

Full Length Research Paper

Investigation of *Argania spinosa* L. (Skeels) polyphenols growing in arid and semi-arid conditions

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***Argania spinosa* L. Skeels, belonging to the *Argania* genus of the Sapotaceae family, is a species native to Morocco and Algeria. Due to its perfect adaptation to soil and climate, this tree plays an important ecological role in a constantly threatened encroached desert region. To understand the biological role of polyphenols in making the argan tree adapts to its natural habitat, we conducted a comparative study in two of the tree development stations: Tindouf located in South-western Algeria and Stidia located in Northwest Algeria. High performance liquid chromatography (HPLC) and Gas chromatography-mass spectrometry (GC-MS) used for the analysis of flavonoids led to the identification of six flavonols (two types of myricetin, rutin, hyperoside, quercetin, kaempferol) in the leaves of Tindouf argan tree, and two molecules (myricetin and quercetin) in the leaves of Stidia argan tree. Other molecules presented are few. The determination of flavonoids by spectrophotometry revealed the richness of Tindouf argan in these compounds (20%) compared to that of Stidia argan tree (8.7%). Histolocalisation of the flavonoids, tannins and anthraquinone in the leaves and stems of the tree was done using fluorescence microscope to understand the role of these molecules in the protection of this tree in its environment.**

Key words: *Argania spinosa* L. (Skeels), histolocalisation, polyphenols, high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS).

INTRODUCTION

Argania Spinosa L. (Sapotaceae) or commonly known as 'argan' is an endemic tree of Morocco and it grows also in

the Southwestern Algeria, which is one of the most continental positions (Baumer and Zerraia, 1999). A.

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spinosa (L.) Skeels is the only species of the tropical family Sapotaceae growing in Nord Africa. Its oil extracted from the seeds is highly exploited by Morocco population. In addition to oil, the argan tree is traditionally used for other services such as wood for fuel (Charrouf and Guillaume, 1999). Also, the nutritional properties and culinary value of argan oil recently emerged from obscurity, and it has become the most expensive oil in the world (Lybbert et al., 2011). Today, argan oil of Morocco is employed in numerous cosmetic patents in fields in USA and Europe (Lybbert, 2007).

In Algeria, during the last two decades, the argan tree has been the subject of intensive investigation. We indicate the evaluation of the area of the argan tree in its natural habitat in Southwest Algeria estimated to 90.644 ha (Kechairi, 2009); the study of the seed structure and lipid composition (Errouane et al., 2014), the endocarp structure of the fruit to better understand its influence on germination capacity (Sebaa and Harche, 2014) and its role in the bio-sorption value of heavy metals (Hachem et al.; 2011 and Hachem et al., 2016). The argan tree was introduced in the Algerian coast (Stidia) (Baumer and Zerraia, 1999). This tree involves a combination of morphological, physiological and metabolic mechanisms that reflect different types of adaptations. The purpose of this paper is to evaluate the polyphenols role in Argan relations in both development sites (Tindouf and Stidia). Stidia is located on the west coast of Northern Algeria. Tindouf is in the westernmost of southern Algeria. Indeed, these metabolites are widely distributed in plants either as soluble or cell wall bound compound as a result of interaction of a plant with its environment (Matern et al., 1995, Khallouki et al., 2005).

MATERIALS AND METHODS:

The healthy plant materials (leaves and stems) used for this investigation were collected in June 2011, from well-developed 30 trees in Tindouf location and 6 trees in Stidia location. Tindouf is located in South western Algeria at 27° 40N 8° 09W, 433 m altitude; it has an arid climate, its maximum temperature is 48°C from July to August, and its annual precipitation does not exceed 100 mm. Stidia site is located on the west coast of northern Algeria at 104 m altitude. It is characterized by a semi-arid climate, its maximum temperature is 25°C from July to August, and its annual precipitation is 524 mm. In this location, the number of argan trees is only 6.

Determination of the relative water content:

The relative water content is determined according to Hachem et al. (2011).

Qualitative analysis of flavonoids

Plant materials were dried and milled into uniform powders using a knife crusher of the type RETCH; they were placed in a mesh filter and then stored carefully until use. 100 mg plant materials were extracted with 10 ml Me OH 80%. After agitation (15 mn), the

solvent is degassed in an ultrasonic bath (15mn) and then filtered (Millipore filter: 0.5 µm porosity). 30 µL of extract is injected for HPLC analysis.

