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Chemical composition, *in vitro* antioxidant and anti-inflammatory activities of *Juniperus oxycedrus* subsp. *oxycedrus* extracts from Algeria

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ABSTRACT: This study was conducted to examine chemical compositions, antioxidant and anti-inflammatory properties of methanolic and aqueous extracts from aerial parts of *Juniperus oxycedrus* subsp. *oxycedrus* growing in Mascara, Algeria. The quantitative assessment indicated that methanol extract was the most concentrated in phenolic, flavonoid and tannin contents (167.77±5.12 mg GAE/g DW, 90.56±2.23mg QE/g DE and 110.21±2.38 mg CE/g DE respectively). The chromatographic analysis by HPLC showed quantitative differences in phenolic constituents, noting that Chlorogenic acid was the major compound of both extracts. Moreover, the methanolic extract exhibited the highest antioxidant activity than the aqueous extract when tested by the 1,1-diphenyl-2-picrylhydrazyl (IC50 4.45±0.001 µg/mL) and phosphomolybdenum (328.52±0.071 mg of GAE/g DW) assays. Furthermore, the in vitro anti-inflammatory activity showed strong inhibition of albumin denaturation by the methanolic extract at different concentrations when compared to the standard drug diclofenac sodium. These findings confirm the richness of Algerian *Juniperus oxycedrus* extracts in bioactive compounds with antioxidant and anti-inflammatory capacities.

Keywords: Anti-inflammatory; Antioxidant; *Juniperus oxycedrus*; HPLC; In vitro; Phenolic extracts.

1. INTRODUCTION

The developments concerning human health and its treatments have been increasing every passing day. Research of natural drugs with any side effects on human health has been a growing interest among scientists. Traditional medicine is used widely throughout the world including Algerian population. It is a system that relies on a wide range of practices. Recently there has been an upsurge of interest in the therapeutic potentials of plants, as antioxidants by interacting with free radicals, chelating, catalytic metals, and functioning as oxygen scavengers, in order to reduce oxidative damage produced by free radicals [1, 2]. Oxidative damage is considered as a pathogenic mechanism contributing to aging and the development of different chronic diseases

including diabetes, cancer, atherosclerosis, arthritis, and neurological disorders [3] and frequently is associated with inflammation [4]. Inflammation is a normal protective response to tissue injury and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair [5]. Inflammation is a defense process that occurs when the body reacts to a variety of stimuli including infections, irritants, or various cellular and tissue damages [6]. As well, inflammation is associated with pain, and it involves an increase in protein denaturation, an increase in vascular permeability, and membrane alteration, among others [7]. Several synthetic anti-inflammatory and antioxidant products are widely available (non-steroidal and steroidal anti-inflammatory drugs, propyl gallate, butylated hydroxytoluene). However, their overuse can cause gastrointestinal, renal or cardiovascular complications, ulcers and osteoporosis [8, 9]. In this context, the anti-inflammatory and antioxidant properties of various medicinal plants are being investigated throughout the world to find out newer, effective, and safe drugs, in order to use them in foods and pharmaceutical preparations to replace synthetic ones [10]. Plants have a large number of secondary metabolites such as alkaloids, terpenoids and phenolic compounds such as phenolic acids, flavonoids, tannins, lignins, quinones, coumarins and others. These phenolics are responsible for biological properties like antimicrobial, anticarcinogenic, anti-inflammatory and other therapeutic effects [11]. *Juniperus oxycedrus* subsp. *oxycedrus* belonging to the *Cupressaceae* family is a shrub or small tree. It is endemic to the Mediterranean area and near East countries [12]. From the entire *Juniperus* genus, this tree is the most abundant subspecies in Algeria [13]. Commonly known as «*taga*» *Juniperus* has been used in folk medicine to treat bronchitis, cough, common colds, gynecological diseases, fungal infections on foot, hemorrhoids [14], bloating, wound healing [15], abdominal pain, stomach disorders, as digestive, hypoglycemic, against urinary inflammations and to pass kidney stone [16]. Extracts from the leaves, resin, bark, and berries have anti-cancer [17], analgesic [18], and antibacterial properties [19]. Moreover, cade oil, distilled from the wood of *J. oxycedrus*, is widely used in human and veterinary dermatology for treating skin diseases such as eczema [20].

