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Phytochemical study and antioxidant activity of methanolic extracts and alkaloids of the leaves of pistachio Atlas (*Pistacia atlantica*)

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ABSTRACT

Medicinal plants are considered essential raw material source for the discovery of new molecules necessary for the development of future drugs. In this context, the aim of this work contributes to an enhancement of phytochemical pistachio Atlas. This study focused on the extraction and / or assay of secondary metabolites and the antioxidant activity of *Pistacia atlantica*. The yield of essential oils leaves female feet is more important (between 0,1423% and 0,3092%) than males feet (between 0,1173% and 0,1217%). The yield of essential oils galls *Forda marginata* and *Pemphigus utricularius* male feet is greater (1,7584%) than female feet (0,8304%). The leaves are rich in total phenols (216,04 mgEAG/gMS) and flavonoïds (between 102,75 and 117,89 MgEC/gMS). However tannins are quite low (between 0,45% and 1,59 %).The alkaloids are higher among female's feet (1,2 % and 1,3 %) than male's feet (0,4 % and 0,6 %). The methanol extracts have a lower antioxidant activity (0,82 mg/ml) compared ascorbic acid (0,013 mg / ml) against they are quite important than alkaloids total (between 1,03 ; 2,06 mg/ml).

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INTRODUCTION

Algeria, for its location, offers rich and diverse vegetation, estimated at more than 3000 species belonging to different botanical families. These species are mostly spontaneous with a significant number (15%) of endemic species (Ozenda, 1977), which gave the traditional medicines invaluable wealth.

Since ancient times, this vegetation has always been an important source of therapeutic agents. According to the World Health Organization (WHO), approximately 65-80% of the world population in developing countries because of poverty and lack of access to modern medicine depend essentially traditional medicinal plants their primary health care (Lhuillier, 2007). Despite remarkable progress in synthetic organic chemistry of the twentieth century, more than 25% of prescribed drugs in developed countries derive their origins directly or indirectly plants (Newman and *al*., 2000 ; Calixto, 2005). Urgent attention should be given to plants that have not yet been scientifically studied to determine not only their potential pharmacological and phytochemical properties but also assess their qualifications and their efficiencies. This green heritage represents an enormous reservoir of compounds waiting to be discovered (Hosttemann Marston, 2003). They contain a significant proportion of compounds involved in all enzymatic or biochemical reactions taking place in the organism. We can distinguish two metabolites groups: primary metabolites, secondary metabolites.

Secondary metabolites are the subject of much research, they have multiple interests, and they are put to use both in the food industry, cosmetics that pharmaceutical. They are widely used in therapy as vascular-protective, anti-inflammatory, enzyme inhibitors, antioxidants and anti-free radicals (Epifano and *al*., 2007)

Essential oils are volatile and odorous substances obtained from the plant by steam distillation of water. They are formed in a large number of plants as products of secondary metabolism (Sanon and *al*., 2002)

Any substance which, when present in low concentrations compared to that of the oxydable substrate, delays or prevents a significant oxidation of this substrate is called antioxidant. The organism is able, under certain measures to limit the damage caused by free radicals, through defense mechanisms developed during evolution.

By the range of research areas currently involved, we were interested in this work the following objectives:

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 Quantitative analysis of essential oils of leaves and galls of *Pistacia atlantica*

 Quantitative analysis of the content of total phenols, flavonoids, tannins and alcaloïdes of methanol extracts of the leaves of *Pistacia atlantica*.

 Evaluation of the antioxidant activity of different extracts of leaves from *Pistacia atlantica* and total alkaloids using the DPPH test.

MATERIALS AND METHODS

Plant material:

The adult leaves pistachio Atlas subject of extraction and / or assay of secondary metabolites and antioxidant activity were collected in December 2012 in the sites of the wilaya of Mascara (Bouhnifia and Aïn Fekan). The galls induced on the Atlas pistachio were harvested in a stand in the Benbadis town in July 2012.

Once in the laboratory, the plant material was dried in the shade and darkness.

Dosage and / or extraction of secondary metabolites:

Extraction of essential oils from the leaves and galls:

The essential oils were extracted by steam distillation. Given that the yield of essential oil was low, two cycles for three-hour each were conducted for each sample. The recovered oil is stored at 4°C for subsequent analysis.

