Fatty acids, essential oil and phenolics composition of *Silybum marianum* seeds and their antioxidant activities

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Abstract: The presentstudydescribes the biochemical evaluation of *Silybum marianum* seed. The analysis of essential oil composition of *Silybum marianum* seed by Gas Chromatography-Mass Spectrometry GC-MS showed the presence of14 volatile components with the predominance of γ-cadinene (49.8%) and α-pinene (24.5%). Whereas, the analysis of fatty acids composition, showed the predominance of linoleic (50.5%) and oleic (30.2%) acids. *Silybum marainum* presented also an important polyphenol contents with 29mgGAE/g DW, a good antiradical activity (CI_{50}=39µg/ml) but a lower reducing power ability. Flavonoid and condensed tannin contents were about 3.39mg EC/g DW and 1.8mg EC/gDW, respectively. The main phenolic compounds identified by RP-HPLC, were silybin A (12.2%), silybin B (17.67%), isosilybin A (21.9%), isosilybin B (12.8%), silychristin (7.9%) andsilydianin (7.5%).

Keywords: *Silybummarianum*, essential oil, linoleic acid, antiradical activity, silybin, isosilybin,

INTRODUCTION

Silybum marianum (Asteraceae family) is one of the oldest known plantsoriginated in the Mediterranean and the North African regions. Its leaves, flowers, roots and fruits have historically been used in European vegetative diets. The seeds have been used as a coffee substitute. While, *the* fruits were traditionally employed to stimulate milk production. *Silybum marianum* has been shown to have clinical applications in the treatment of toxic hepatitis, fatty liver, cirrhosis, ischemic injury, radiation toxicity, and viral hepatitis.

The main compounds were flavonolignans (silibinin, isosilybin, silydianin and silychristin), flavonoid (Škottová *et al*., 2003)**.** Silibinin has a wide range of pharmacologic effects such as inhibition of cell proliferation, cell cycle progression, and induction of apoptosis in various cell lines including fibroblasts and breast cancer cells (Ebrahimnezhad *et al*., 2013). While,silymarin (a mixture of at least 4 closely related flavonolignans extracted from *S. marianum),* offers good protection in various toxic models of experimental liver diseases in laboratory animals. It acts by antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating mechanisms (Amiridumari *et al*., 2013). It has been also revealed that silymarin may be helpful in slowing down the progression of neurodegeneration in focal cerebral ischemia (El Sherif *et al*., 2013).

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It has been also demonstrated that silymarin treatment was associated with a reduction of insulin resistance and a significant decrease in fasting insulin levels, suggesting an improvement of the activity of endogenous and exogenous insulin (Cacciapuoti *et al*., 2013).

The objectives of our study were first of all, the determination of *Silybum marianum* seeds composition through their fatty acids, essential oil, phenolic analysisthen the study of the antioxidant activity of their methanolic extracts.

MATERIAL AND METHODS

Chemicals

Sulfuric, acetic/tricloroacetic acids, and anhydrous sodiumsulfate (Na_2SO_4) were purchased from Sigma-Aldrich (Steinheim, Germany). Diethyl ether, acetonitrile, ethanol, acetone, and methanol were purchased from Merck (Darmstadt, Germany). However, butylatedhydroxytoluene (BHT), β-carotene, linoleic acid, ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), iron(II) chloride ($FeCl_2$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and polyvinyl polypyrolidonethe, were purchased from Sigma. The main supplier of Folin-Ciocalteu reagent, aluminum chloride, sodium nitrite, and sodium carbonate was Aldrich. Sigma and Fluka are the main suppliers of authentic standards of phenolic compounds.

Plant material

Fully ripened *Silybummarianum* seeds used in this study were collected randomly in Juin 2009 and repeated in

june 2010 from Amdoun region (North Western of Tunisia, latitude: 36° 45' 55. 02'' (N), longitude: 9°5'22. 5344 (E), altitude: 40m). The annual rainfall of Amdoun region is1600 mm and the annual temperature is16°C.To avoid variability; collection always took place in the same geographic area from 20-30 plants having the same development stage (same maturity stage of seeds). The room temperature was used to dry the *Silybum marianum* seeds during two weeks. 2.5g of dry organ powder were mixed with 25ml of solvent and shaked for 30min, then kept at 4°C for 24h, filtered through a What man No. 4 filter, and evaporated to dryness under vacuum at 4°C, The final extracts were stored until analysis.