Flavonoids are identified using their physicochemical properties: the comparison of their UV spectra with the spectra of known sample.

Standards

Methanolic solutions (200 µg mL⁻¹) of the following pure commercial substances were employed: Rutin, quercetin, myricetin, and kaempferol from Sigma. The purity of standard samples was assessed by HPLC (contents were greater than 98%). HPLC grade water was prepared by Milli-Q. All solvents were filtered through 0.5 µm (Millipore) membranes. Methanolic extracts were passed through C⁻¹⁸ Cartridge before use (Dohou et al., 2003). Liquid chromatography LC-MS mass spectrometry was used to confirm the analyses by HPLC

HPLC condition

HPLC separation was performed with an Ultimate 3000 Dionex system. The instrument was equipped with a RHEODYNE (100 µL) injector and a PAD UV/Vis detector. A NovaPak water filled with the same material was used. Two solvent mixtures were employed for elution A=water 0.1% formic acid and B=methanol. The solvent gradient consisted of linear increase in solvent B as follows: Initial conditions, 10%; 40 min, 60% and 43 min, 90%.

Separation was achieved at ambient temperature with a flow rate of 0.8 mL/min.

LC-MS condition

LC-MS analyses were performed with an Acquity UPLC coupled to a Xevo-G2Q-ToF (waters) mass spectrometer. Chromatographic separations were performed on a 100 mm × 2.1 mm × 1.7 µm Acquity UPLC BEH C 18 column (Water) maintained at 35°C. The binary solvent system included A=water +0.1% HCOOH and B=acetonitrile. The solvent gradient consisted of linear increase in solvent B as follows: Initial conditions, 10%; 12 min, 100%. Flow rate was 300 µL/min.

Phenolic compounds were detected using negative mode with the following settings: Capillary voltage, 2 KV; cone voltage, 30 V; source temperature, 130°C; desolvation temperature, 450°C. Nitrogen was used as both cone gas (20 L/h) and desolvation gas (750 L/h). Argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution. To ensure accuracy and reproducibility, analyses were acquired using the leucine-enkephalin in real-time as the lock mass at a concentration of 500 pg/µL and flow rate of 2 µL/min.

The samples for the assays and the identification of flavonoids were collected in June 2011. They were dried in an oven at a temperature of 45°C. and then powdered and stored from light until use.

Determination of total flavonoids

2 mL of alcoholic was mixed with 100 µL of Neu. Absorbance at 404 nm was determined and compared to quercetin standard (0.05 mg/mL) treated with the same amount of reagent. The percentage of total flavonoids is calculated by quercetin equivalent using the following formula (Lebreton et al., 1967):

$$F\% = (0.05 \times A_{\text{ext}} / A_{\text{q}}) \times 100 / C_{\text{ext}}$$

