

# Unsaponifiable fraction of Tunisian grape seed oils

**Hela Harbeoui**<sup>1,2</sup>

**Walid Yeddes**<sup>1,2</sup>

**Wissem Aidi Wannas**<sup>1,✉</sup>

**Moufida Saidani Tounsi**<sup>1</sup>

<sup>1</sup>Laboratory of Aromatic and Medicinal Plants (LPAM), Center of Biotechnology, Borj-Cedria, Tunisia

<sup>2</sup>Faculty of Sciences of Bizerte, University of Carthage, Tunisia

✉ CORRESPONDING AUTHOR:

Wissem Aidi Wannas,

Laboratory of Aromatic

and Medicinal Plants,

Biotechnologic Center Borj-Cedria

Technopark,

Hamam-lif, Tunisia

Tel: +21679325738

Fax: +2167941263

E-mail: aidiwissem@yahoo.fr

<https://orcid.org/0000-0003-3134-0457>

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In this study, the composition of phytosterols, tocopherols and triterpenoids from four Tunisian grape seed oil varieties (Razagui, Marsaoui, Muscat d'Alexandrie and Khamri) was determined. GC-MS analysis allowed the identification of one triterpenic compound ( $\beta$ -amyirin), five phytosterols (campesterol, stigmasterol,  $\beta$ -sitosterol,  $\beta$ -sitostanol, cholesterol) and three tocopherols isoforms ( $\alpha$ ,  $\beta$  and  $\gamma$ -tocopherols). Furthermore, grape seed oils had potent antioxidant activities using DPPH and chelating iron assays. Multivariate analysis and principal component analysis (PCA), based on compounds profiling data and chemotypes found in this fraction, revealed distinguished groups of varieties associated to specific composition patterns.

**Keywords:** Antioxidant activity. Grape seed oils. Phytosterols. Tocopherols. Triterpenoids. Unsaponifiable fraction.

## 1. INTRODUCTION

Seeds are the richest part of the fruit in bioactive molecules representing, as by-products, a double loss for the agriculture food industry due to the cost of disposal and the loss of profits for their reuse and valorisation [1]. Recently, developing strategies for reducing agriculture food by-products, derived from the fruit processing industry and partially in seed form, are occurred to create good impact for the food security, economy, and the climate [2].

Among the different grape (*Vitis vinifera*) fruit parts, seeds show the highest antioxidant activity as compared with the skin and the flesh [3]. Grape seeds are by-products of wineries and are often referred as important agricultural and industrial waste [4] with potentials to be used in pharmaceutical, food, and cosmetic applications [5]. Seeds alone make up around 15% of the solid waste produced in wine industries. They are generally burnt and sometimes used for cattle feed, despite they are the source of excellent oil for human consumption [6]. As grape seeds are discarded of the wine making process, the extraction and sale of grape seed oil and grape seed extract can be a gainful sideline as well as a resourceful by-product [7]. Grape seeds can give 10-20% of oil and 5% of by-product residue rich of antioxidant compounds. Grape seed oil is produced in many areas as Italy, Spain, Chile, United States, Australia, and France [8]. So, the recovery of oil from grape seeds is probably the main application due to the huge amount of seeds produced worldwide and because of the health benefits of grape seed oil intake in our diet, as well as its potential use in other non-food industries [9]. Nowadays, grape seed oil is gaining popularity as a culinary oil [9] and it has been examined for promoting health and preventing diseases owing to its anti-inflammatory [10], anticancer [11], antidiabetic [12], antimicrobial [13], cardioprotective [14], hepatoprotective [15] and neuroprotective [16] properties. The benefits of grape seed oil are essentially related to its high content of polyunsaturated linoleic acid [17]. Additionally, several studies reported that this oil contains minor bioactive

components as phenolic compounds, phytosterols and tocopherols that are characterised by a high antioxidant activity [18-24]. However, little effort has been given to characterise the unsaponifiable fraction of grape seed oils. This fraction represents about 1.5-2% of the oils and is an important source of interesting minor compounds as phytosterols, tocopherols and triterpene compounds [25]. All these bioactive compounds afford important practical benefits such as antihyperlipidemic and antioxidant activities [26].

The aim of this paper is to evaluate the composition and the antioxidant activity of the unsaponifiable fraction of grape seed oils obtained from four native Tunisian varieties (Razagui, Khamri, Marsaoui and Muscat d'Alexandrie).