Oil extraction

The maximum seed oil was extracted by a Soxhlet apparatus (50 ml capacity; 23 100mm cartridge) using 100 ml of n-hexane during 6 hours. Furthermore, to ensure that the resultant oil carried no water, the extracted samples were passed over anhydrous sodium sulphate under vacuum in a G1 sintered glass filter, and evaporated in a rotary evaporator at 30°C. The oil was then transferred to speed vacuum tubes and dried by centrifugal evaporation. The oil yield is determined as the mass of oil extracted from 100g of dried *Silybum marianum* seeds.

Total lipid extraction

In order to inactivate phospholipases, samples of one gram of seeds were fixed during 3 min with 10ml of boiling water (Douce, 1964), then ground into chloroform-methanol-hexane (3:2:1, v/v/v) mixture in a china mortar (Marzouk and Cherif, 1981).

The organic phase containing total lipids was recovered, dried under nitrogen steam and kept at -20°C until analysis.

Fatty acid methylation and analysis

According to the method presented by (Cecchi *et al*., 1985); using sodium methylate at 3% in methanol; total fatty acids were converted into their methyl esters. The internal standard was used to quantify fatty acids Methyl heptadecanoate (C17:0). The analysis of methyl esters were made by gas chromatography using a Hewlett-Packard 6890 chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (FID), an electronic pressure control (EPC) injector, and a capillary column (HP Innowax): 30mx0.25 mm x 0.25mm with a stationary phase made of polyethylene glycol. Analyses were performed at split mode (60:1) under the following oven temperature program: isothermal at 150°C during 1 min, raised from 150 to 200°C at the rate of 150°C/min, raised from 200 to 242°C at the rate of 2°C/min, and isothermal at 225°C during 2min. The injector and detector temperatures were, respectively, held at 250 and 275°C. Carrier gas was nitrogen at a flow rate of 1.6mL/min.

Essential oil extraction

The extraction of essential oil (EO) was made by hydro distillation during 90 min using 100g of dry seeds. The distillated was exacted with diethyl ether (v/v) and dried over anhydrous and the organic phase was concentrated with a Vigreux column at 35°C. The obtained EO was finally analyzed. All experiments were done in triplicates.

Gas Chromatography-Mass Spectrometry (GC-MS)

A gas chromatograph HP 6890 (II) interfaced with a HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA) with electron impact ionization (70eV) was used to effect the analysis of *Silybum marianum* seeds compositions. The capillary column used was A HP-5MS.The column temperature was programmed to rise from 40 to 280°C at a rate of 5°C/min. The carrier gas was helium, with a flow rate of 1.2mL/min. The scan time and mass range were 1 s and m/z 50-550, respectively. The injected volume was 1µL, and the total run time was approximately 63 min.

Compound identification

The identification ofthe oil components was effected by two /methods: (a) a comparison of their retention indices relative to C8-C22 n-alkanes to those of the literature or to those of authentic compounds available in our laboratory, (b) matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data system and other published mass spectra (Adams, 2001).

Polyphenols extraction

1g of min, the extracts were then kept at 4°Cfor 24 h, filtered through a What man N°4 filter and evaporated under vacuum to dryness. Finally, the extracts we restored at 4°C until analyzed (Mau *et al*., 2001)

Total phenolic contents

Total phenolic contents were determined by Singleton's method slightly modified by Dewanto *et al*. (2002) using the Folin-Ciocalteu reagent. 0.125ml of a diluted methanolic seeds extract (0.25mg ml^{-1}) was added to 0.5 ml of deionized water and 0.125ml of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 1.25ml of 7% Na_2CO_3 solution. The solution was then adjusted with deionized water to a final volume of 3ml and mixed thoroughly. After incubation for 90 min at 23°C, the absorbance versus prepared blank was measured at 760nm. Total phenolic contents of seeds (three replicates per treatment) were expressed as mg gallic acid equivalents (GAE) per gram of dry weight.

Total flavonoid contents

Total flavonoid contents were determined by method described by Dewanto *et al.* (2002). 250µl of the methanolic seed extract appropriately diluted were mixed with 75µl $NaNO_2$ (5%). we added 150µl of 10% aluminium chloride after 6 minutes. 500µl of NaOH (1M)

were added to the mixture 5 min later and finally the mixture was adjusted to 2.5ml with distilled water. The absorbance versus prepared blank was measured at 510 nm. Total flavonoid contents of seeds (three replicates per treatment) were expressed as mg catechin equivalents (CE) per gram of dry weight.