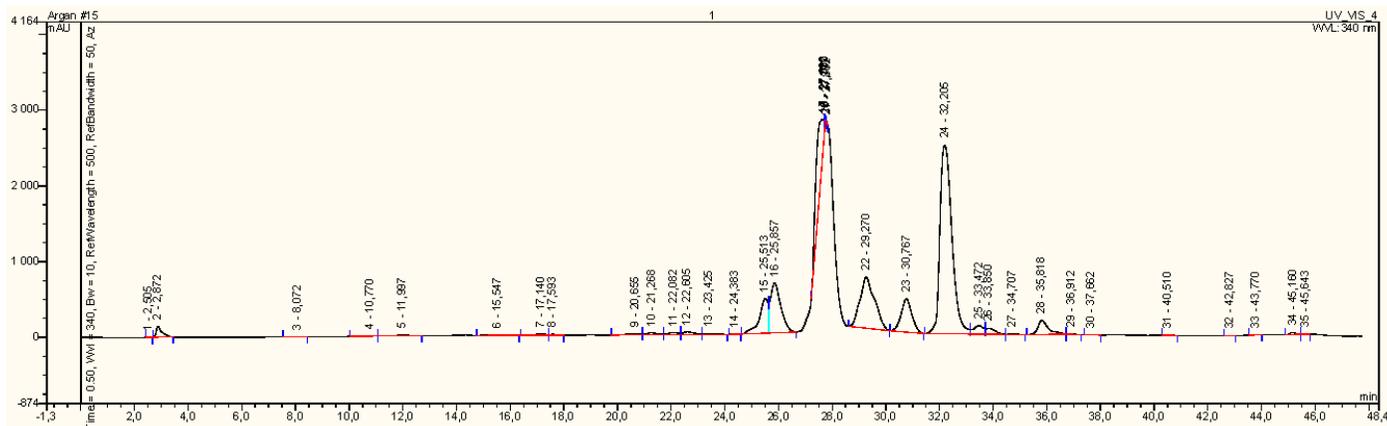


Figure 1. HPLC analysis of flavonoids of Tindouf argania leaves. The numbers corresponding to the different flavonoid (Stable 1).

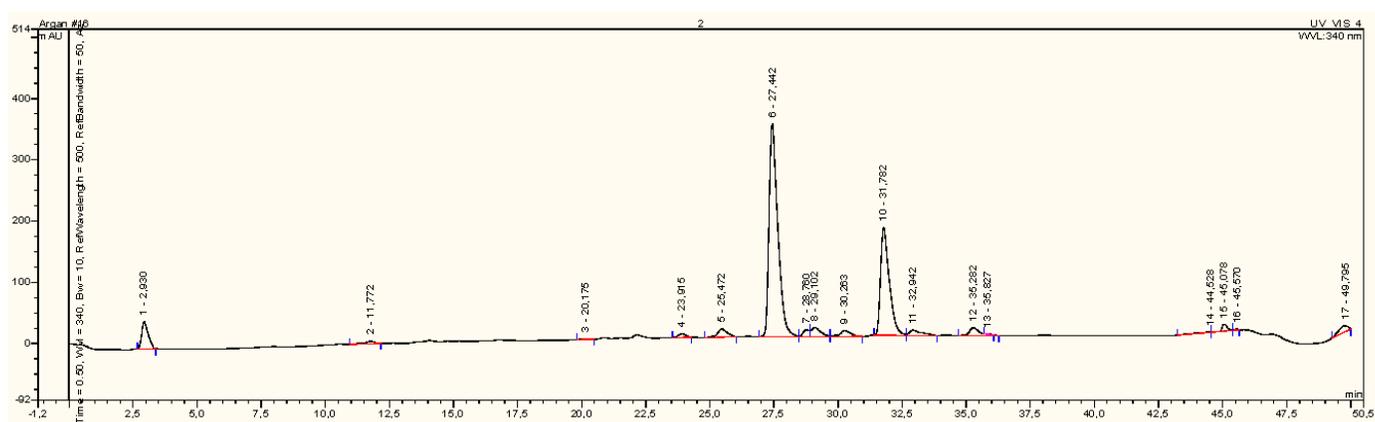


Figure 2. HPLC analysis of flavonoids of Stidia argania leaves.

A ext: Absorption of the extract; Aq: Absorption of the quercitin.

Histolocalisation of phenolic compounds studied on the leaves and stems section

Flavonoids

The leaf and stem sections are immersed in Neu reagent (1956) for 2 min and then placed between slide and cover slip. They were observed using a fluorescence microscope. The flavonoids had dull yellow fluorescent colour .

Tannins

Tannins are detected by vanillin sulfuric acid reagent: The sections were placed for 10 min in this reagent. The tannins are colored red.

Anthraquinones

The sections were placed for 10 min in a solution of 1% KOH and mounted between slide and cover slip. They were observed under light microscope. Anthraquinones color is orange.

RESULTS AND DISCUSSION

The water content in the leaves of Tindouf argan tree is greater than that in the leaves of Stidia (85% against 55% respectively). The relative water content of leaves of the argan tree is higher than the leaves of the argan tree growing in Tindouf, where aridity is strong. The water retention is probably due to the strategy used by the stomata to curb evapotranspiration. Indeed, similar observations were described by Epstein and Grant (1973) and El Aboudi (1990). The total flavonoids in fractions expressed as quercitin equivalents are 20% in the leaves of Tindouf argan tree; they are 8% in the leaves of Stidia. This difference observed between the two samples indicates that the phenolics content varies based on weather conditions. The Argan tree growing in Tindouf is located in extreme conditions of stress; it reacts by increasing its phenolic pool. These observations are similar to those of Domingo et al. (2003) on other species.