The present study aimed to investigate the chemical composition of methanolic and aqueous extracts from aerial parts of *Juniperus oxycedrus subsp. oxycedrus*, and then assess their *in vitro* antioxidant properties and anti-inflammatory effect against protein denaturation.

2. MATERIAL AND METHODS

2.1. Plant material

Juniperus oxycedrus subsp. *oxycedrus* aerial parts (leaves and stems) were collected in March 2018 from the forest of Nesmoth (latitude N 35°14', longitude E 0°22'52") in the Mascara region, north-western Algeria. The sample was identified in the laboratory of the University of Mascara, then the referenced specimen (J.O: 01) was air-dried in the shade at room temperature. The powder of the dried plant was stored away from heat, air and light until the moment of use.

2.2. Preparation of extracts

2.2.1. Infusion in water

Powdered plant material (500 g) was infused in 1L of boiling water and incubated at dark and at room temperature on a rotating shaker (300 rpm) for 1 hour. Then, the aqueous extract was filtered with Whatman filter paper No. 1 and dried at 40°C [21].

Figure 1. Aerial parts (leaves stems and fruits) of *Juniperus oxycedrus* subsp. *oxycedrus* from Mascara, Algeria.

2.2.2. Maceration with methanol

The methanolic extract was prepared using maceration of 100 g of powder in methanol-water (70:30 v/v) at room temperature for 24 h. The filtrate was evaporated in a vacuum at 45°C by a rotary evaporator, and then the residue was dried in an oven at 40°C to yield dry extract.

The percentage of yield was calculated according to the following formula: (weight of extract/weight of dried plant material) × 100 [13].

2.3. Total phenolic content determination (TPC)

The total phenolic content was determined using the Folin-Ciocalteu method with some modifications as described by Ismail et al. [22]. Briefly, 100 µL of a sample (1 mg/ml) was mixed with 750 µL of Folin-Ciocalteu reagent (diluted to 10% in distilled water). After 5 minutes, 750 µL of a sodium carbonate solution (6% w/v) was added and the mixture was incubated in the dark at room temperature for 90 min. The absorbance of the resulting color solution was measured at 725 nm using a UV-Vis spectrophotometer. The calibration curve was prepared with gallic acid solutions. TPC was expressed as mg gallic acid equivalent (GAE) per grams of the dry weight of plant extract (DW) ± standard deviation (SD).

2.4. Total flavonoid content determination

Total flavonoid content was determined by a colorimetric assay described by Attia et al. [23] with some modifications. A volume of 1000 µL of each sample was mixed with 1000 µL of aluminum chloride solution (2%) and allowed at room temperature for 30 min. the absorbance of the resulting solutions was then determined at 430 nm. Total flavonoid content was expressed as mg quercetin equivalent (QE) per gram extract (DW) ± SD.

2.5. Total tannins content determination (TTC)

The total tannins content in samples was determined using the vanillin-methanol solution as described by Chaouche et al. [24]. 250 µL of sample (1 mg/ml) were mixed with 1500 µL of vanillin solution (4% in methanol) and 750 µL hydrochloric acid. The mixture was incubated at room temperature for 15 min. The absorbance of the reaction mixture was measured at 500 nm. TTC was expressed as mg catechin equivalents (CE) per gram of dry weight extract (DW)± SD.

2.6. High-performance liquid chromatography analysis (HPLC)

Phenolic compounds present in extracts were identified using a Shimadzu model prominence liquid

chromatography, thermostatic column compartment, online degasser and a UV-visible detector model SPD-20A (operating at 268 nm) using Shim-pack VP-ODS C18 column (4.6 mm×250 mm, 5 µm), (Shimadzu Co., Japan). The mobile phase consists of acetonitrile (A) and 0.2% acetic acid in water (B). The gradient linear method was: starting with 90% (B); then decreasing to 86% (B) in 6 min, to 83% (B) in 16 min, to 81% (B) 23 min, held at 77% (B) in 28-35 min, to 60% (B) in 38 min, to 90% (B) in 50 min [25]. The flow rate was 1 ml/min and the injection volume was 20 µL. All the samples and mobile phase were filtered through a 0.45 µm membrane filter (Millipore). Stock solutions of standards references were prepared by dissolving 10 mg of purified polyphenol in a 50 mL volumetric bottle containing a sufficient volume of methanol (HPLC grade) to dissolve the polyphenol, it was sonicated for about 10 min and then brought to volume with the mobile phase. Identification of the chromatography peaks was performed by comparing their retention time and UV absorption spectrum with those of the reference standards: gallic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, vanillin, rutin, naringin, and quercetin. The quantification of separated peaks was performed by calibration with curves of standards. All chromatography operations were carried out at ambient temperature.