Assessment of condensed tannins

50µl of the methanolic seed extract appropriately diluted were mixed with 3ml of 4% methanol vanillin solution and 1.5ml of H_2SO_4. In fact, in presence of concentrated H_2SO_4, condensed tannins were transformed by the reaction with vanillin to anthocyanidols (Sun *et al*., 1998). The absorbance was measured at 500nm after 15min and condensed tannin contents of seeds (three replicates per treatment) were expressed as mg catechin equivalents (CE) per gram of dry weight.

DPPH assay

The electron donation ability of the obtained methanol extracts was measured according to the method of Hanato *et al.* (1988) by bleaching of the purple-coloured solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). In fact, 2ml of methanolic extracts at different concentrations were added to 0.5ml of a 0.2mmol l^{-1} DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min. The absorbance of the resulting solution was then measured at 517 nm after 30 min. The antiradical activity (three replicates per treatment) was expressed as IC_{50} (mg ml^{-1}), the concentration required to cause a 50% DPPH inhibition. A lower IC_{50} value corresponds to a higher antioxidant activity of seed extract (Patro *et al*., 2005).

Reducing power

The method of Oyaizu (1986) was used to assess the reducing power of *Silybum marianum* seed extracts. Seed methanol extracts (1 ml) were mixed with 2.5ml of a 0.2 M sodium phosphate buffer (pH=6.6) and 2.5ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$), and incubated in a water bath at 50°C for 20min. Then, 2.5ml of 10% trichloroacetic acid were added to the mixture and centrifuged at 650*g* for 10min. The supernatant (2.5ml) was then mixed with 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride solution. The intensity of the blue green colour was measured at 700nm. The EC_{50} value mg ml^{-1}) is the extract concentration at which the absorbance was 0.5 for the reducing power and was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used as a positive control.

Phenolic compound identification

Dried samples from *Silybum marianum* seeds were hydrolysed according to the method of Proestos *et al*. (2006), which was slightly modified. The acidic hydrolysis was used to release the aglycones in order to simplify the identification process since the free forms of phenolic compounds are rarely present in plants and they occur as esters, glycosides or bound to the cell wall (Nuutila *et al*., 2002). 20 ml of methanol containing BHT (1g/l) were added to 0.5g of a dried sample. Then 10 ml of 1M HCl were added. The mixture was stirred carefully and sonicated for 15 min and refluxed in a water bath at 90°C for 2 h. The obtained mixture was injected to HPLC. The phenolic compound analysis was carried out using an Agilent Technologies 1100 series liquid chromatography (RP-HPLC) coupled with an UV-Vis multiwavelength detector. The separation was carried out on a 250×4**.**6-mm, 4-µm Hypersil ODS C18 reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was kept at 0.5 ml/min. The gradient programme was as follows: 15% A/85%B 0-12 min, 40%A/60% B 12-14 min, 60%A/40%B 14-18 min, 80%A/20% B 18-20 min, 90%A/10%B20-24 min, 100% A 24-28 min (Bourgou *et al*., 2008). The injection volume was 20µl, andpeaks were monitored at 280 nm. Samples were filtered through a 0.45µm membrane filterbefore injection. Phenolic compounds were identified according to their retention times andspectral characteristics of their peaks against those of standards, as well as by spiking the sample with standards. Analyses were performed in triplicate.

STATISTICAL ANALYSIS

Data were subjected to statistical analysis using statistical program package STATISTICA (Stasoft, 1998). Values are represented as means ± SD of three experiments. The one- way analysis of variance (ANOVA) followed by Duncan multiple range test was employed and the differences between individual means were deemed to be significant at $P<0.05$.

RESULTS

Seed yieldoil

Silybum marianum seeds provide a linoleic oil with a yield of 50% expressed as a dry matter at full maturity. This relative high content oflinoleic oil made possible to classify our studied plant among the oleaginous plants. Although, other oleaginous seeds can produce more than 50% of oil, like *Pistacia vera* (52.8%), *Prunusamygdalus* (56.7%), *Corylusa vellana* (60.2%) (Kornsteiner *et al*., 2006). Other seeds like that of *Carthamus tinctorius* showed oil yield between 25% and 40%, *Boragoofficinalis* (33.8%) (Mhamdi *et al*., 2009).