The observation of chromatographic profiles of the

Table 1. Qualitative analysis of flavonoids of the argan tree leaves.

Pic No. UV	Compounds	TR (min)	The absorption in the spectrum
1	Myricetine a	25.85	209, 258, 353
2	Myricetine b	27.80	209, 261, 351
3	Rutine	29.27	205, 256, 355
4	Hyperoside	30.76	206, 256, 353
5	Quercetine	32.20	207, 258, 350
6	Kaempferol	35.81	196, 266, 350

TR, Time retention.

extracts of the leaves by HPLC shows that the leaves of Tindouf argan tree have six flavonoid molecules (Myricetin a, myricetin b, rutin, hyperoside, quercetin, kaempferol) (Figure 1), while those of *Stidia* have two predominant molecules (myricetin b and quercetin) (Figure 2). Other molecules are present in trace (Table 1).

HPLC analysis confirms the existence of two flavonoids in the extracts of the leaves of Tindouf argan tree (quercetin, hyperoside) and their absence in the argan tree of *Stidia*. These biomolecules are characterized by their ability to absorb UV radiation in the wavelength range from 280 to 315nm (Reuber et al., 1996). This enables the adaptation of Tindouf argan tree to high altitude (530 m) where UV radiation is intense. The absence of these molecules in *Stidia* leaves suggests that UV radiation is less intense at altitude where *Stidia* is located. The wavelength of 310nm radiation penetrates less deeply into the leaves rich in glycosides of flavones (Ryan et al., 2002). This property of *o*-diphenols is related to their antioxidant properties besides the direct effect of their filter screen, a second level of action that traps and neutralizes the activated forms of the oxygen and free radicals formed by the action of UV-sensitive molecules (Macias et al., 2003).

In the the histolocalisation of leaf phenolics, due to their fluorescence property, their accumulation sites that are related to biotic and abiotic factors are shown. Flavonoids are present mainly in the upper epidermal seat (Figure 3). Chlorophyll, which appeared red in the presence of Neu reagent, can hide flavonoids present in the palisade parenchyma Chlorophyll.

Tannins (Figure 4) and anthraquinones (Figure 5a and b) are present in the palisade and spongy parenchyma.

In the stem, flavonoids are located in the epidermal cell and phloem. They are adsorbed in cell walls (Figure 6). The presence of these molecules in the outermost cells of the leaves and stems reduces UV penetration in the parenchyma.

A kinetic study of the radiation wavelength of 310 nm on barley leaf using optical fibers (Reuber et al., 1996) showed that the radiation penetrates less deeply into the leaves of a variety rich in flavonoids than in a poor mutant containing little flavonoids (Stapleton and Walbot, 2004).

These metabolites play the role of UV filter; protect

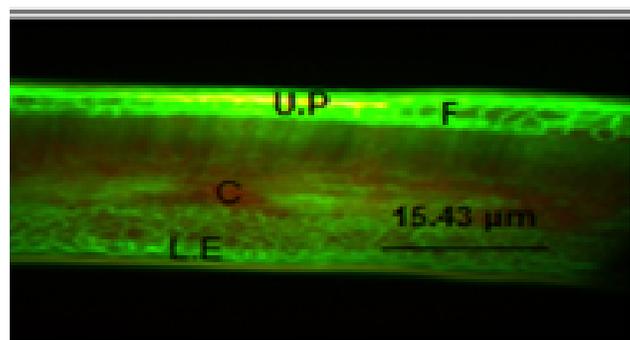


Figure 3 Location of flavonoid in leaves. C, cortex; F, fluorescence; L.E, lower epidermis; UP, upper epidermis.

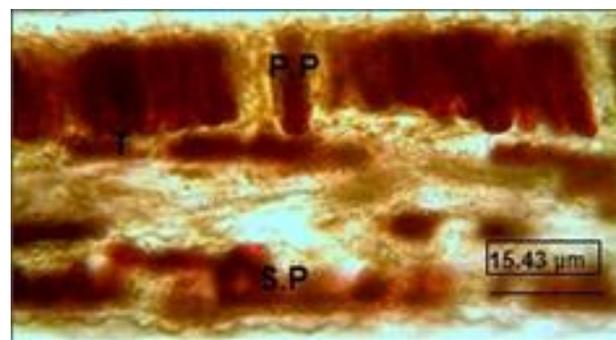


Figure 4. Location of tannins. SP, spongy parenchyma; P.P., palisade parenchyma; T, tannin.

