# GC/MS Analysis of Some Bioactive Constituents from *Carthamus lanatus* L.

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Sterols, triterpenes, volatiles, polar and other constituents in aerial parts of *Carthamus lanatus* were analyzed by gas chromatography-mass spectrometry. Over 90 compounds were identified most of them new for the species. Sitosterol and stigmasterol were the most abundant of 10 sterols identified in the sterol fraction. Taraxasterol, α- and β-amyrine prevailed in the triterpene fraction. Volatiles, sterols and a fraction of the dichloromethane extract showed strong cytotoxicity (*Artemia salina* assay).

*Key words: Carthamus lanatus*, GC/MS Analysis, Cytotoxic Activity

## Introduction

*Carthamus lanatus* L. (Asteraceae) is a biennial plant growing in the Mediterranean, which possesses sedative, anti-tumor and interferon-inducing activities (Benedi *et al.*, 1986; Yasuhuko *et al.*, 1979). Phytochemical studies of the species resulted in isolation of sesquiterpene glycosides (Feliciano *et al.*, 1990), flavonoids (El-Shaer *et al.*, 1998; Novruzov and Shamsizade, 1998), aromatic acids, serotonins (Lahloub *et al.*, 1993), lipids (Demir *et al.*, 1978), amino acids, carbohydrates (Yasuhuko *et al.*, 1979). Sterols and triterpenes isolated from *C. tinctorius* species were found to possess important biological activities, which encourages studies on these compounds. GC/MS studies on the composition of sterols, triterpenes, volatiles and other constituents in aerial parts of *C. lanatus* collected in Bulgaria are presented. Most of the compounds were found for the first time for this species. The cytotoxicity of the main fractions was studied and the biological activity of many individual compounds shortly reviewed.

## Experimental

### *Plant material*

Aerial parts of *Carthamus lanatus* were collected in July at the Losen village region. A voucher specimen (No 156639) is deposited in the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM). The plant was identified by Dr. Rilka Taskova.

### *Isolation and GC/MS analysis of sterols and triterpenes*

1.5 kg dry and ground aerial parts of *C. lanatus* were consecutively extracted with 15 l dichloromethane and 15 l methanol. The concentrated dichloromethane (29 g) was partitioned between upper and lower (12 g) layer of hexane-methanol-water (19:19:2, v/v/v). Part (5 g) of the lower layer of the partitioned dichloromethane extract was separated on silica gel (Merck) column with hexane and hexane/ethylacetate (20:1 to 1:10, v/v) (20 ml fractions were collected). Crude fr. 43–49 (28 mg; fraction A; triterpene mixture: on TLC with $R_f$ of β-amyrine), fr. 53–57 (31 mg; sterol mixture) and fr. 62–64 (90 mg; fraction B; on TLC with $R_f$ of oleanolic acid) were separated and further purified by SEP-Pak $C_{18}$ cartridges for rapid sample preparation (Waters, Milford, USA) with methanol. The purified samples were analysed by GC/MS.

For GC/MS a Hewlett Packard gas chromatograph 6890 series II Plus linked to Hewlett Packard mass spectrometer system equipped with a capillary column HP5-MS (30 m × 0.25 mm, 0.25 μm film thickness) was used. The temperature was programmed from 230 °C to 300 °C at a rate

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of 4 °C $min^{-1}$ with 10 min hold. Injector was at 280 °C. Helium was used as a carrier gas with a constant flow at 0.8 ml $min^{-1}$. The ionization voltage was 70 eV. Fraction B was analyzed also after silylation at the conditions given for the silylated polar compounds mentioned below.

Quantitative analysis of sterols was performed on a Hewlett Packard gas chromatograph 5890 equipped with FID and capillary column HP5-MS (30 m × 0.25 mm, 0.25 μm film thickness), at 230 °C and programmed to 300 °C at 4 °C $min^{-1}$ and 10 min hold. Injector and detector were at 280 °C. 1 μl of each sample were injected triplicate split/spiltless and quantities represented as relative area% as derived from the intergrator.

*Isolation and analysis of volatiles*

Two samples of *C. lanatus* of fresh minced aerial parts and flowers were subjected for 4 h distillation-extraction in a Likens-Nickerson apparatus. The volatiles were collected in diethyl ether and dried over anhydrous $Na_2SO_4$ (0.06% and 0.02% of dry wt). The GC/MS analysis was performed with GC/MS equipped with a capillary column HP5-MS (30 m × 0.25 mm, 0.25 μm film thickness). The temperature was programmed from 40 °C to 280 °C at a rate of 6 °C $min^{-1}$. The ion source was set at 250 °C. Helium was used as a carrier gas with a constant flow at 0.8 ml $min^{-1}$.