Total lipids

A high percentage of unsaturated fatty acids (UFA) characterized the full ripening *Silybum marianum* seed swith 81.6% of TFA but the percentage of saturated fatty acids are present at a rate of 19.4%. The major fatty acid observed in *Silybum marianum* seeds was linoleic acid

(LA, C18: 2n-6) with 50.5% followed by oleic acid (C18: 1 n-6) with 30.2% (fig. 1). Previous studies of Hasanloo *et al*. (2008) have shown that the main fatty acids detected in the Iranian *S. marianum* seeds were: linoleic acid (45.36%) followed by the oleic acid (31.58%). We have also detected the presence of palmitic acid (C16:0), stearic acid (C18:0), Arachidic acid C20:0, γ-linolenic acid C18: 3 (n-6) and Cis-11- Eicosenoic acid C20:1n-9 in *S. marianum* seeds.

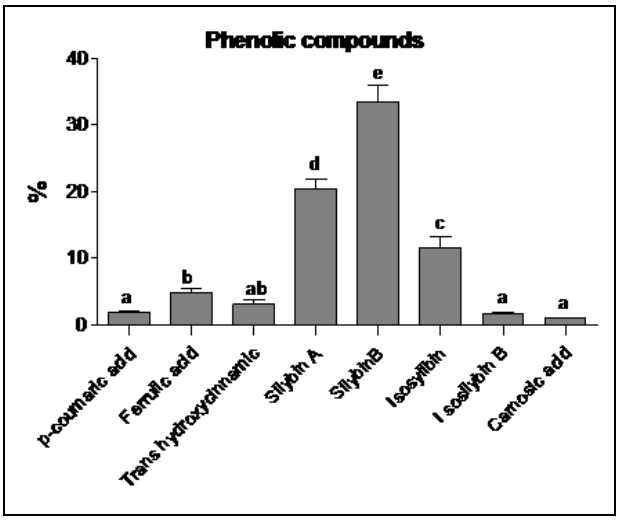

Fig. 1: Phenolic compounds composition (%) of *Silybum marianum* seed

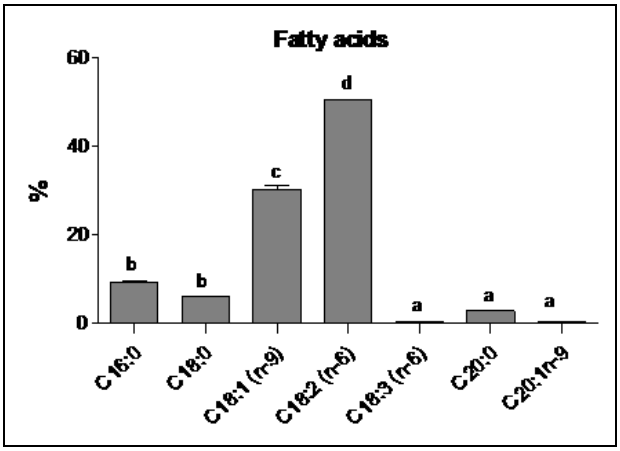

Fig. 3: Fatty acid composition (%) of *Silybum marianum* seed

Essential oil composition

Various previous studies have demonstrated the biological activities of the milk thistle (*S. marianum* extracts. However there are not works, which studied the essential oils composition of *S. marianum* seeds. Gas chromatography (GC)/MS analysis revealed that13 compounds were detected in essential oilsof *Silybum* seeds (table 1). The essential oil yield was 0.12% expressed in dry matter. The most abundant components of the oil from the seed were γ-cadinene (49.8%) and α-pinene (24.5%). Other representative compounds were identified and showed lowest values like β-caryophyllene (0 .61%), terpinene-4-ol (1.42%), limonene (0.5%), α-humulene (4.7%). *Achyrocline flaccid* belong to Asteraceae family showed the predominance of α-pinene and β-caryophyllene (Daiana *et al.*, 2009).