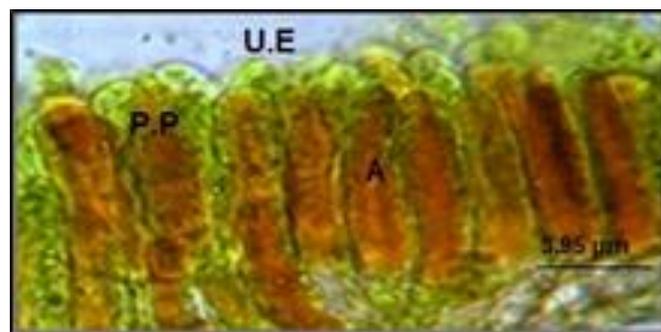


Figure 5a. Location of anthraquinones at the palisade parenchyma. U.E, upper epidermis; PP, palisade parenchyma; A, anthraquinones.

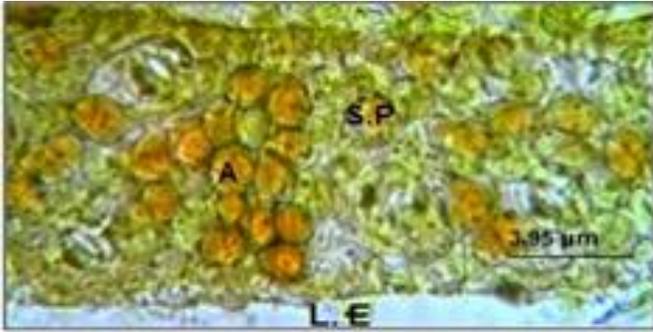


Figure 5b. Location of anthraquinones at the spongy parenchyma. A, anthraquinones; LE, lower epidermis; SP, spongy parenchyma.

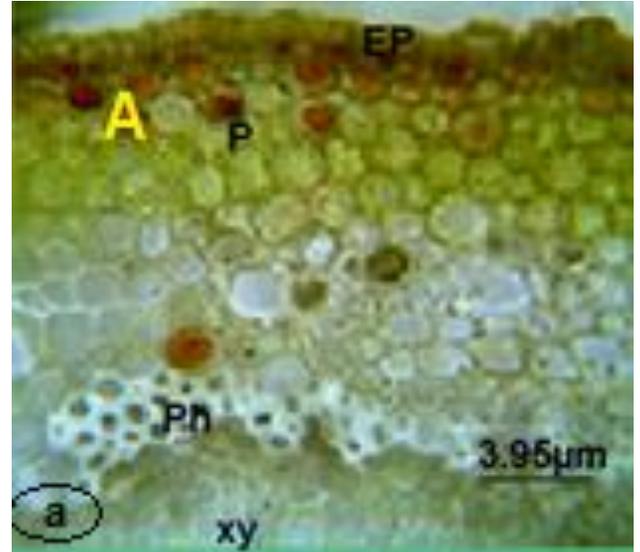


Figure 8a. Location of anthraquinones in the stem. A, anthraquinones; P, parenchyma; Ph, phloem; xy, xylem.

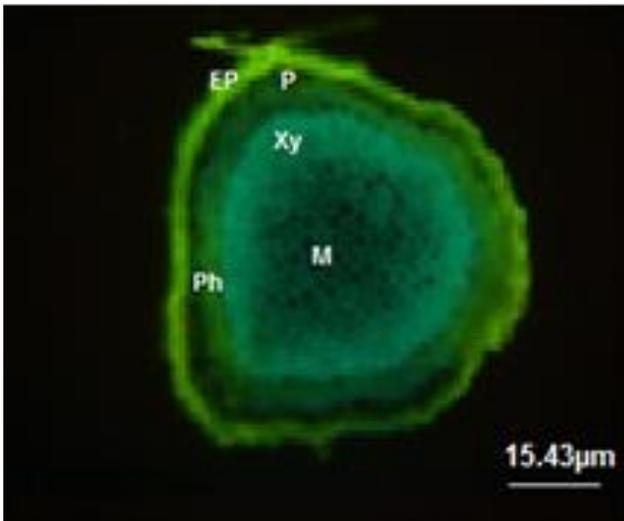


Figure 6. Location of flavonoids in stem.