## 2. MATERIALS AND METHODS

### 2.1. PLANT MATERIAL

Four autochthonous grape varieties (*Vitis Vinifera* L.) in Tunisia were chosen for this investigation: Razagui, Marsaoui, Muscat d'Alexandrie and Khamri. 1 Kg per variety, have been harvested from Takelsa (North-Eastern Tunisia) in September 2017. Seeds were manually separated and dried at room temperature for 7 days then stored at -30°C until extraction.

### 2.2. SEED OIL EXTRACTION

Oil was extracted according to the method of Saidani & Marzouk [27]. Twenty grams of each grounded seed were retained in a Soxhlet extractor with hexane (100 ml) for 12 h. Extracts were evaporated, and the oil contents were determined

### 2.3. UNSAPONIFIABLE FRACTION

The analysis of sterol, triterpene and tocopherol compounds was piloted according to Fedeli, et al. [28] with some modification. Five grams of grape seed oil and Betulin (20 mg/100g), used as internal standard, were saponified with a potassium hydroxide ethanolic solution. After boiling, water was added and the unsaponifiable fraction extraction was completed with diethyl ether.

The solution was rinsed several times with water and the unsaponifiable fraction was extracted with diethyl ether then evaporated by distillation on a rotary evaporator at 30°C under vacuum. Then the water phase was detached, and the organic sample was dried with anhydrous sodium sulphate, and the obtained residue was weighed.

The fractions of sterols, triterpenic alcohols, and tocopherols were separated, by thin-layer chromatography, on a basic silica gel plate. After the evaporation of extract to dryness, several components were converted to trimethylsilyl ethers by adding 50 µl pyridin and 50 µl BSTFA-TMCS (90:1 v/v) for 30 min at 60°C.

### 2.4. GC-MS ANALYSIS

GC-MS analyses of silylated sample (2 µl) were made

by gas chromatograph HP 5890 (II) joined to HP 5972 mass spectrometer with electron impact ionisation (70 eV). The carrier gas was helium with a flow rate of 1.2 ml/min; split ratio was 60:1. Scan time and mass range were 1s and 40-300 m/z respectively. A HP-5MS capillary column (30 m × 0.25 mm) was utilised and the column temperature was programmed to rise from 50 to 240°C at a rate of 5°C/min.

### 2.5. DPPH ASSAY

The electron donation ability of unsaponifiable fraction was measured by decolorising of the purple-coloured solution of DPPH [29]. 2 mL of unsaponifiable fraction of grape seed oil was added to 0.5 mL of 0.2 mM DPPH methanolic solution. Before reading the absorbance at 517 nm, samples were incubated at room temperature for 30 min.

### 2.6. CHELATING POWER ACTIVITY

According to Zhao *et al.* [30], 0.1 ml of unsaponifiable fraction of grape seed oil was added to 0.05 mL of 2 mM FeCl<sub>2</sub>. The reaction was started by adding 0.1 mL of 5 mM ferrozine and 2.75 mL of distilled water. The mixture was left at room temperature for 10 min and the absorbance of the solution was measured at 562 nm.

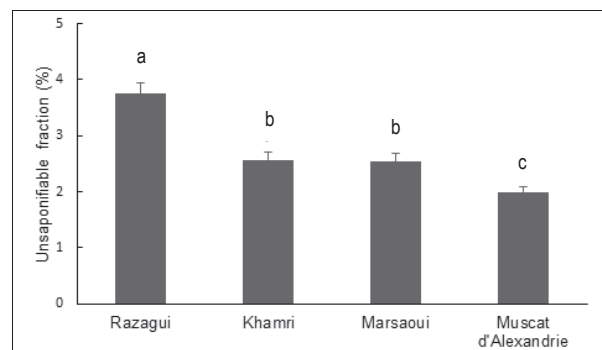
### 2.7. STATISTICAL ANALYSIS

The data were recorded as mean ± standard deviation (n = 3) and analysed by multivariate analysis, clustering and ANOVA analysis using XLSTAT software (Addinsoft, www.xlstat.com). For mean comparison, the Tukey HSD test was used at  $p \leq 0.05$  to identify significant differences. To determine accession groups, the principal component analysis (PCA) was achieved. Hierarchical cluster analysis (HCA) was performed using the Lance & Williams [31] method.