*Isolation and analysis of polar compounds*

Fresh aerial parts were minced and extracted with methanol. Water was added to the concentrate and successive extraction with trichloromethane and *n*-butanol was carried out. 5 mg of the concentrated butanol extract were subjected to silylation with 50 μl pyridine and 75 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture was heated at 80 °C for 30 min and analyzed by GC/MS equipped with a capillary column HP5-MS (30 m × 0.25 mm, 0.25 μm film) at 100 °C and programmed to 300 °C at 5 °C $min^{-1}$ and 10 min hold. Injector temperature 280 °C.

*Identification of compounds by GC/MS analysis*

The identification was accomplished using computer searches by NIST98 Wiley MS Data library. In some cases where identical spectra were not found only the structural type of the component was proposed based on the MS fragmentation. When possible reference compounds were co-chromatographed to confirm GC retention times.

*Cytotoxicity assay*

The brine shrimp (*Artemia salina*) assay was performed in triplicate with appropriate amounts of samples dissolved in DMSO (1% final volume) using 10 freshly hatched larvae, suspended in 5 ml artificial sea water (Solis *et al.*, 1993; De Rosa *et al.*, 1994). Concentrations of 1, 0.1, 0.01 and 0.001 mg/ml were used. For each dose tested deaths and survivors were counted after 24 h and data statistically analyzed by the Finney program, which affords $LC_{50}$ values with 95% confidence intervals. Caffeic acid phenetyl ester (CAPE) was used as active reference substance.

## Results and Discussion

*Sterol composition*

Sterols are important constituents of all eukaryotes and play vital role in plant cell membranes. Plant sterols possess valuable physiological activities, they are biogenetic precursors of many hormones and oviposition stimulants of some insects (Harborne, 2001).

The sterol fraction was analyzed by GC/MS. The data are summarized in Table I. Three groups of sterols were found together, sterols with double

Table I. Sterol composition (% of the total sterol fraction)*.

| Sterol | Abundance % |
|---|---|
| Cholesterol | 0.5 |
| Ergost-5-en-3β-ol (campesterol) | 3.4 |
| 24-Methylcholestan-3-ol | 2.1 |
| 24-Ethylcholesta-5,22-dien-3β-ol (stigmasterol) | 25.1 |
| Stigmast-22–3β-ol | 0.7 |
| Ergost-7-en-3β-ol | 1.8 |
| Stigmasta-7,25-diene-3β-ol | 1.5 |
| Stigmast-5-en-3β-ol (sitosterol) | 45.1 |
| Stigmastan-3β-ol (fucostanol) | 5.1 |
| Stigmast-7-en-3β-ol | 13.8 |

* Values obtained from three parallel measurements. The standard deviations (related to peak proportion on the chromatograms) are as follows: ± 0.3 for cholesterol and ± 0.1 for the others.

bonds at C-5 or C-7 and stanols with $\Delta^5$-sterols prevailing. The typical plant sterols, sitosterol and stigmasterol, appeared as main sterol components, while cholesterol was present in negligible concentrations. Sitosterol possesses antihyperlipoproteinaemic, antibacterial and antimicotic activity and has been shown to act as inhibitor of tumor promotion *in vivo* (Yasukawa *et al.*, 1991) and to inhibit carcinogenesis (Raicht *et al.*, 1980). Stigmasterol was found to markedly inhibit tumor promotion in two-stage carcinogenesis in mice (Kasahara *et al.*, 1994; Yasukawa *et al.*, 1991) and to exhibit significant inhibitory effect on HIV reverse transcriptase (Akihisa *et al.*, 2001). A mixture of stigmasterol and sitosterol was shown to possess anti-inflammatory activity after topical application (Gomez *et al.*, 1999). Therefore, the presence of these sterols in *C. lanatus* is of practical importance.

The sterol composition of *C. lanatus* differed to some extent from those found in other higher plants, because usually stigmasterol is not among the main sterols in plants. However, it is the main sterol in *C. lanatus* and in *C. tinctorius*, another species of the genus (Kasahara *et al.*, 1994). Hence high concentrations of stigmasterol might be a distinguishing characteristics of the genus *Carthamus*.

Two stanols, 24-methylcholestan-3-ol (campestanol) and fucostanol, were identified within this study. Campestanol is distributed in plant oils consumed in human diets, which is not accumulated when fed and incorporated into the diet may block cholesterol absorption (Xu *et al.*, 1999). Stigmastan-3-ol has been reported to inhibit HIV-1 reverse transcriptase (Akihisa *et al.*, 2001). Sterols with C-7 double bond occur relatively seldom in plants. They could be used for treatment of some prostate problems (Gomez *et al.*, 1999).