2.7. Evaluation of the antioxidant activity

2.7.1. Phosphomolybdenum antioxidant assay

The total antioxidant capacity was determined according to the phosphomolybdenum techniques. It is based on the reduction of Mo (VI) to Mo (V) by plant extract and the formation of a green-colored phosphate/Mo(V) complex at acidic pH. A volume of 300 µL of various extracts was added to 3000 µL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 M ammonium molybdate). Test tubes were incubated at 95°C for 90 min. After the samples were cooled at room temperature, the absorbance was measured at 695 nm against the blank, which contained 3000 µL of methanol instead of the extracts. Ascorbic acid served as the reference standard. The total antioxidant capacity was expressed as milligrams gallic acid equivalent per gram dry weight (mg GAE/ DW) [26].

2.7.2. DPPH• Free radical scavenging assay

The antioxidant potential of plant extracts was investigated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method as described by Wannes et al. [27]. Briefly, 500 µL of a 0.2 mmol/L DPPH methanolic solution was added to 1000 µL of sample solution prepared in methanol at different concentrations (1000, 500, 250, 125, 62.5 µg/mL). After agitation, the mixture was incubated at room temperature for 30 min. The absorbance was then measured at 517 nm against the blank. Ascorbic acid was used as a positive control. The percent inhibition of DPPH radical was calculated as follows: % inhibition = [Ac – As) / Ac] × 100, where Ac and As are the absorbance of the control sample and the test sample respectively.

The antiradical activity was expressed as IC_{50} (µg/mL): the concentration of extract required to reduce 50% of the initial DPPH concentration. Values were estimated using linear regression.

2.8. Evaluation of the *in vitro* anti-inflammatory activity

This assay was evaluated by the protein denaturation method according to the protocol described by Sen et al. [28] with small modifications. Reaction mixtures were prepared using 0.2 mL of an aqueous solution of egg albumin (5%) and 2.8 mL of phosphate-buffered saline (pH 6.4). Then 2 mL of AJE, MJE or standard diclofenac sodium at different concentrations (125, 250, 500, 1000 µg/ml) was added. These mixtures were incubated at 37°C for 15 min. and kept in a water bath at 70°C for 10 min to induce denaturation. After cooling, the turbidity was measured at 660 nm. A similar volume of distilled water served as a control. The percentage

inhibition of protein denaturation was calculated using the following formula:
% inhibition = $(A_{test\ sample} - A_{control}) / A_{control}$ x 100.

The extract/drug concentration for 50% inhibition (IC50) was determined by plotting percentage inhibition with respect to control against treatment concentration.

2.9. Statistical analysis

Data were expressed as the average of triplicate values of three independent experiments ± standard deviation. Statistical comparisons were performed by analysis of variance (ANOVA) and Tukey post-hoc test. Differences were considered significant at $p < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Total phenolic, total flavonoid and total tannins contents

Table 1 summarized extraction yield, total phenolic, total flavonoid and total tannins contents of the extracts prepared from *Juniperus oxycedrus* ssp. *oxycedrus* aerial parts (AJE: aqueous extract, MJE: methanolic extract). The extraction with methanol gave a higher yield (14.21%). Moreover, MJE exhibited the highest values of total phenolic content (167.77 mg AGE/g DW), total flavonoid content (90.56 mg QE/g DW) and total tannins content (110.21 mg CE/g DW) compared to aqueous extract AJE (148.84 mg AGE/g DW, 74.06±3.71 mg QE/g DW, and 86.51 mg CE/g DW).

The difference between these results may be attributed to the polarity of the solvent. According to Do et al. [29], chemicals are more soluble in a mixture of water and organic solvents (methanol, ethanol, and acetone) than in water.

Compared to previous studies on the same plant, total phenolic, total flavonoid and total tannins levels in MJE and AJE are higher than those reported by Chaouche et al. [13] for the hydro-methanolic extracts in needles and roots bark from the plant collected in Tlemcen, Algeria (133.08 ± 4.1 mg AGE/g DW, 61.52 ± 3.1 mg CE/g DW and 26.43 ± 2.6 CE/g DW respectively). However, TPC values are lower than those recorded by Ben Mrid et al. [30] for the same plant collected in Morocco.