## 3. RESULTS AND DISCUSSION

### 3.1. UNSAPONIFIABLE FRACTION YIELD

The unsaponifiable fraction yields of the four grape



**Figure 1** - Unsaponifiable fraction yields in four Tunisian varieties. The data represent the mean values of three experiments ± SE. Data marked with (a - c) are significantly different ( $p < 0.05$ ).

seed oil varieties are given in Figure 1. Results obtained showed that the yields of unsaponifiable fraction were significantly ( $p < 0.05$ ) affected by the variety. Muscat d'Alexandrie had the lowest unsaponifiable fraction yield (1.98%) whereas Razagui had the uppermost unsaponifiable fraction yield (3.75%). Khamri achieved 2.57%, followed by Marsaoui (2.55%). In an earlier study, Harbeoui *et al.* [24] reported that the unsaponifiable fraction yield of Tunisian grape seed oil was 4.05% in Carignan, 3.98% in Syrah and 3.48% in Merlot. Comparable results were detected by Millan-Linares *et al.* [32] concerning Spanish grape seed cultivars with an unsaponifiable fraction yield ranging from 2.2% to 2.4%.

### 3.1.1. Phytosterol content

Phytosterols, specifically  $\beta$ -sitosterol, are known for their capacities to reduce the serum level of cholesterol concentration and reduce atherosclerotic risk [33]. These bioactive compounds are one of the most studied chemical oil groups due to their positive effect on human health. Analysis of phytosterols, applying GC-MS, led to identify five compounds in unsaponifiable fraction of grape seed oil such as cholesterol, campesterol, stigmasterol,  $\beta$ -sitosterol and  $\beta$ -sitostanol.

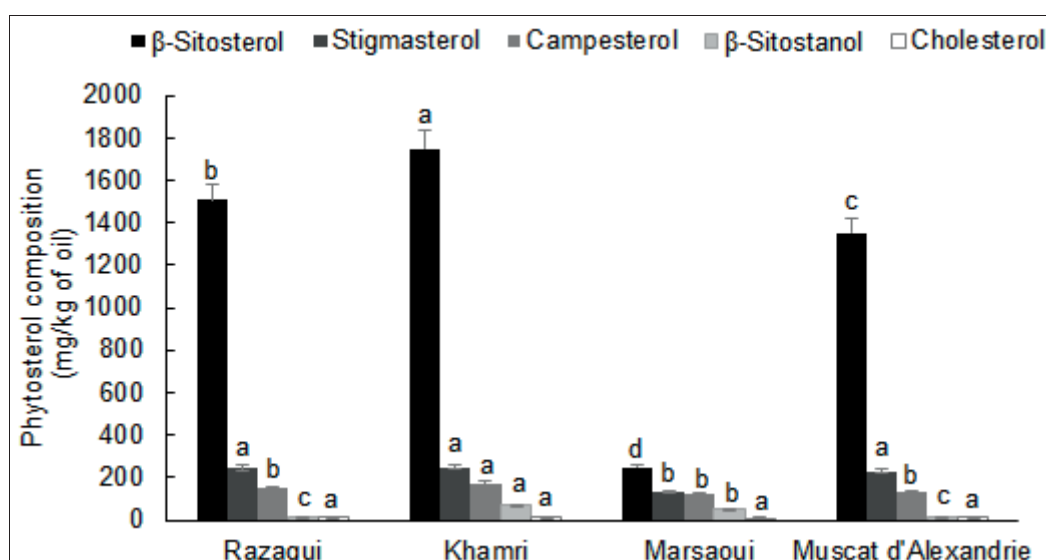
The total phytosterol contents of the four seed oil varieties varied from 1609.87 mg/kg of oil to 3616.67 mg/kg of oil. The higher total phytosterol content was detected in Razagui (3616.67 mg/kg of oil), followed by Muscat d'Alexandrie (1933.70 mg/kg of oil) and Khamri (1609.87 mg/kg of oil). These results were in accordance with the data previously reported; the content ranged from 1860 to 3160 mg/kg of oil [9, 19, 21, 34].