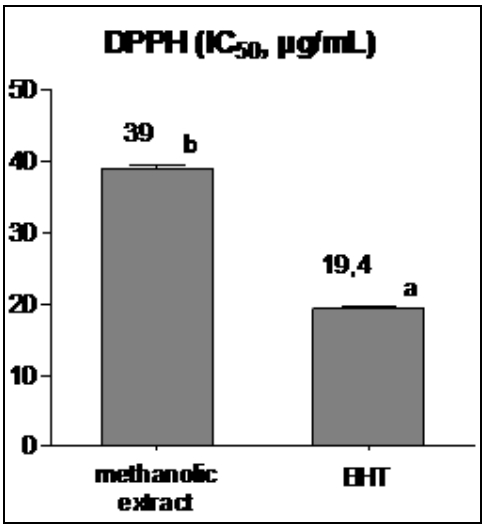

Fig. 2: Antiradical activity of *Silybum marianum* seed expressed as IC50 values (µg/mL). Means of three triplicates followed by a least one same letter are not significantly different at P‹0.05

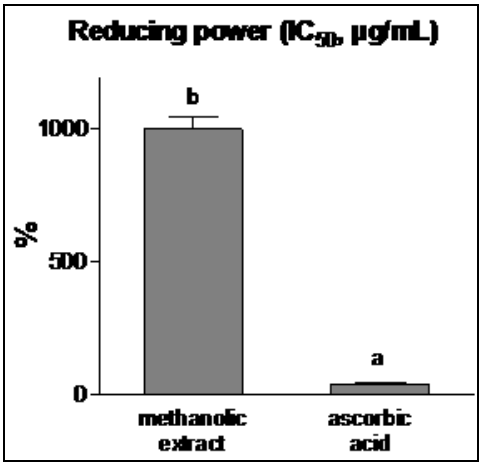

Fig. 4: Reducing power expressed as EC50 values (µg/ mL) of *Silybum marianum* seed methanolic extract

Sesquiterpene hydrocarbons represented the main fraction of the seed oil (55.4%) followed by the monoterpene hydrocarbons (33.29%) but oxygenated monoterpenes are presented minor proportions with 5.41%. The essential oils of *Silybum marianum* are particularly rich on mono and sesquiterpene hydrocarbons, which could have specific usages in pharmaceutical industry (table 1).

The polyphenol contents in *Sylibum marianum* seeds were about 29mg GAE/g DW. In comparison to several

species, this content was more important (fig. 2). In fact, Djeridane *et al.,* (2006) found that Asteraceae family was characterized by this abundance. The biosynthesis of secondary metabolites such as polyphenols may be related to the hard climate conditions of Asteraceae usual habitat (hot temperature, high solar exposure, drought, salinity). In the other hand, the total polyphenol contents provided by *Sylibum marianum* seeds is more important than those measured from *Cynara cardunculus* seeds belonging to the same family (Asteraceae) (Faleh *et al*., 2008). The flavonoid and condensed tannins contents were respectively 3.39mg EC.g^{-1} DW and 1.8mg EC.g^{-1} DW.

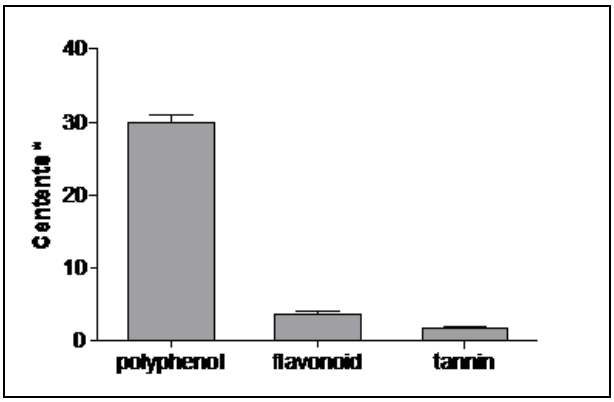

Fig. 5: Total phenolic, tannin and flavonoid contents

Antioxidant capacity

DPPH▪ assays demonstrated the antiradical activity of seed was important (IC_{50} was 39µg ml^{-1}). In fact, in comparison with the value of IC_{50} of BHT (19.40µg ml^{-1}), we can deduce that the seed methanolic extract is capable for scavenging free radicals (fig. 3).

Reducing power

We have also determined the reducing capacity of seed extracts that may serve as indicator of its potential antioxidant activity (Meir *et al.,* 1995). Our results demonstrated that *Silybum marianum* seeds exhibited a moderate reducing capacity with an EC_{50} value of reducing power of methanol seed extracts of about 1mg ml^{-1} for as compared with value obtained for ascorbic acid (38µg ml^{-1}) (fig. 4).

Identification of phenolic compounds by RP-HPLC

The different phenolic acids and flavoligans identified are illustrated in fig. 5. A8 phenolic compounds were successfully identified in the seeds, where the major phenolic compounds were silybin A (12.3%), silybin B (17.6%), isosilybin A (21.9%), isosilybin B (12.8%), silychristin (7.9%), silydianin (7.5%) which are the most active phytochemical and are largely responsible for the claimed benefit of the sylimarin complex (fig. 5).