Table 1. Yield percentage, total phenolic (TPC), total flavonoid (TFC) and total tannin content (TTC) in *Juniperus oxycedrus* ssp. *oxycedrus* extracts.

	Yield (%)	TPC (mg GAE/g DE)	TFC (mg QE/g DE)	TTC (mg CE/g DE)
AJE	9.6±2.04	148.84±2.99	74.06±3.71	86.51±0.64
MJE	14..21±0.33	167.77±5.12	90.56±2.23	110.21±2.38

Legend: AJE*: J. oxycedrus* aqueous extract, MJE: *J. oxycedrus* methanolic extract. Values were expressed as means± standard deviation of three separate experiments. Significant difference at ($p < 0.001$).

3.2. HPLC analysis of the extracts

The chemical composition of MJE and AJE was further investigated by HPLC analysis (Fig. 2, Fig. 3 and Table 2). Eight phenolic compounds including gallic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, banillin, rutin, naringin and quercetin were identified by matching their retention time values and UV spectrum with those of the corresponding commercial standards. Our results revealed that the total contents of the identified compounds varied among *Juniperus* extracts. MJE was most concentrated in phenolic compounds than AJE. This was in accordance with the results obtained by the colorimetric methods.

Phenolic acids are the major constituents of the aqueous extract, whereas flavonoids are the major components in *Juniperus* methanolic extract. Chlorogenic acid was detected as the most abundant phenolic compound in both AJE and MJE extract (13737.44 and 19872.01 µg/g respectively), followed by naringin and rutin in methanolic extract. While in aqueous extract, gallic acid and p-coumaric acid were the main ones identified.

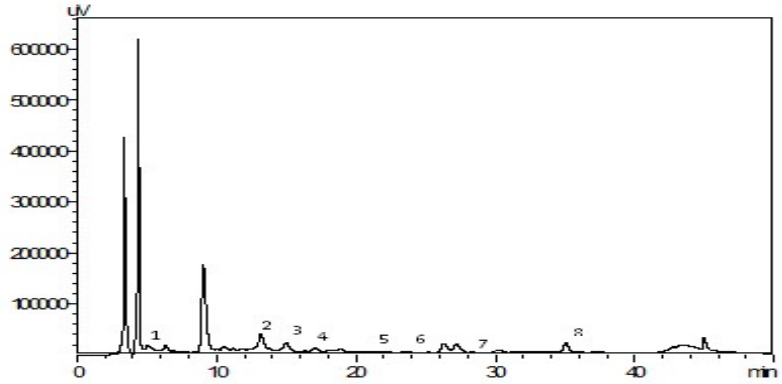

Figure 2. HPLC chromatogram of *J. oxycedrus* aqueous extracts detected at 268 nm.1: gallic acid, 2: chlorogenic acid, 3: vanillic acid, 4: p-coumaric acid, 5: caffeic acid, 6: vanillin, 7: rutin, 8: naringin.

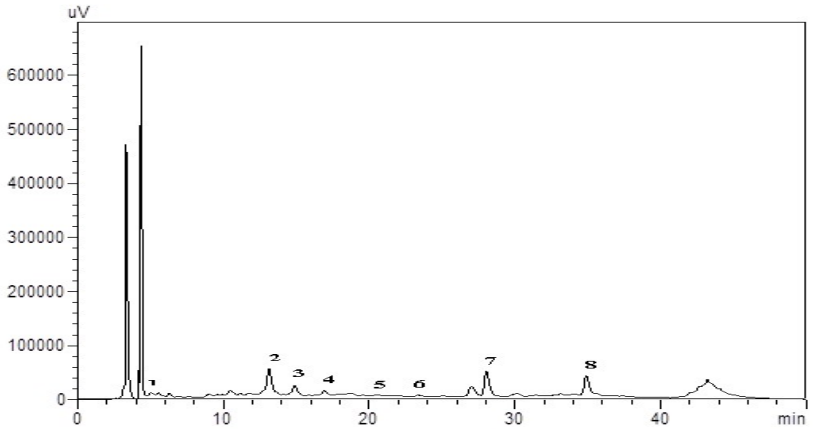

Figure 3. HPLC chromatogram of *J. oxycedrus* methanolic extracts detected at 268 nm. 1: gallic acid, 2: chlorogenic acid, 3: vanillic acid, 4: p-coumaric acid, 5: caffeic acid, 6: vanillin, 7: rutin, 8: naringin.

