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# FATTY ACID, TOCOPHEROL, AND STEROL CONTENT OF THREE *Teucrium* SPECIES FROM TUNISIA

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*In the present study analyses concerning the composition of vitamin E, sterols, triglycerides, and fatty acids of three* Teucrium *species (*Teucrium alopecurus, T. nabli, *and* T. polium*) seed oil were performed. Linoleic, linolenic, and palmitic were the major fatty acids. The oil was characterized by a high amount of phytosterol, wherein clerosterol, sitosterol, and stigmasterol are the main constituents. The amount of tocopherol is nearly 550 mg/kg of oil, with* α*-tocopherol as the major isomer.*
*Information concerning the composition of* Teucrium *seed oil is very important for evaluating the therapeutic effect of this oil.*

**Key words:** *Teucrium*, seed oil, fatty acids, tocopherol, triglycerides, sterols.

Natural seed oils are mixtures of triacylglycerides (95–98%) and a wide range of compounds like wax esters, sterols, tocopherols, phospholipids, phenolics derivatives, etc. (2–5%) [1].

Phytosterol and tocopherol are among the most interesting lipophilic constituents, and they have multiple beneficial effects. Tocopherol plays an important role in the prevention of some chronic diseases and certain kinds of cancer [2], while phytosterol and specifically beta-sitosterol are known to lower serum cholesterol concentration and atherosclerotic risk [3, 4].

*Teucrium* genus (Labiatae) is found through the world but mainly in the Mediterranean basin with 220 taxa [5], 19 of which grow in Tunisia [6–8]. *Teucrium* species have been used for more than 2000 years as medicinal herb for a great range of actions: antispasmodic, hypolipidemic, anti-inflammatory, and hypoglycemic [9–14].

Phytochemical investigations have shown that the aerial part of *Teucrium* species contains various compounds such as flavonoids, diterpenes, iridoids, etc. *Teucrium alopecurus* and *Teucrium nabli* are two endemic species limited to south Tunisia [6, 8]. The seeds of this plant and of *Teucrium polium* have not been explored. The objective of our work was to characterize the chemical composition of seed oil (fatty acids, triglycerides, vitamin E, and sterols) from three *Teucrium* species (*Teucrium alopecurus*, *Teucrium nabli*, and *Teucrium polium*), which could have very interesting cosmetic and/or pharmaceutical uses.

*Teucrium* seeds averaged 1% yield, with *T. nabli* exhibiting the highest yields (1.2%). According to the results shown in the Table 1, eleven fatty acids were identified; hydrocarbons were also detected but not included in Table 1.

The fatty acid content of the seed oil of the three *Teucrium* species showed big quantitative differences (Table1). The most abundant fatty acids were linoleic, linolenic, and palmitic acids, which together comprised 89.2%, 74.2%, and 62.6%, respectively, in *T. nabli, T. alopecurus*, and *T. polium* total fatty acids. The analysis of FAME of different species showed that lauric acid was found in relatively high amounts (9%) in *T. polium*; this acid is less important in *T. alopecurus* and *T. nabli* (2% and 1.6%). *T. polium* and *T. alopecurus* seed oil presented the highest amounts of C28:0, 9% and 5.9%, respectively, whereas *T. nabli* had the lowest value, 3%. The ratio of unsaturated to saturated fatty acids is more important in *T. nabli* (3.89), while it constituted only 1.54 in *T. alopecurus* and 0.79 in *T. polium*.

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TABLE 1. Fatty Acid Composition (mass %) and Tocopherol Contents (mg/kg) of *T. polium*, *T. nabli*, and *T. alopecurus* Seed Oil

| Compound | *T. nabli* | *T. alopecurus* | *T. polium* | Compound | *T. nabli* | *T. alopecurus* | *T. polium* |
|---|---|---|---|---|---|---|---|
| 12:0 (La) | 1.6 | 2 | 9 | 24:0 (Li) | 0.4 | 2.0 | 2.2 |
| 14:0 (M) | 1.5 | 1.8 | 3.2 | 26:0 | 0.8 | 8.9 | 3.8 |
| 16:0 (P) | **9.7** | **13.5** | 17.9 | 28:0 | 3 | 5.9 | 9.0 |
| 18:2 (L) | **44.2** | **31.7** | 19.4 | $\alpha$-Tocopherol | 296.04 | 316.25 | 277.25 |
| 18:3 (Ln) | **35.3** | **29.0** | 25.3 | $\beta$-Tocopherol | 14.95 | 19.96 | 14.58 |
| 18:0 (S) | 2.3 | 2.8 | 4.5 | $\gamma$-Tocopherol | 142.0 | 141.26 | 89.49 |
| 20:0 (A) | 0.7 | 1.3 | 2.7 | $\delta$-Tocopherol | 115.78 | 73.11 | 152.96 |
| 22:0 (B) | 0.5 | 1.0 | 2.9 | Total, mg/kg | 568.77 | 550.58 | 533.92 |