Zao *et al.* (2005) and Škottová *et al.*(2003) have also indicated that *Silybum marianum* contains flavonolignans.

DISCUSSION

The analysis of *S. marianum* total lipids demonstrated that linoleic and oleic acid, from the Tunisian variety collected from Amdoun region, are the major fatty acids of the plant. The comparison of these fatty acid levels with those obtained from the Pakistan *S. mariamun* variety (khan *et al*., 2007), showed differences in the composition of total lipids between the two varieties. This difference in fatty acid levels and composition can be explained by the effect of genetic factors as well as environmental conditions. Indeed, like many secondary metabolites, fatty acids and phenolic acid are known to be affected by biotic and abiotic stresses (Malekzadeh *et al*, 2011).

Linoleic acid is one of essential fatty acids (EFAs) that it cannot be synthesized by the human body, and must be ingested as part of the diet (Malekzadeh *et al*., 2011) because of its nutritional value due to its metabolism at the tissue levels (produce the long chain polyunsaturated fatty acids (PUFA) and prostaglandin). As a result, the use of this fatty acid has become increasingly popular in the cosmetic and pharmaceutical products.

Interest in the polyunsaturated fatty acids, as health promoting nutrients has expended in recent years. In fact, polyunsaturated fatty acids participate in the treatments of certain chronic diseases such as diabetes, cardiovascular, inflammatory, atherosclerosis (Finley and Shahidi, 2001). The degree of fatty acid unsaturation is also the most important factor intervening in the maintenance of membrane fluidity and provides the appropriate environment for the membrane functions and the processes of plant adaptation under constraining conditions. Palmitic and stearic acids were present in comparable proportions with value means of 9.25% and 6.22%, respectively. The minor fatty acids were γ-linolenic (C18: 3n-6), arachidic (C20:0), which found at low percentages form with 3.76% of TFA.

We have also observed that *S. marianum* seed extract seems to contain powerful inhibitor compounds, which may act as primary antioxidants that react with free radicals as efficient hydrogen donors. Due to their interesting antioxidative properties, silybin compounds complex can be very effective in skin protection, cosmetic and food industry, in cardiovascular diseases, diabetes, inflammation and in the prevention and the treatment of many neurodegenerative and neurotoxic process (Dixit *et al.,* 2007, Pérez-H *et al*., 2014).

In our study, we noted also the presence of others phenolic compounds such as gallic, 4-hyroxybenzoic, vanillic and p-coumaric acids with minor percentages. These phenolic acids are known to play important roles in plant resistance to pathogens and herbivores, allelopathy, oxidative stress and plant growth regulation. Also, they

Table 1: Essential oil composition of *Silybummarianum* seed

Compound	RI[a]	RI[b]	%
α-pinene	939	1032	24.5±2.1
Camphene	954	1076	6.6±0.9
Limonene	1030	1203	0.5±0.04
γ-terpinene	1062	1255	1.13±0.02
terpinolene	1092	1290	0.56±0.01
Linalool	1098	1553	3.22±0.2
Terpinene-4-ol	1178	1611	1.42±0.2
P-cymene-8-ol	1183	1864	0.21±0.01
α-terpineol	1189	1709	0.56±0.01
β-caryophyllene	1419	1612	0.61±0.02
α-humulene	1454	1687	4.7±0.1
germacrene -D	1480	1726	0.49±0.01
γ-cadinene	1492	1766	49.6±2.4

* Components are listed in order of elution in apolar column (HP-5); RI[a], RI[b]: retention indices calculated using respectively an apolar column (HP-5) and polar column (HPInnowax); volatile compound proportions were calculated from the chromatograms obtained on the HP-Innowax column.

have been shown to have beneficial effects on human health.

In conclusion, essential oils and plant extracts are of great interest in food, cosmetic and pharmaceutical industries. The biological compounds present in these products, can be use as natural additives emerged from a growing tendency to replace synthetic preservatives by natural ones. Our study can be considered as the first one which analyzed the fatty acids (linoleic acid), essential oil (sesquiterpene and monoterpene hydrocarbons) and phenolic compositions of the endemic Tunisian *S. marianum* and its *in vitro* antioxidant features. Therefore, the traditional hepatoprotective effects of *Silybummarianum* can be due to its important antioxidant capacity. This antioxidant activity may correlate with its higher phenolic contents.

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