The complex composition and the variety of biological activities of sterol compounds in *C. lanatus* show the potential of future investigations on chemotaxonomy and practical application of this species.

### *Triterpene composition*

Triterpenes attract attention because of their biological activities. Two fractions of different polarity (fractions A and B corresponding to zones with $R_f$ of amyrine and oleanolic acid) were separated and investigated. The fractions were isolated after column chromatography and purified by SEP-Pak $C_{18}$ cartridges. The analysis of fraction A was performed without derivatization by means of GC/MS (Table II). In fraction A prevailed taraxasterol, followed by β-amyrine and α-amyrine. Taraxasterol was shown to exhibit considerable activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory ear oedema in mice and tumor promotion in mouse skin (Akihisa *et al.*, 1996). Triterpene alcohols from Compositae flowers were demonstrated to possess marked anti-inflammatory activity (Akihisa *et al.*, 1996). Taraxerol possesses antiulcer properties. α-Amyrine, lupeol and cycloartan-type triterpenes are cytotoxic agents (Banskota *et al.*, 1999).

The more polar fraction B was analysed as such and after silylation by GC/MS (Table II). Different type of compounds were found in this fraction. Dehydroabietic acid was present in considerable amounts. Dehydroabietic acid is an antibacterial (Soderberg *et al.*, 1990), antiinflammatory (Li and McChesney, 1992) and potential antitumor-promoting (Kinouchi *et al.*, 2000) agent. The identified betulin is antineoplastic agent. Inhibitory effect on TPA-induced inflammation and inhibitory activities against tumor promotion in mice of this triterpenoid was shown (Yasukawa *et al.*, 1991). Three flavone aglycons, three anthraquinones and vitamin E were identified. Flavonoids possess anticarcinogenic and anti-inflammatory properties. Chrysin is anti-inflammatory and antibacterial agent and recently have been found to exhibit anti-HIV-activity (Wang *et al.*, 1998). Two of the main constituents of this fraction with molecular mass 410 were not identified because of the lack of reference compounds. Among the carboxylic acids prevailed hexadecanoic acid. The fatty acids are well known active metabolites. They serve as an important energetic substrate for the cells. Linoleic acid is essential for maintenance of growth and α-linolenic acid for neural functions. Both acids were shown to be potent cycloxygenase-2 (COX-2) catalyzed prostaglandin biosynthesis inhibitors (Ringbom *et al.*, 2001).

### *Volatiles*

Volatile compounds often possess valuable biological activities. They serve as allelochemicals defending plants from bacteria, fungi and viruses and

Table II. Triterpene composition (fraction A, zone with $R_f$ of amyrine) and composition of fraction B (zone with $R_f$ of oleanolic acid) analyzed by direct GC/MS and after silylation (%)*.

| Compound | Abundance % |
|---|---|
| ***Triterpene composition**** | |
| Eicosanol*** | 2.3 |
| Helianol | 0.5 |
| Tarax-14-en-3β-ol (taraxerol) | 0.3 |
| Olean-12-en-3β (β-amyrine) | 12.0 |
| Urs-12-en-3β-ol (α-amyrine) | 9.6 |
| Lup-20(29)en-3β-ol (lupeol) | 0.3 |
| 24-Methylenecycloartan-3β-ol (24-methylenecycloartanol) | 3.8 |
| Hop 22(29)-3-one | 0.8 |
| Hop-22(29)-3-ol | 2.0 |
| Taraxast-20(30)-en-3β-ol (taraxasterol) | 65.2 |
| ***Composition of fraction B**** | |
| *Acids* | |
| Eicosanoic acid | 2.5 |
| *Terpenes* | |
| Dehydroabietic acid | 20.9 |
| *Flavonoids* | |
| 5-Hydroxy-6,7-dimethoxy-flavone | 5.3 |
| 5-Hydroxy-7-methoxy-flavone | 0.5 |
| Chrysin | 5.2 |
| *Others* | |
| Vitamin E | 0.2 |
| Unidentified M 410 | 14.1 |
| Unidentified M 410 | 16.1 |
| Betulin isomer | 3.7 |
| Betulin | 2.7 |
| ***Composition of silylated fraction B*** | |
| *Carboxylic acids* | |
| 2-Hydroxy-propanoic acid | 0.7 |
| Dodecanoic acid | 0.3 |
| Tetradecanoic acid | 0.9 |
| Pentadecanoic acid | 0.8 |
| Hexadecanoic acid | 24.3 |
| Heptadecanoic acid | 1.2 |
| Linoleic acid | 7.9 |
| α-Linolenic acid | 3.4 |
| Octadecanoic acid | 2.8 |
| Eicosanoic acid | 0.5 |
| *Terpenes* | |
| Dihydroactinidiolide | 0.2 |
| Dehydroabietic acid | 11.6 |
| *Phenolics* | |
| 3-Methoxy-4-hydroxy-benzaldehyde | 0.2 |
| 2-Methoxy-4-(1-propenyl)-phenol | 0.8 |
| 3-Methoxy-4-(1-propenyl)hydroxy-benzaldehyde | 0.2 |
| *Anthraquinones* | |
| 1-Hydroxy-3-methoxy-6-methylanthraquinone | 0.5 |
| 1,6-Dihydroxy-3-methylanthraquinone | 3.5 |
| 1,6-Dihydroxy-8-methoxy-3-methylanthraquinone | 0.5 |
| *Others* | |
| Unidentified M 410 | 17.3 |
| Unidentified M 410 | 9.0 |

