅ H ₁₄ O ₆
9	27,897	Acide 3 hydroxy 4-methoxycinamic	C ₁₀ H ₁₀ O ₄
10	28,933	Methyldigallate (isomer II)	C ₁₅ H ₁₂ O ₉
11	29,663	Methyldigallate	C ₁₅ H ₁₂ O ₉
12	30,710	Rutin	C ₂₇ H ₃₀ O ₁₆
13	31,587	Myricetin O-rutinose	C ₂₇ H ₃₀ O ₁₇
14	32,143	Myricetin O-hexoside II	C ₂₁ H ₂₀ O ₁₃
15	37,183	Myricetin	C ₁₅ H ₁₀ O ₈
16	38,443	Quercetin O-hexoside	C ₂₁ H ₂₀ O ₁₂
17	39,513	Gentisic acid pentoside	C ₇ H ₆ O ₄

4. CONCLUSION

Quantitatively our results lead us to conclude that the aqueous extract of *Pistacia atlantica* contains a considerable amount of total polyphenols and flavonoids. Our study showed that this extract has a good activity which could be due to compounds considered as antioxidant agents. Also the inhibition of the growth of the RD and Hep2 cancer cell lines observed during the antiproliferative evaluation indicates that this extract could be a new alternative for the production of new anti-cancer agent and this should be done by isolating the active principle. In conclusion we observe that the antioxidant capacity of a plant also reflects its antiproliferative capacity. It should be pointed out that these results are only obtained *in vitro*, further *in vivo* studies are still necessary to correlate these observations.

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Conflict of interest statement

The authors report no conflict of interest

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