TABLE 2. Identified Triglycerols of *T. polium*, *T. nabli*, and *T. alopecurus* Seed Oil

| Triglycerides | *T. nabli* | | *T. polium* | | *T. alopecurus* | |
|---|---|---|---|---|---|---|
| | ECN | % | ECN | % | ECN | % |
| LnLnLn | 36 | 6.7 | 36 | 6.3 | 36 | 9.1 |
| LaLnLn | 36 | 3.1 | 36 | 5.8 | – | – |
| LaLaLa | 36 | 2.9 | – | – | – | – |
| LLnLn | 38 | 10 | 38 | 1.7 | 38 | 3.4 |
| MLnLn | 38 | 5.7 | 38 | 1.7 | 38 | 3.1 |
| MLaLa | 38 | 4.9 | 38 | 1.1 | 38 | 3.9 |
| LLLn | 40 | 9.4 | 40 | 0.5 | 40 | 5.8 |
| LnLnP | 40 | 10.8 | 40 | 0.7 | 40 | 6.9 |
| LLL | 42 | 7 | 42 | 6.2 | 42 | 16.7 |
| LLnP | 42 | 4.4 | 42 | 5.5 | - | – |
| SLnLn | 42 | 8.6 | 42 | 13.6 | 42 | 11.7 |
| LLP | 44 | 7.5 | 44 | 4.1 | 44 | 11.0 |
| LPM | 44 | 4.5 | 44 | 10.0 | 44 | 5.3 |
| PLnP | 44 | 1.8 | 44 | 1.9 | 44 | 5.2 |
| SLL | 46 | 3.1 | 46 | 8.3 | 46 | 2.4 |
| PLP | 46 | 2.9 | 46 | 2.8 | 46 | 3.1 |
| SLnP | 46 | 1.5 | 46 | 14.1 | 46 | 5.5 |
| ALL | 48 | 2.8 | 48 | 5.3 | 48 | 5.1 |
| PPP | 48 | 2.4 | 48 | 10.6 | 48 | 1.9 |

Although the oils obtained from various species showed quantitative differences, in all species we note the absence of MUFAs (monounsaturated fatty acids) and the presence of PUFAs (polyunsaturated fatty acids: C18:2 and C18:3) in high percentages especially for *T. nabli*. The high ratio of linoleic and linolenic acids (omega-3 and omega-6) may have many beneficial health effects such as reduction of serum cholesterol and atherosclerosis, prevention of heart diseases, and other diseases [15, 16].

The qualitative and quantitative determination of triacylglycerols (TGs) by HPLC with refractive index allows high separation of triglycerides according to increasing ECN.

The results obtained are summarized in Table 2 where the relative peak areas of all identified TGs are calculated from RID chromatograms and identified by comparison with soybean, olive, and rapeseed oils.

The contents of TGs differed between species: *T. nabli* shows high percentages of LLnLn, LLLn, and LnLnP while *T. polium* is characterized by SLnLn, SLnP, and PPP, and *T. alopecurus* by LLL, SLnLn, and LLP).

TABLE 3. Sterol and Triterpenic Alcohol Composition of *T. polium*, *T. nabli*, and *T. alopecurus* Seed Oil, %

| Compound | RRT** | *T. nabli** | *T. polium** | *T. alopecurus** |
|---|---|---|---|---|
| | | Sterol | | |
| Campesterol ($\Delta^5$) | 1.22 | 7 | 8.4 | 3.6 |
| Stigmasterol ($\Delta^5$) | 1.31 | 20.7 | 25.8 | 21.8 |
| Clerosterol ($\Delta^5$) | 1.45 | 44.3 | 47.0 | 58.9 |
| Sitosterol ($\Delta^5$) | 1.48 | 24.6 | 10.4 | 12.0 |
| Obtusifoliol (methylsterol) | 1.75 | 3.2 | 8.4 | 3.7 |
| | | Triterpenic alcohol | | |
| $\beta$-Amyrine | | 10.2 | 11.6 | 4.0 |
| Lanosterol | | 4.0 | 6.2 | 3.6 |
| Not identified | | 8.4 | 16.3 | 6.5 |
| A′, Neogammacer-22(29)-en-3-ol | | 57.7 | 43.5 | 50.0 |
| 24-Methylenecycloartanol | | – | – | 6.7 |
| Fern-7-en-3$\beta$-ol | | 19.7 | 22.4 | 29.2 |

*Quantification of the sterol was based on the area of the GC peaks.