As can be shown in Figure 2,  $\beta$ -sitosterol is the most

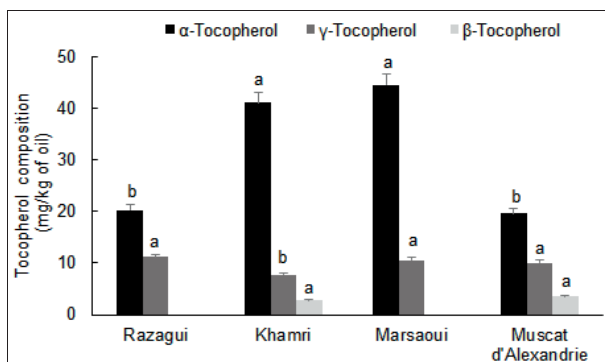
abundant sterol in all seed oil varieties which varied from 250 mg/kg to 1353.77 mg/kg. Additionally, an important presence of stigmasterol (132.55-250 mg/kg), campesterol (123.55-175 mg/kg) and cholesterol (9.82-16.02 mg/kg). Similar results were obtained by Hassanein & Abedel-Razek [20], Crews *et al.* [21], Harbeoui *et al.*, [23] and Pardo *et al.* [34] who reported that  $\beta$ -sitosterol was a predominant phytosterol in all oil samples. The most important biological effect of a dietary intake of phytosterols in human health is lowering blood cholesterol and reducing risks of cancer [35]. In particular,  $\beta$ -sitosterol, with polyphenols from the winery industry, have revealed a cardioprotective activity, preventing the release of pro-inflammatory and pro-atherogenic molecules [9, 36].

### 3.1.2. Tocopherol content

The total tocopherol contents of four grape varieties displayed a considerable variation (Figure 3). The tocopherols, known as vitamin E, in grape seed oils ranged from 33.37 to 181.35 mg/kg of oil. These levels are almost similar to the data on ten oil samples from France, Italy and Spain (63-1208 mg/kg oil) reported by Crews *et al.* [21] and those on the mature seeds of seven Turkish cultivars reported by Demirtas *et al.* [37]. Tocopherols, fat soluble vitamin complexes, are indispensable antioxidant sources for human nutrition and healthy diets. Although all tocopherols have vitamin E activity in the human body, they have hypocholesterolemic, antithrombotic and antitumor effects [38], antioxidant activity in cells, although their effect on the oxidative stability of oils is weak [39]. Overall, our results suggest that unsaponifiable fraction of grape seed oil is a good source of vitamin E which provide a unique potential for human health according to Martin *et al.* [9].



**Figure 2** - Phytosterol composition (mg/kg of grape seed oil) determined by GC-MS in unsaponifiable fraction of four Tunisian varieties. The data represented the mean values of three experiments  $\pm$  SE. Values highlighted with different superscript are significantly different ( $p < 0.05$ ).



**Figure 3** - Tocopherol composition (mg/kg of grape seed oil) determined by GC-MS in four Tunisian varieties. The data represented the mean values of three experiments  $\pm$  SE. Values highlighted with different superscript are significantly different ( $p < 0.05$ ).

Mean values of individual tocopherol contents of the studied samples were 44.04; 8.68 and 22.88 mg/kg of oil for  $\alpha$ ,  $\beta$  and  $\gamma$ -tocopherols, respectively (Figure 3). These values are overly similar to the previous reports by Sabir *et al.* [40] who analysed the tocopherol contents of grape seeds from 21 different varieties grown in Turkey and to research realised by Wei *et al.* [41] who determine the tocopherol contents of grape seeds from fourteen varieties grown in Korea. Our data revealed that in terms of the tocopherol composition of different varieties  $\alpha$ -tocopherol was the most abundant, followed in order by  $\gamma$  and  $\beta$ -tocopherol. This composition is in agreement with that reported by Hassanein & Abedel-Razek [20], Demirtas *et al.* [37], Shiozaki & Murakami [42] and Harbeoui *et al.* [25]. In addition,  $\alpha$ -tocopherol, the most abundant tocopherol among the samples analysed, ranged from 19.58 to 44.51 mg/kg of oil (Figure 3). The highest  $\alpha$ -tocopherol content was in Marsaoui (44.51 mg/kg of oil), followed by Khamri (41.06 mg/kg of oil), Razagui (20.25 mg/kg of oil) and finally Muscat d'Alexandrie (19.58 mg/kg of oil).  $\gamma$ -Tocopherol was the second most abundant constituent, ranging from 7.69 mg/kg of oil (Khamri) 11.15 mg/kg of oil (Razagui).  $\beta$ -Tocopherol were found in low concentrations compared to  $\alpha$ - and  $\gamma$ -tocopherols.