We define the efficiency of the essential oils (R_{HE}) as the ratio between the mass of the oil in grams (M_{HE}) obtained after distillation and the mass of the plant material in grams (M_{MV}). It is expressed in percentage (%) and given by the following formula:

$$\mathbf{R_{HE}\ (\%) = (M_{HE} / M_{MV})*100}$$

Methanolic Extract:

Before polyphenols dosage, hydro-alcoholic extracts of different samples to be analyzed are prepared as follows:

An amount of 0.2 g of the leaves is crushed in cold (4°C) in a mortar with 10 ml of methanol 80%. The result undergoes an agitation with vortex. The mixture was centrifuged by a centrifuge (type SIGMA ROTOR 12024 max 10 000 rpm) at a speed of 4000 rev / min for a period of 10 min, the first supernatant (Su1) is thus recovered. This operation is repeated 2 times to deplete the content of soluble phenolic compounds of the sample. The three supernatant (Su1, Su2 and Su3) are grouped together and formed a hydro-alcoholic extract where stored at -20°C for further analysis**.**

Determination of total phenols :

The assay of total polyphenols was performed with the Folin Ciocalteu colorimetric reagent (Wang and *al.*, 2006 ; Li and *al.*, 2007)

The concentration of total polyphenols is calculated from the calibration range of the regression equation developed with gallic acid and is expressed as milligrams of gallic acid equivalents per milligram of dry matter (mgEAG / gDM).

Dosage flavonoids :

The dosage of flavonoids is performed by the method of aluminum trichloride ($AlCl_3$) (Bahorun and *al.*, 1996)

The results are expressed as milligrams of catechin equivalents per milligram of dry weight (mgCE / mgDM).

Determination of Tannins:

Hydrolysable Tannins:

The method of Mole and Waterman (1987) is based on a reaction with iron trichloride, the mixture of tannic extract more ferric trichloride reagent ($FeCl_3$) causes a purple red color of which the formation of complex ions (Fe^{3+}).

The hydrolysable tannins are expressed by the following formula:

$$\mathbf{Th\ (\%)} = (D.O \times M \times V) / E\ mole \times P$$

Th: hydrolysable tannins; **D.O**: optical density

E mole: 2169 gallic acid (constant expressed in moles)

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M: constant mass =300; **V**: volume of the extract used
P: sample weight

Condensed Tannins (vanillin test with H_2SO_4):

The determination of the content of condensed tannins is achieved by the method proposed by Swain and Hillis (1959). It is based on the condensation of the polyphenolic compound with vanillin under acidic conditions. (Price and *al*., 1978)

Condensed tannins are expressed by the following formula:

$$\mathbf{Tc\ (\%)} = (5{,}2 \times 10^{-2} \times D.O \times V) / P$$

Tc: condensed tannins, **OD**: optical density
5,2 x 10^{-2}: constant expressed in equivalent cyanidins
V: volume of the extract used, **P**: sample weight.

Extraction of alkaloid:

Alkaloids are obtained by liquid-liquid extraction (Harbone, 1998), 10 g of crushed leaves are extracted with 150 ml of ethanol by Soxhlet for 5 hours. The extract is evaporated with Rota-vapor (type HEIDOLPH LABORATA 4000) at 40°C, 20 ml of chloroform is added to the dry residue. The mixture was acidified to pH3 with HCl (5%). After incubation for 30 min at room temperature, the aqueous phase was recovered, 20 ml of chloroform are added. This mixture was basified to pH9 with $NaHCO_3$ (5%). After a rest period of 15 min at room temperature, the chloroform phase is recovered and separated by Rota-vapor, the dry residue is alkaloids.

Antioxidant activity:

DPPH Test:

DPPH radical is a stable organic free radical with a maximum absorption band between 515-528 nm. It is one of the most commonly used substrates for quick and direct evaluation of the antioxidant activity due to its radical form stability and simplicity of the analysis (Bozin and *al.*, 2008). In this test, the antioxidants reduced and decolorized radical DPPH • (2.2 diphenyl picrylhydrazyl 1) purple in 2.2 1 diphenyl picryl hydrazine yellow. The results can be expressed as a percentage of the radical-scavenging activity, as a percentage of remaining DPPH • and also using the IC50 parameter, which is defined as the substrate concentration that causes a loss of 50% of the activity of DPPH (Markowicz Bastos and *al.*, 2007).

Preparation of the methanol extract:

Maceration of 1g crushed leaves in 20 ml methanol 96.6° is performed at room temperature for 24H, followed by filtration (vacuum) through Whatman paper N° 0.45µm, the solution obtained is separated by a Rota-vapor at 60°C. The residue is dissolved in 3 ml of methanol and kept at 40°C.

Sample Preparation:

50 µl of various concentrations (1 to 1/16) of methanolic extracts are added to 1950 µl of the DPPH solution (0,025g/L (DPPH/methanol)), after 30 min of incubation at room temperature, and in the dark, absorbance is read at UV spectrophotometer at a wavelength of 515 nm.