**Gas chromatographic retention times of silinazed sterol are relative to cholesterol.

There are not many studies reported on the isolation of sterols. H. Gaspar et al. [17] reported the isolation of clerosterol, poriferasterol, and (24*S*)-24-ethylcholesta-5,22,25-trien-3$\beta$-ol from *T. abutiloides* and *T. betonicum*. Sitosterol and clerosterol were detected in *T. chamaedrys* [17]. G. Fontana [18] reported also the isolation of 6′-fatty acid esters of (24*S*)-24-ethylcholesta-5,25-dien-3-yl-glucopyranoside from *T. fruticans*. All these studies have been conducted on the polar chromatographic fraction of the acetone extract of aerial parts of different species of *Teucrium*, but the seed of these plants has not been investigated. GC analyses of the sterol isolated from *T. polium, T. nabli*, and *T. alopecurus* seed oil showed that it has the same chromatographic profile of phytosterol but with quantitative differences.

The sterol composition was determined by GC/FID and GC/MS and reported in Table 3. *Teucrium* seed oil comprised four $\Delta^5$ sterols (campesterol, stigmasterol, clerosterol, sitosterol), wherein the sterol marker was clerosterol, which comprised 44.3%, 47%, and 58.9%, respectively, in *T. nabli*, *T. polium,* and *T. alopecurus* of the total sterol content, followed by stigmasterol and sitosterol, which percentages are different according to the species.

*Teucrium* seed oil is a rich source of phytosterol, with the highest concentration of 363.6 mg/100 g of oil in *T. nabli*, 330.4 mg/100 g of oil in *T. polium*, and 349.7 mg/100 g *in T. alopecurus*. We notice the presence of methylsterol (obtusifoliol) in a relatively high amount (8.4%) in *T. polium* seed oil, while it was only 3% in the other species.

Many studies have reported the hypolipidemic effect [12, 13] of *Teucrium* species. The presence of phytosterol in this plant in high amounts may be the cause of this effect. Indeed, phytosterols have long been known to reduce serum LDL-C level by competing with dietary and biliary cholesterol for intestinal absorption [4].

We also notice in the gas chromatographic profile of sterols the presence of another peak, which was identified by GC/MS as triterpenic alcohol. The major compound is A′, neogammacer-22(29)-en-3-ol and fern-7-en-3$\beta$-ol in the three species. The role of these triterpenic alcohols is not very clear, but it seems to be a necessary precursor for biosynthesis of sterols and saponins.

There are four natural tocopherols ($\alpha$, $\beta$, $\gamma$, $\delta$). The most commonly used method for their analysis is normal phase HPLC with fluoresence detection. Using this method, we can detect the four tocopherols at nearly the same amounts among the three species (see Table 1).

The major tocopherol in *Teucrium* seed oil was the $\alpha$-isomer, at 296, 316, and 277 mg/kg in, respectively, *T. nabli*, *T. alopecurus*, and *T. polium*, while $\beta$-tocopherol is the lowest tocopherol detected. *Teucrium* seed oil is a potential source of tocopherol, with a total content ranging from 534 to 569 mg/kg of oil.

The presence of sterols and polyunsaturated fatty acids C18:2 and C18:3 is favorable for the reduction of serum levels of cholesterol and triglycerides. The amount of vitamin E, ranging from 534 to 569 mg/kg of oil, can also prevent chronic diseases and certain types of cancer. Although *Teucrium* provides low yields of oil, the results obtained show that the seed oil of *Teucrium* species can be very important in pharmaceutical use.

## EXPERIMENTAL

**Materials.** Mature seeds of *Teucrium alopecurus* were collected from Matmata; the seeds of *Teucrium nabli* and *Teucrium polium* were collected from Gafsa (center of Tunisia) in April, 2005. The plants were identified by Prof. Z. Ghrabi, National Agronomic Institute of Tunis, University of Tunis. Voucher specimens of these plants have been deposited at the Herbarium of the National Agronomic Institute of Tunis.

**Reagents**. Potassium hydroxide (KOH) pellets were from Scharlau; 95% sulfuric acid ($H_2SO_4$) was from Riedel-de Haen. Standards used for sterol characterization were purchased from Fluka, and those used for vitamin E ($\alpha$, $\beta$, $\delta$, and $\gamma$-tocopherol) were purchesed from Roche.