### 3.1.3. Triterpene content

The interest of the unsaponifiable fraction as functional and biological agents is due to the presence of triterpenes, these compounds showed anti-inflammatory [43], antioxidant [44], antidysrhythmic and vasodilatory activities [45]. The triterpene contents of grape ranged from 8.88 to 45.10 mg/Kg of oil. The two triterpenes founded in unsaponifiable fraction of grape seed oil are lanosterol and  $\beta$ -amyrin. Because triterpenes are concentrated mainly in the skin of fruits, their content is about 10 times higher than in other types of oils as seed oil [46]. These

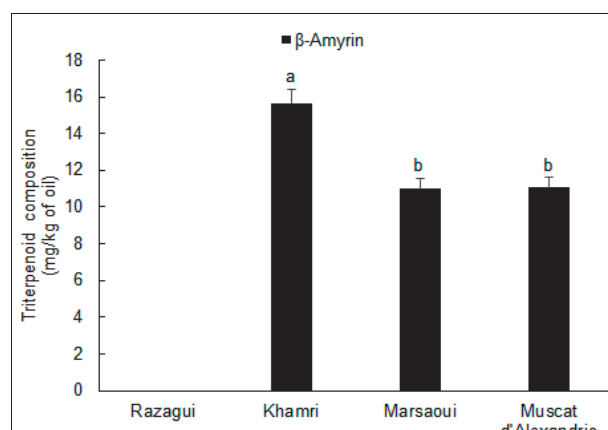
compounds have not been previously described in the unsaponifiable fraction of grape seed oil, perhaps due to the genuine absence of these compounds in other varieties previously studied but most probably because of their presence at low levels that were difficult to detect. From Figure 4,  $\beta$ -amyrin was founded in all varieties except Razagui, among all analysed grapes, Khamri (15.65 mg/Kg of oil) exhibited the highest content compared to Muscat d'Alexandrie (11.05 mg/Kg of oil) and Marsaoui (10.98 mg/Kg of oil). According to our results and those previously reported by Harbeoui *et al.* [25], the presence of  $\beta$ -amyrin in grape appears to be a common feature in *Vitis vinifera* varieties studied to date.

### 3.2. DPPH AND CHELATING IRON ASSAYS

Generally, the aim of in vitro radical scavenging experiments is to provide a preliminary appraisal of the antioxidant capacity of unsaponifiable fraction. The results of the antioxidant activity assays with the reference standard BHT and Trolox are presented in Table I.

In the DPPH assay, Khamri ( $IC_{50} = 3.65 \mu\text{g/g}$ ), Marsaoui ( $IC_{50} = 4.09 \mu\text{g/g}$ ), Muscat d'Adexandrie ( $IC_{50} = 4.22 \mu\text{g/g}$ ) and Razagui ( $IC_{50} = 4.72 \mu\text{g/g}$ ) exhibited a significant and a higher activity than BHT ( $IC_{50} = 12.69 \mu\text{g/g}$ ). Harbeoui *et al.* [24] reported that the unsaponifiable fraction of grape seed oil had a stronger antiradical activity which could be deduced that the minor compounds of this fraction mainly contribute to the potent antiradical activity of grape seed oil. Choi *et al.* [47] revealed that vitamin E and phytosterols, the second- and third-most abundant lipophilic compounds in grape seed oil composition after fatty acids, respectively, are also known to have an antioxidant activity.

From Table I, the  $EC_{50}$  value of metal chelating power ability of Trolox ( $EC_{50} = 266.30 \mu\text{g/g}$ ) is lower than



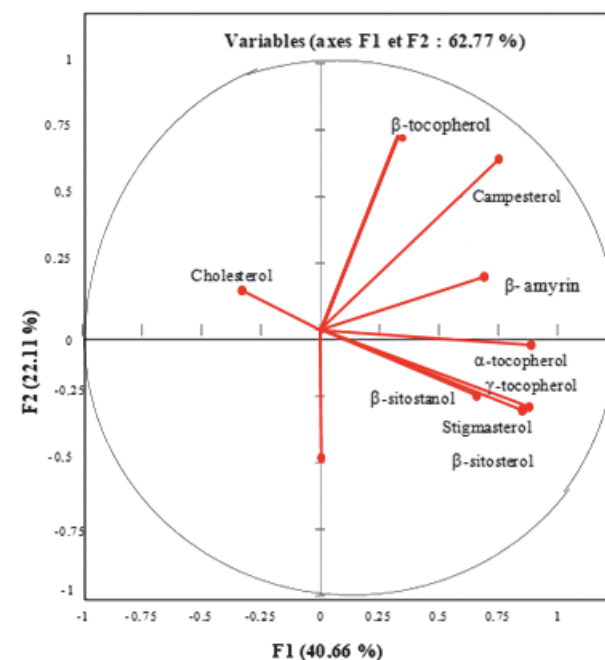
**Figure 4** - Triterpene alcohol composition (mg/kg of grape seed oil) determined by GC-MS in unsaponifiable fraction of four Tunisian varieties. The data represented the mean values of three experiments  $\pm$  SE. Values highlighted with different superscript are significantly different ( $p < 0.05$ ).