The ascorbic acid known for its antioxidant properties was used as control.

The activity of the free radical DPPH (%) is calculated by the following formula:

$$\mathbf{(\%) = (A_{blanc} - A_{échantillon} / A_{blanc}) * 100}$$

The results (IC 50) are expressed in milligrams per millimeter of different concentration (from 1/16 to 1 mg / ml), and this is achieved by our samples.

II. Results:

The yield of essential oil from the leaves of *Pistacia atlantica* shows that it is more important in females feet compared to male's feet (Figure 01). Comparing the performance of the two sites, it appears that the leaves of the AF website have a slightly higher yield.

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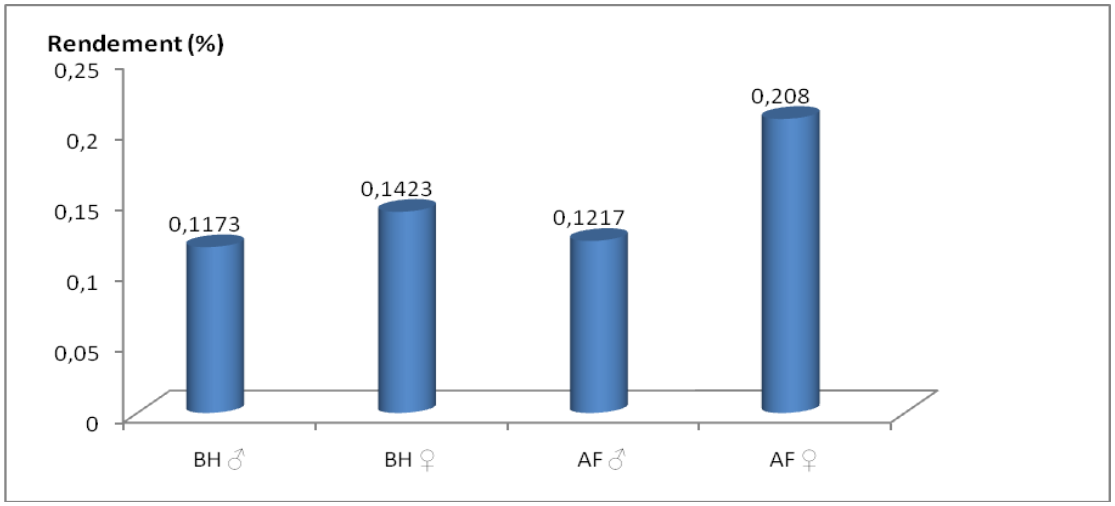

Fig. 1: Essential oils yield (R) of leaves of *Pistacia atlantica* (%)

The yield of essential oils of galls *Forda marginata* of *Pistacia atlantica* is important for the male feet (1.37%) than the female feet (0.83). For galls *Pemphigus utricularius*, yield essential oils is important for the male feet (1.75%) than the female feet (0.83%) (Figure 02).