**Oil Extraction.** Seeds of *Teucrium* species (*T. polium*, *T. alopecurus*, *T. nabli*) were ground in a grinder. Oil was extracted using hexane in a Soxhlet extractor for 8 h (50 g sample in 150 mL hexane). Seed oil was stored at –20°C for further analysis.

**Fatty Acid Methyl Ester Analysis (FAMEs).** Glycerides present in the natural lipid were saponified, and fatty acids were liberated and esterified. Seed samples (350 mg) were saponified with 10 mL of 1 N KOH in methanol, and the mixture was refluxed for 10 min. Then 5 mL of hexane and 1 mL of $H_2SO_4$ in methanol were added, and the whole incubated at 50°C for 6 h. The hydrocarbon layer was washed with a saturated solution of sodium chloride and dried with anhydrous sodium sulfate. The fatty acid methyl ester was analyzed using an Agilent Technologies 6890 gas chromatograph equipped with a flame ionization detector (FID). The analyses were performed on an HP-5 column (30 m × 0.32 mm, film thickness 0.25 μm). Oven temperature program, 150–260°C (8°C/min), 260°C (40.0 min); injector temperature, 270°C; carrier gas, helium at 1mL/min flow rate; split ratio 1/20; detector temperature, 300°C.

For the identification of FAMEs, a 1 μL sample was injected onto a GC-HRMS Autospec M 610 mass spectrometer coupled to an Agilent Technologies 6890 gas chromatograph. The analyses were performed under the same condition.

**Gas Chromatography Analysis of Sterols.** The qualitative and quantitative sterol contents of the samples were determined according to the international ISO (12228: 1999) [19]. The oil sample was saponified with 5 mL ethanolic potassium hydroxide solution. The unsaponifiable fraction was cleaned on an aluminium oxide column chromatography (from Scharlau AL0835) using ethanol and ethyl ether. Then the sterol fraction was separated by TL chromatography. The remaining ethyl ether was evaporated under a stream of nitrogen. Then a mixture containing 50 μL of pure 1-methylimidazole and 1mL of *N*-methyl-*N*-(trimethylsilyl-heptafluorobutyramide) was added. The mixture was shaken and heated at 105°C for 15 min.

The analyses of the silanized sterol fraction was carried out by capillary column gas chromatography on an Agilent Technologies 6890 chromatograph equipped with HP5 (30 m × 0.32 mm i.d.; film thickness : 0.25 μm). The working conditions of the chromatograph were: injector and detector at 270°C, oven temperature program, 240–255°C (4°C/min), 255°C (60 min). The injected quantity was 1μL at a flow rate of 1.2 mL/min using helium as carrier gas, with split ratio 1/20. Sterol peak identification was carried out according to the reference method. Quantification and identification was achieved by addition of an internal standard (cholesterol from Fluka) and comparison of their relative retention time (RRT) with those given by the norm [19]. The same samples were injected onto a GC-HRMS chromatography-mass specrometry (high-resolution mass spectrometry). An Autospec M 610 (Waters) mass spectrometer coupled to an Agilent Technologies 6890 gas chromatograph was used under the same conditions.

**Identification of Triacylglycerols Using High Performance Liquid Chromatography**. For HPLC analysis, the samples of seed oil (500 mg) were dissolved in 10 mL acetone/chloroform (1:1, v/v) according to the AOAC official method for determination of triglycerides in vegetable oil (1996) [20]. A 10 μL sample was injected into the liquid chromatographic system equipped with a differential refractometer and $C_{18}$ (hypersil ODS 250 × 4 mm) column, packed with 5 μm diameter silica. The elution solvent was 65/35 acetonitrile/acetone, and the column temperature 35°C. Reference triglycerides (triolein and tricaproin were purchased from Fluka) and common oils (soybean, olive, and rapeseed oils) were used as external standard. All solvents used were HPLC grade obtained from Merck (Darmstadt, Germany).

**Identification and Quantification of Tocopherol.** Tocopherols were analyzed by ISO (9936:1997) norm [21] with an Agilent 1100 series HPLC system consisting of a quaternary pump, an auto sampler, a fluorescence detector, and a normal phase column (150 mm × 3.2 mm, Pinnacle II Silica 3 μm). The elution solvent was a mixture of 0.5% (v/v) propan-2-ol in *n*-hexane, and the flow rate was 1 mL/min. The fluorescence detector excitation and emission wavelengths were set at 290 and 330 nm, respectively. Tocopherols were identified by comparison with an external standard solution of 95% $\alpha$, $\beta$, $\gamma$ and $\delta$-tocopherol (purchased from Roche). The quantization was based on $\beta$- and $\gamma$-tocopherol (Fluka). Seed oil was diluted with hexane to obtain a concentration of 2 g / 25 mL.

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