the studied unsaponifiable fraction of Razagui ( $IC_{50} = 49.18 \mu\text{g/g}$ ) that confirm the important antioxidant capacity. Interestingly, Khamri ( $EC_{50} = 275.93\mu\text{g/g}$ ) and Marsaoui ( $EC_{50} = 278.59\mu\text{g/g}$ ) showed a good antiradical capacity. Similar results were reported by Harbeoui *et al.* [25] in the case of other grape seed varieties, namely Carignan ( $EC_{50} = 261.60 \mu\text{g/g}$ ), Syrah ( $EC_{50} = 263.01 \mu\text{g/g}$ ) and Merlot ( $EC_{50} = 280.60 \mu\text{g/g}$ ). The stronger Ferrous ions ( $Fe^{2+}$ ) chelating power determined in unsaponifiable fraction of Tunisian grape seeds oil provide a substantial value of this product since reducing power is correlated with antioxidant activity [48]. Compounds with reducing power show they are electron donors, can decrease the oxidised intermediates of lipid peroxidation processes and acting as primary and secondary antioxidants [49].

### 3.3. GRAPE SEED DIVERSITY BASED ON UNSAPONIFIABLE FRACTION CONTENTS

Secondary plant metabolites could be used as a complementary tool for taxonomic studies in plants [50]. Chemotaxonomy aims to precise relationships between the chemical composition of the studied varieties and their systematic classification [51]. To test the efficiency of the contribution of unsaponifiable fraction compounds to the taxonomic classification of grape varieties, the principal component analysis (PCA) was used in this study.

As shown in Figure 5, two principal clusters could be distinguished: one positively correlated with the last axis and grouped Muscat d’Alexandrie, Marsaoui and khamri designated by the presence of  $\beta$ -tocopherol, campesterol, stigmasterol and  $\beta$ -amyrin. The second cluster was negatively correlated with this axis grouping essentially Razagui distinguished by the presence of  $\beta$ -sitostanol,  $\alpha$  and  $\beta$ -tocopherols. The chemical polymorphism in the unsaponifiable fraction of grape seed oil might be attributed to the environmental variation associated with climate change affecting precipitation and thereby plant architecture, flowering, fruiting, phytochemical composition and in competition with intra-species [52]. In fact, Górnas *et al.* [53] reported that vitamin E concentrations are influenced by harvest conditions because they influ-



**Figure 5** - Heatmap cluster based on the normalized quantities of the identified compounds in unsaponifiable fraction across four varieties. Each line in the heatmap represented a metabolite. The deeper the red color, the higher its content in the four grape varieties; similarly, the deeper the black color, the lower its content.

ence vegetation periods and then the degree of maturation of the seeds used to extract oil.

## 4. CONCLUSION

Recently, much attention has been focused to use natural antioxidant compounds rather than synthetic antioxidants. Therefore, the inclusion of unsaponifiable fraction obtained from grape seed oil in food products may be a useful way to obtain natural antioxidants.

### Conflict of interest

The authors declare there is no conflict of interest.

**Table I** - Antioxidant activities of unsaponifiable fraction.  $IC_{50}$  ( $\mu\text{g/g}$ ): the concentration of the extract generating 50% inhibition;  $EC_{50}$ : the effective concentration at which the absorbance was 0.5. The data represent the mean values of three experiments  $\pm$  SE. Values marked with (a - c) are significantly different ( $p < 0.05$ ).

	Ferrous ions ( $Fe^{2+}$ ) chelating assays	DPPH
Khamri	275.93 $\pm$ 0.89 <sup>c</sup>	3.65 $\pm$ 0.49 <sup>b</sup>
Marsaoui	278.59 $\pm$ 2.12 <sup>c</sup>	4.09 $\pm$ 0.53 <sup>c</sup>
Razagui	401.38 $\pm$ 1.08 <sup>a</sup>	4.72 $\pm$ 0.65 <sup>c</sup>
Muscat d’Alexandrie	310.59 $\pm$ 0.69 <sup>b</sup>	4.22 $\pm$ 1.06 <sup>c</sup>
Trolox	266.30 $\pm$ 1.89 <sup>c</sup>	-
BHT	-	12.69 $\pm$ 0.25 <sup>a</sup>



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