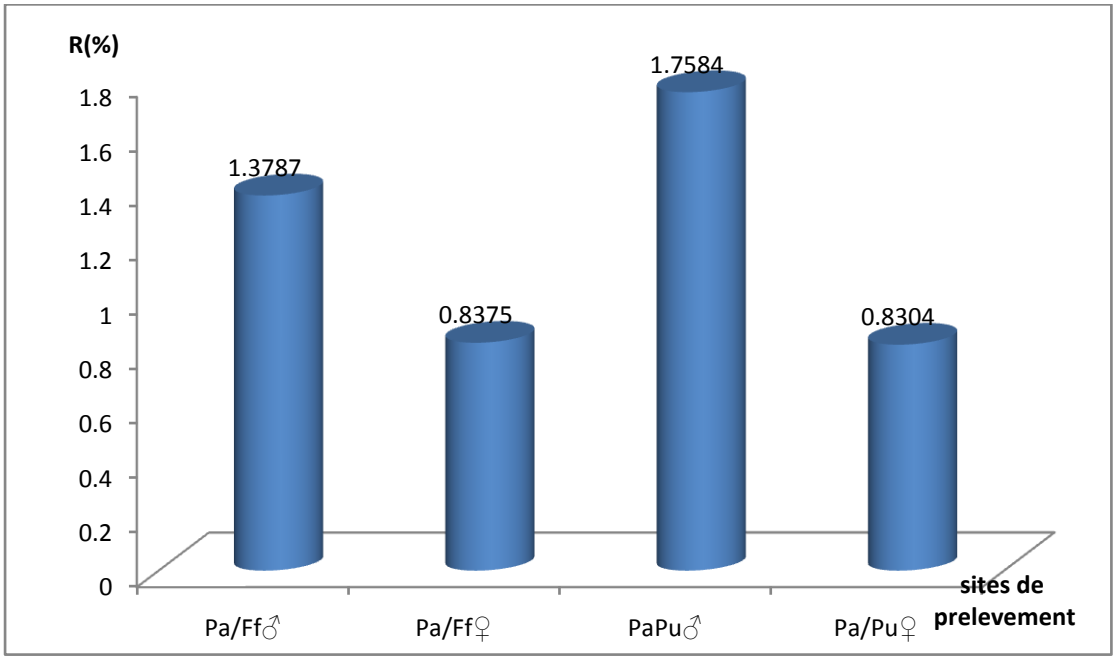

Fig. 2: Essential oils yield (R) of galls of *Pistacia atlantica* (%)

For the determination of total phenols, gallic acid is considered as a positive control for performing the calibration curve where the total phenols content was calculated and expressed as mg gallic acid equivalent per gram of dry matter (mg EAG / g DM). According to the results of the assay of total phenols, the concentration of 216.04 ± 0.00 mg EAG / g DM was recorded identically for all samples analyzed (Figure 03).

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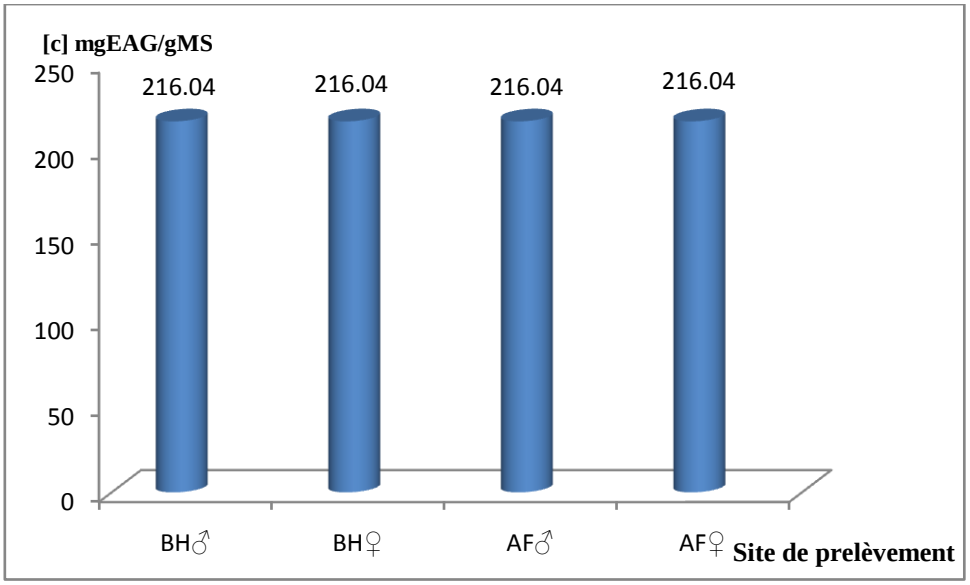

Fig. 3: Average levels of total phenols (mAG / gMS)

For the determination of flavonoids, the catechin is considered as a positive control for performing the calibration curve which the flavonoid content was determined and expressed in mg equivalent of catechins per gram of dry matter (mgEC / gDM).

The concentrations of flavonoids is greater in leaves of female feet, registering 117.89 ± 0.01 mgCE / gDM for BH site, and 115.75 ± 0.07 mg CE / g DM for the AF site. In the leaves of male plants, we recorded the contents of 113.27 ± 0.04 mg CE / g DM and 102.75 ± 0.01 mg CE / g, respectively, for AF and BH sites (Figure 04).