In a previous study, Seca and Silva [31] proved that coumarins (umbelliferone), flavonoids (amentoflavone, cupressuflavone, hinokiflavone, rutin), are found in various parts of *Juniperus oxycedrus*. According to Yaglioglu and Eser [32], catechin and rutin are the most abundant metabolites found in the leaves of plants growing in Turkey. On the other hand, Miceli et al. [33] have found that amentoflavone was the most abundant flavonoid compound, however protocatechuic acid was the only phenolic acid observed. Also, recent studies proved the occurrence of salicylic acid as the main compound in needles of the plant collected in Morocco (3398.1 mg/100 g and 2942.7 mg/100 g for aqueous and methanolic extracts respectively) followed by rutin, whereas gallic acid was not identified [30]. Quercetin hexose and quercetin are detected in *J. oxycedrus* root bark from Algeria [24], however, quercetin was not detected in MJE and AJE.

The difference between the results reported previously and the current results may probably be influenced by genetic factors [34] and environmental conditions like sunlight, temperature and precipitation [35].

3.3. In vitro antioxidant activity

According to previous studies, it has been reported that the extracts from different parts (needles, berries and root bark) of *J. oxycedrus* possessed antioxidant effect. *In vitro* antioxidant activity results are summarized in Table 2.

Table 2. Phenolic compounds (µg/g DE) identified in aqueous (AJE) and methanolic (MJE) extracts of *Juniperus oxycedrus* subsp. *oxycedrus* aerial parts collected from Mascara, western Algeria.

N°	Phenolic compounds	Rt (min)	AJE	MJE
1	Gallic acid	5.29	2747.97	1374.514
2	Chlorogenic acid	13.39	13737.44	19872.01
3	Vanillic acid	15.53	329.30	770.77
4	Caffeic acid	16.28	566.40	2161.58
5	p-Coumaric acid	23.82	1217.81	237.58
6	Vanillin	21.46	653.09	1067.96
7	Rutin	28.37	789.98	12078.10
8	Naringin	34.79	887.87	13950.33
9	Quercetin	45.05	ND	ND

Legend: AJE*: J. oxycedrus* aqueous extract, MJE: *J. oxycedrus* methanolic extract, Rt: Retention time (minute), ND: Not detected.

The phosphomolybdenum assay is a simple quantitative method to evaluate the total antioxidant capacity. It was determined by measuring the absorbance of the green phosphomolybdenum complex at 695 nm [36]. The total antioxidant capacity ranged from 195.73 mg GAE/g DW to 328.52 mg GAE/g DW for AJE and MJE respectively in comparison with ascorbic acid 374.08 GAE/g DW. The results obtained were comparatively higher than those obtained by Chaouche et al. [13] for the hydro-methanolic extracts (115.32 mg of GAE/g DW).

The DPPH assay is a simple and widely used method for testing the antioxidant activity in vitro. It is based on the ability of extracts to transfer electrons or H atoms to the free radical DPPH to become a stable molecule. Table 2 showed that the radical scavenging activity of *Juniperus oxycedrus* extracts increased in a dose-dependent manner suggesting that both extracts have greater antioxidant potential. MJE was found to be more active than AJE. IC_{50} values of the methanolic extract (4.45±0.001 µg/mL) were lower than ascorbic acid (10.34±0.008 µg/mL), indicating a strong ability of this extract to scavenge free radical DPPH. Although IC_{50} value of aqueous extract (155.32±0.04 µg/mL) was higher, the extract still showed good free radical scavenging activity. The IC_{50} value of MJE was lower compared to those reported by Chaouche et al. [13] for *J. oxycedrus* needle extracts with an IC_{50} 10.95 µg/mL. In studies on the antioxidant activity of Moroccan and Turkish *J. oxycedrus* extracts [30, 33, 37], IC_{50} values obtained are much higher compared to our results.