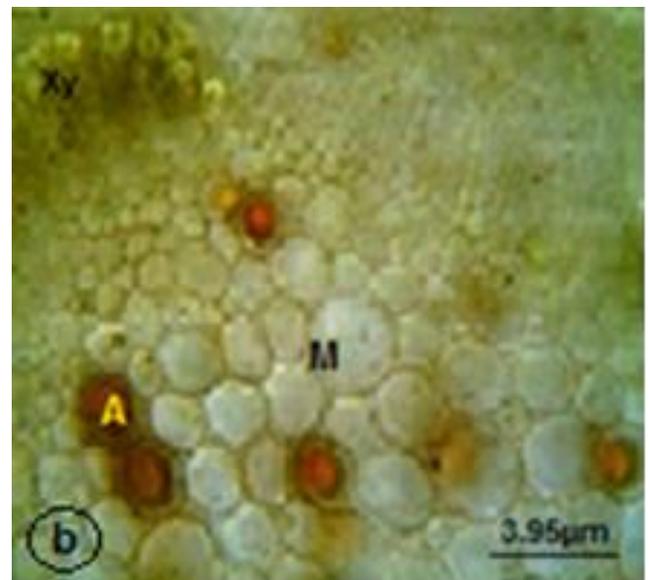


Figure 8b. Location of anthraquinones in the stem. A, anthraquinones; M, pith; Xy, xylem.

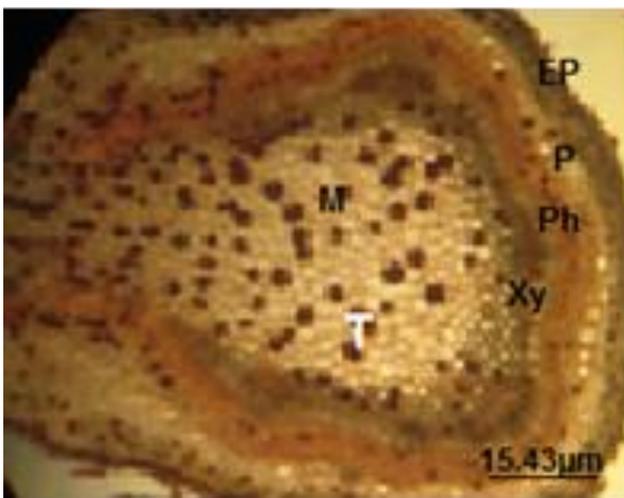


Figure 7. Location of tannins in stem. Ep, epidermis; M: pith; Ph, phloem; P, parenchyma; Xy, xylem.

some macromolecules such as DNA, RNA, photosynthetic pigments, proteins and lipid membran (Moiseev et al., 2011). Tannins are observed in the cortical parenchyma, phloem and pith (Figure 7).

The tannins are present as sufficient hydrosolubility to be accumulated in the vacuole. The presence of these molecules in various tissues shows their importance for defense: It protects the tree against all attacks from animals or microorganisms due to their capacity to bond to proteins in solution and to precipitate them.

Anthraquinones is located in epidemic cells and

cortical parenchyma (Figures 8a and b). Anthraquinones are often present in the plant under heteroside (ose and aglycone), and it is only after hydrolysis that the quinone is free (Jignasu and Mehta, 2012). Quinone is an aromatic compound that bounds to isoprenique chain and has essential biological functions, in particular the transfer of electrons.

The peripheral location of flavonoids and tannins at the level of the sheets of the Argan tree constitutes a protective screen for the most internal tissues against UV radiations and other attacks of the external environment.

Conclusion

This preliminary study on the explanation of the role of secondary metabolites (flavonoids, tannins and anthraquinones) in making the argan tree adapts to its environment shows that the tree has a strategy to adapt to drought. This is due to its ability to retain water and synthesize secondary metabolites. Quercetin and myrecitine b are present in Tindouf argan leaves and *Stidia* argan leaves but myrecitine a, rutine, hyperoside are present only in Tindouf argan leaves. This difference in the composition of Tindouf argan tree located in arid zone and *Stidia* argan tree located in semi- arid zone indicates the adaptation of *A. spinosa* to its environment.

Conflict of Interests

The authors have not declared any conflict of interests.

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