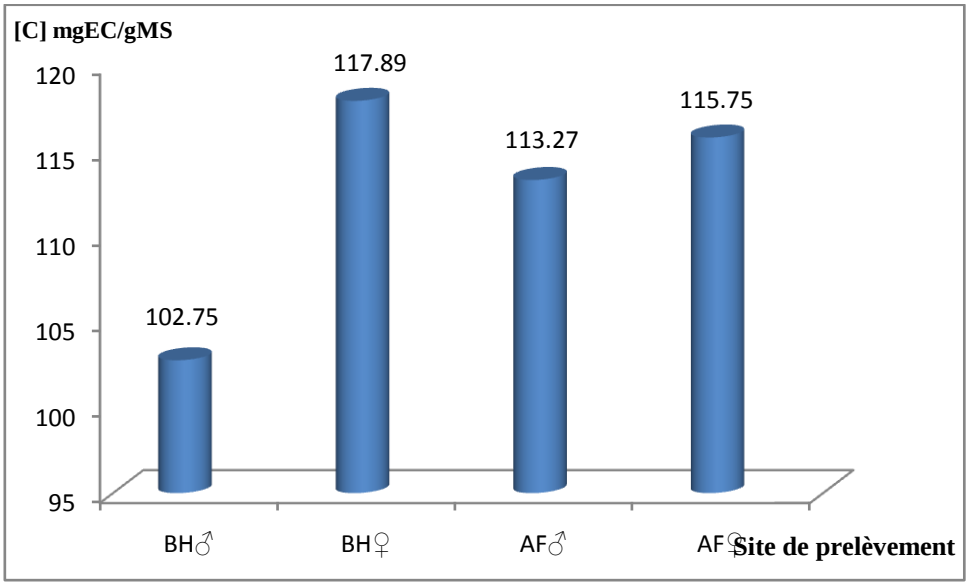

Fig. 4: Average levels of flavonoids (mg EC / g DM)

As shown in Figure 05, the content of tannins is higher in mals feet for two sites BH and AF respectively (1.5909 ± 0.04% ; 1.4166± 0.06%) than female feet (1.3142 ± 0.01% ; 1.4006± 0.17%).

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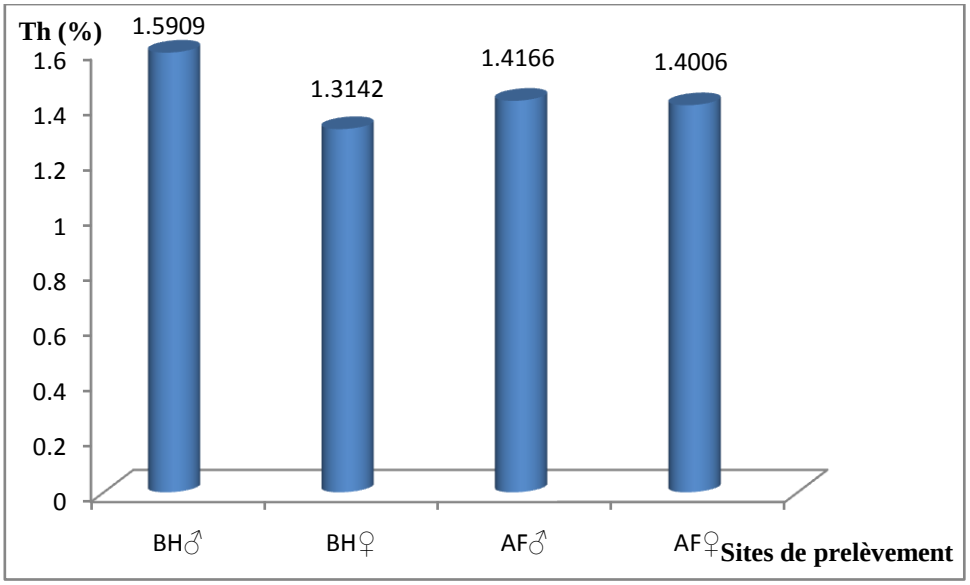

Fig. 5: Content of hydrolysable tannins in leaves of *Pistacia atlantica* (Th%)

The observed concentrations of condensed tannins of different samples ranged from 0.45± 0.13% to 0.63 ± 0.23%. The levels were slightly higher in the leaves of male's feet than among female's feet (Figure 06).

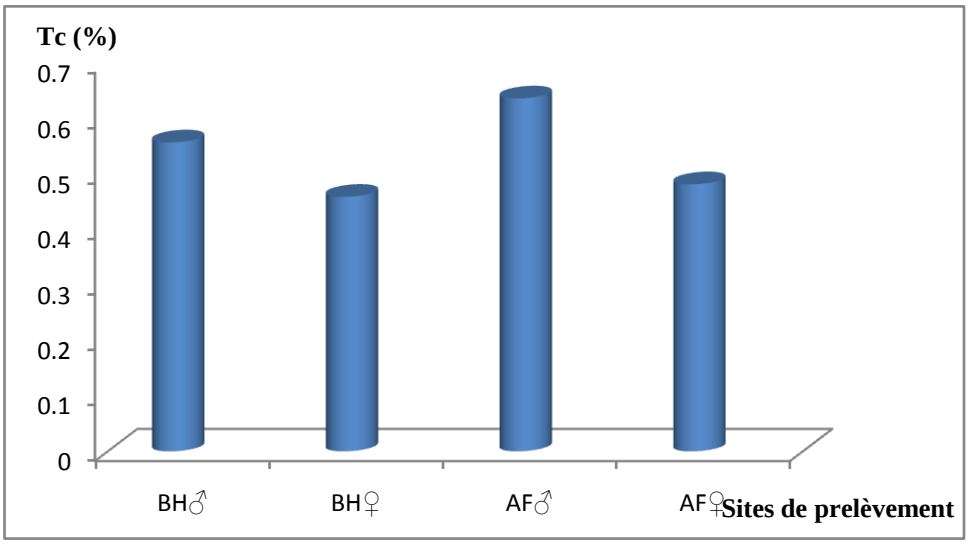

Fig. 6: content of condensed tannin in leaves of *Pistacia atlantica* (Tc%)