In the aforementioned studies, methanolic extracts exhibited higher antioxidant properties than the aqueous extracts which is in accord with our results. This could be due to the difference in phenolic and flavonoid contents between the extracts. According to El Jemli et al. [37], extracts prepared to utilize a pure and aqueous organic solvent have a higher radical scavenging capacity.A strong correlation between antioxidant activity and total phenolic content has been proven. In addition, Krishnaveni et al. [38] suggested that free

radical scavenging activities of plants depend on the presence and the concentration of secondary metabolites. Moreover, Yang et al. [39] indicated that rutin is a powerful free radical inhibitor. More specifically, several flavonoid compounds such as catechol, quercetin, catechin, rutin and gallic acid contribute significantly to free radical reducing power [40, 41].

Table 3. DPPH* scavenging effect (percentage %), inhibition DPPH*concentration (IC50) values (µg/mL) and total antioxidant capacity of *J. oxycedrus* aqueous and methanolic extracts.

Concentration (µg/mL)	% Inhibition of DPPH*					$IC50_{(DPPH)}$ (µg/mL)	TAA (mg of GAE/g DW)
	31.25	62.5	125	250	500		
AJE	16.44	39.48	48.54	59.91	66.97	155.32±0.04[a]	195.73±0.07[b]
MJE	64.05	70.05	72.5	79.88	84.02	4.45±0.001[b]	328.52±0.071[a]
Ascorbic acid	56.84	66.67	68.36	72.81	78.49	10.34±0.008[b]	374.08±0.18[a]

Values were expressed as means± standard deviation of three separate experiments. Significant difference at (p <0.001).

3.4. *In vitro* anti-inflammatory activity

The *in vitro* anti-inflammatory effect of *J. oxycedrus* was investigated by the egg albumin denaturation method. Table 3 shows a significant (p <0.001) inhibitory effect on protein denaturation caused by the heat of Sample extracts and diclofenac sodium (reference drug) in a dose-dependent manner displayed at different concentrations.

The percent inhibition of albumin denaturation observed in MJE was important and comparable to diclofenac sodium (81.95 and 91.51% respectively at a concentration of 1000 µg/mL), whereas the aqueous extract (AJE) has shown the least inhibitory activity with 32.48% at the same concentration. The richness of MJE in phenolic compounds may explain its high ability to prevent thermal protein denaturation. In many previous studies, the anti-inflammatory properties could be related to the concentration of phytochemicals contained in plant extracts such as luteolin, rutin quercetin and to their antioxidant capacity [42, 43]. It is therefore expected that phytochemicals compounds such as alkaloids, saponins, phytosterols, tannins and flavonoids present in medicinal plants reduced inflammatory events [44]. Furthermore, Torres-Rêgo et al. [45] revealed that rutin and chlorogenic acid operate synergistically to suppress inflammatory processes.

In a study conducted by Moreno et al. [46], *J. oxycedrus* extracts prepared with methanol and dichloromethanol could partially antagonize the contractile response to histamine, serotonin and acetylcholine in a concentration-dependent manner. Therefore, the stem and leaves of the same plant significantly reduced edema induced by carrageenan in rat hind [47].

Table 4. *In vitro* anti-inflammatory activity of AJE, MJE and diclofenac sodium.

Concentration (µg/ml)	Percent inhibition (%)		
	Diclofenac	AJE	MJE
1000	91.51±0.025	32.48±0.027	81.95±0.03
500	61.99±0.017	23.35±0.011	62.85±0.002
250	46.49±0.012	3.82±0.01	49.89±0.016
125	24.2±0.016	1.69±0.15	43.31±0.006

Values were expressed as means± standard deviation of three separate experiments. Significant difference at (p <0.001).

4. CONCLUSION

The results of the present study suggest that the aerial parts of *Juniperus oxycedrus ssp. oxycedrus* growing in Mascara, Algeria are rich in phenolic compounds. HPLC analyses identified eight phenolic compounds. Consequently, the methanolic extract exhibited the highest phenolic concentration which could explain its excellent antioxidant and anti-inflammatory activities *in vitro*. Therefore, these findings may justify the popular use of this species in traditional medicine and support their application as a natural antioxidant in the food industry and the development of phytopharmaceutical products with a critical role in resolving inflammatory troubles. Further investigations should be carried out to ensure the efficacy of this plant *in vivo*.

Authors' Contributions: SD, BM designed the study. SD performed the experiments. AR did the chromatographic identification. SD wrote the paper with input from of BM, KS and PS. All authors read and approved the final version of the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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