From the results, the yields of alkaloids extracted from the leaves of the female feet are quite significant (1.2 ± 0.19% for the BH site and 1.3 ± 0.01% for AF site) than those of the feet males (0.4 ± 0.09% for the BH site and 0.6 ± 0.10% for AF site) (Figure 07).

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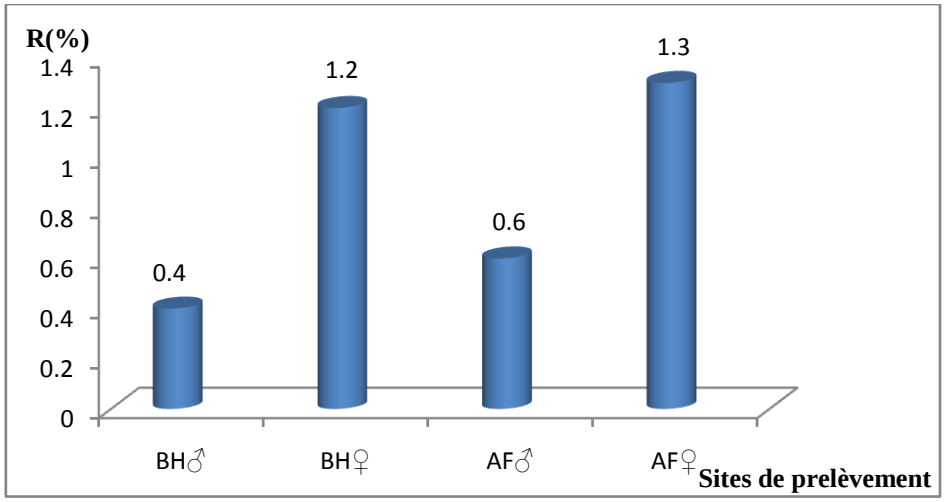

Fig. 7: Yields of total alkaloids (R%)

The antioxidant activity of different extracts of *Pistacia atlantica* compared with the DPPH radical was assessed by spectrophotometry following the reduction in this group which is accompanied by its transition from purple to yellow to 515 nm. The antioxidant capacity of different extracts was determined from the IC 50 is the concentration required to reduce 50% of DPPH radical.

The methanol extracts have a lower antioxidant activity in BH sites (0.68 mg / ml) and AF (0.82 mg / ml) compared ascorbic acid (0.013 mg / ml) against they are pretty important the total alkaloids AF♀, BH♀, AF♂ and BH♂ which are respectively 1.03; 1.04; 1.21 and 2.06 mg / ml.

The IC50 values found for all the extracts tested are shown in Figure 08.

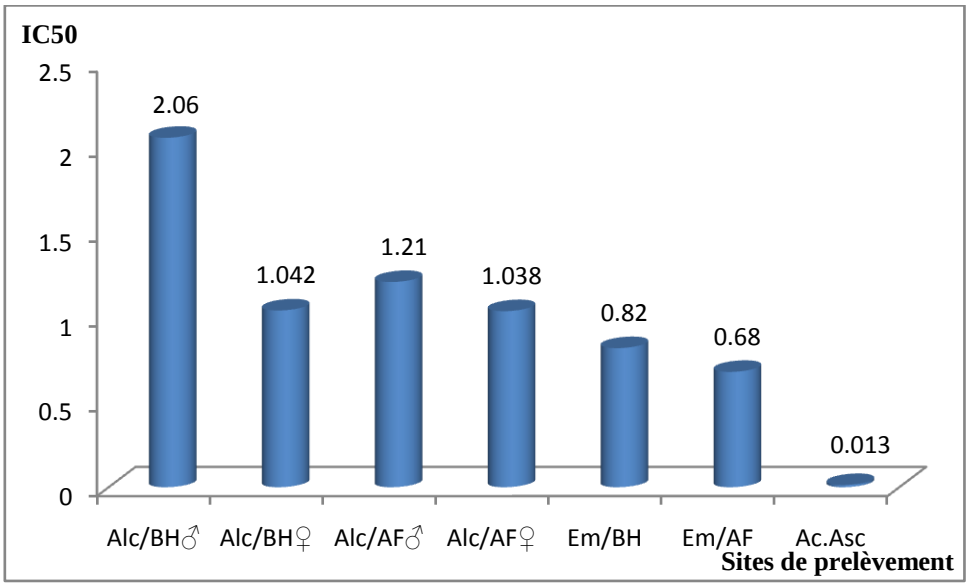

Fig. 8: IC50 methanolic extracts of total alkaloids and ascorbic acid.

Discussion:

To essential oils of the leaves, the yield is of the order of 0.1423% and 0.208% in the leaves of male feet and 0.1217% to 0.1773% in the leaves of the female feet. This yield is similar to those denoted by (Gourine and *al.*, 2009) (0.12%) and (Mechrara-Idjeri and *al.*, 2008) (0.08 to 0.12%). The *Mentha pulegium* offers a higher yield (1.54%) (Stoyanova and *al.*, 2005). At *Thymus vulgaris*, Porte and Godoy (2008) obtained 1.1%.

The difference in yield essential oils from the leaves of *Pistacia atlantica* can be explained according to the stage of maturity of the leaves, the interaction with the environment (soil type, climate), the period of harvesting and extraction method.

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The yield of essential oils of galls *Forda formicaria* and *Pemphigus utricularius* is more important for the male feet for the female feet. These yields are higher than that obtained by (Mechrara-Idjeri and *al.*, 2005) on *Pistacia atlantica* galls of Ain Ouassara.

Studies of *Pistacia atlantica* show that galls caused mutations of genetic information in the leaves that directly affect the metabolism of the plant (Fabre, 1993), This explains the difference in performance of essential oils between galls.

For the determination of total phenols, the concentration of 216.04 ± 0.00 mgEAG / gDM obtained is the same for all our samples (BH, AF). By comparing the assay results with those of the literature, we found that the concentration of total phenols methanol extracts of the leaves of *Pistacia atlantica* is much higher than that in *Crataegus pinnatifida* is 8.17 mgEAG / mgDM (Yoo and *al.*, 2009), at *Rosmarinus officinalis* 162 mgEAG / mgDM (Erkan and *al.*, 2008), at cumin (*Cuminum cyminum*) 75 mgEAG / gDM (Ho and *al.*, 2008), in *Myrtus communis* var.italica 33.67 mgEAG/gDM (Wannes and *al.*,2010), in the Portuguese Myrtle 31.2 mgEAG/gDM (Amensour and *al.*, 2009), in *Malva sylvestris* L. 28 ± 0.35 mgEAC/gDM (Conforti and *al.*, 2008), at verbena Sage (7, 2 ± 0.04 mgEAG/gDM) (Yousfi and *al.*, 2006). However, the content of total phenols leaves of Greece Myrtle is significantly higher compared to our plant; it is of the order of 373 mgEAG/gDM (Chryssavgi and *al.*, 2008).

We can say that the extracts of the leaves of *Pistacia atlantica* are a promising source of phenolic compounds.

Flavonoids are the most important class polyphenolic, with more than 5 000 compounds already described (Gomez-Caravaca and *al.*, 2006). The flavonoids concentration of leaves of *Pistacia atlantica* (102.75± 0.017 to 117.89 ± 0.014 mgCE/gDM) is greater than that found by (Ho and al, 2008) 20.1 mgCE/gDM of the extract of Rosemary (*Rosmarinus officinalis*). The work of (Wannes and *al.*, 2010) showed that the content of flavonoids in the stems and leaves of *Myrtus communis* var. italica is around 1.99 and 1.22 mgCE/gDM, respectively.

The concentration of flavonoids of cumin methanol extract is greater (243.1 mgCE/gMS) (Ho and *al.*, 2008).

The content of tannin (hydrolysable, condensed) is higher in male's feet for two sites BH and AF. The determination of the tannin from the leaves of *Pistacia atlantica* represents values varying between 0.45% and 1.59%. It is small compared to that found in (Wannes and *al.*, 2010) for *Myrtus* var. italica (26.55% in the leaves, 11.95% in the flowers and 3.33% in stems). The presence of tannins give the plant astringent, antiseptic, antioxidant and anti-diarrheal proprieties, the tannins cause a kind of tanning on the skin and a vasoconstrictor action on small blood vessels (Moyse and Paris, 1965).

The Alkaloid yield represent a rate between 0.4± 0.09 % and 0.6 ± 0.10% in the leaves of our male samples who is low compared to the female samples representing attractive yields of 1.2 ± 0.19 and 1.3%± 0.01%. The presence of alkaloids after extraction in leaves is visible in all sites. The leaves and stems of *Atriplex halimus* offer a yield about 0.2% (Benhammou and *al.*, 2009).

For the performance of female feet are always more important than the performance of the listed species.

If the plant is young and has not yet completed the development of important stages of secondary metabolism, the synthesis of these offers different yields as much as other external environmental conditions (pollution) affect the presence of alkaloids in the leaves. The presence of these alkaloids could involve interesting biological activities, including anti-proliferative and analgesic (Kaboré and *al.*, 1995). It should be noted the method used to extract the alkaloids are not pure products that are obtained but rather total alkaloids.

The distribution of secondary metabolites can sing during the development of the plant. This may be related to severe weather conditions (high temperature, sun exposure, drought, salinity), which stimulate the biosynthesis of secondary metabolites such as polyphenols (Falleh and *al.*, 2008). Indeed, the phenolic content of a plant depends on a number of intrinsic factors (genetic) and extrinsic factors (geographical and climatic conditions, cultural practices, maturity at harvest and storage conditions) (Falleh and *al.*, 2008; Podsedek, 2007). For a given plant species, different organs may have very different phenolic equipment (Macheix and *al.*, 2005). A Recent studies have shown that the degree of maturation of the plant and the storage period have a strong influence on the content of polyphenols (Aganga and Mosase, 2001; Pedneault and *al.*, 2001).

The test of antioxidant activity of the extracts according to the method of trapping free radical DPPH showed that both methanol extracts have antioxidant activity which nevertheless remains significantly lower (0.82 and 0.68 mg / ml) than that of the ascorbic acid (0.013 mg/ml). It is, against, more interesting than that of the total alkaloids.

On the one hand, results of our methanolic extracts are more interesting to those found by (Benhammou and *al.*, 2009) in the methanolic extract of the leaves of *Atriplex halimus* (4.55 ± 0.79 mg/ml) and also on the extract methanol of *Satureja calamintha* ssp. nepeta L. (2.075 ± 0.208 mg / ml) (Bougandoura and Bendimerad, 2012).

Concerning the activity of alkaloids, it is more interesting (between 1.038 and 2.06 mg / ml) than that recorded on the alkaloids from the leaves of *Atriplex halimus* (6.71 ± 0.44 mg / ml) (Benhammou and *al.*, 2009). The essential oils from the leaves of Pistacia atlantica have low antioxidant activity (20,61 to 27.48 mg / ml). This can allow us to say that activity should be sought more in phenolic compounds.

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The responsible substances for this activity are polyphenolic compounds (tannins, flavonoids, carotenoids...), Good sensors peroxyl and hydroxyl radicals that act by inhibiting the chains of lipid peroxidation. The presence of flavonoids and tannins in the leaves can justify this activity. (Monique-Gardès and *al.*, 2003)

The extracts are mixtures of several compounds, with different functional groups, polarity and chemical behavior. This chemical complexity extracts could lead to scattered results according to used assay. Therefore, an approach with multiple analyzes to evaluate the antioxidant potential extracts would be more informative and even necessary. (Ozturk and *al.*, 2007)

Conclusion:

This study is proposed to carry out the extraction and / or assay of secondary metabolites from the leaves of Pistacia atlantica, has enabled us to understand that the field of plants is still a field of scientific research approvable.

The yield of essential oil from the leaves of *Pistacia atlanticase* shows low (0.11% to 0.20%), especially by comparing it to that of aromatic plants. However, the yield of oil galls is more important (from 0.83% to 1.75%).

Quantitative determination of total phenols by Folin-Ciocalteu revealed that the leaves of *P.atlantica* are very rich in total phenols (216.04) and constitute a promising source of phenolic compounds.

The quantitative determination of flavonoids using the method trichloride aluminiuma revealed that the leaves of our species show rich in flavonoids (from 102.75 to 117.89). This class dominates the hydrolysable tannins (from 1.31 to 1.59), the condensed tannins (0.45 to 0.63) and alkaloids (0.4 to 1.3).

The results of DPPH test show that the antioxidant activity recorded in methanolic extracts (0.68 to 0.82) is higher than that of the total alkaloids (1.03 to 2.06). However, the activity recorded for ascorbic acid is remarkably very important compared to our extracts.

At the end of this contribution, we think it would be interesting to conduct further detailed phytochemical studies such as the chemical composition of the essential oils and characterization of major chemical groups (nitrogen compounds, phenolic compounds). This may allow identifying compounds having a pharmacological interest. Here we propose to push testing biological activities to a higher valuation.

This same interest could be verified in other plant organs (root, flower, bud) hence the usefulness of other complementary contributions.

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