* The ion current generated depends on the characteristics of the compound and is not a true quantitation.
** Analyzed by direct GC/MS.
*** The non-triterpene alcohol eicosanol present in this fraction is also included.

take part in plant-insect relationships. The volatiles from flowers and aerial parts of *C. lanatus* were obtained by distillation-extraction and analyzed by GC/MS. The data obtained are sumarized in Table III.

As it was expected, the hydrocarbons were present in higher concentrations in the volatiles from flowers. Saturated straight chain hydrocarbons with 21–31 carbon atoms were identified. The main hydrocarbons in the flowers contained 23–27 carbon atoms and, respectively, 25–31 carbon atoms in the aerial parts. Hydrocarbons with odd number of carbon atoms predominated in both samples, especially in the flowers. Acetylenic compounds are known to be present in *Carthamus* species (Chapman and Hall, 1996). 3-Tetradecene-1-yne was identified in the volatile fraction isolated from flower and aerial parts. Two other constituents showed mass spectral fragmentation typical for such compounds, but their structures could not be determined. Toluene was found only in low concentrations in the flowers.

Terpenoids are an important part of volatiles from plants. Most of them possess different allelochemical functions. In *C. lanatus* were identified two sesquiterpenes, α-bisabolol and caryophyllene oxide. α-Bisabolol fucopyranoside is a main constituent of *C. lanatus* (Feliciano *et al.*, 1990). Now the free sesquiterpene alcohol was found. α-Bisabolol possesses antibacterial and antifungal activities, which indicates defensive functions in the investigated plant. Caryophyllene oxide is well known as preservative in food, drugs and cosmetics. It is an antibacterial and antifungal agent (Yang *et al.*, 1999), suggested as potential anticar-

Table III. Volatile compounds from *C. lanatus* (% from the total volatiles)*.

| Compounds | Flowers (%) | Aerial parts (%) |
|---|---|---|
| ***Hydrocarbons*** | | |
| 3-Tetradecen-5-yne | 0.3 | 0.2 |
| Heneicosane | 0.6 | 0.2 |
| Docosane | 2.3 | 0.5 |
| Tricosane | 7.7 | 3.0 |
| Tetracosane | 8.8 | 2.1 |
| Pentacosane | 9.6 | 3.3 |
| Hexacosane | 6.6 | 2.7 |
| Heptacosane | 8.7 | 3.5 |
| Octacosane | 4.5 | 3.1 |
| Nonacosaner | 3.5 | 3.6 |
| Triacontane | 2.6 | 4.1 |
| Hentriacontane | 2.0 | 4.7 |
| ***Aldehydes and ketones*** | | |
| 2,5-Furandione-3-(1,1-dimethyl ethyl) | | 0.1 |
| Nonanal | 0.3 | 0.1 |
| Decanal | 0.2 | 0.1 |
| Dodecanal | 0.2 | 0.1 |
| 2,5-Cyclohexadiene-1,4-dione-2,6-bis(1,1-dimethyl ethyl) | 0.7 | 0.2 |
| 2,5-Cyclohexadiene-1-one-2,6-bis-(1,1-dimethyl ethyl)-4-ethylidene | 3.2 | 0.6 |
| ***Acids*** | | |
| 2-Methyl butanoic acid | 0.2 | – |
| ***Aromatics*** | | |
| Toluene | 0.1 | – |
| Benzene isocyanate | 0.3 | 0.1 |
| Phenol-4,6-di-(1,1-dimethyl ethyl)-2-methyl | 0.3 | 0.1 |
| Benzene-1,1′-(1,1,2,2-tetramethyl-1,2-ethane diyl)bis | 1.2 | 0.3 |
| 2,4-Diphenyl-4-methyl-1-pentene | 0.5 | 0.3 |
| ***Terpenes*** | | |
| α-Bisabolol | 0.6 | 0.6 |
| Caryophyllene oxide | 0.3 | 0.2 |
| ***Sulfur compounds*** | | |
| Dimethyl disulfide | 0.1 | 0.3 |
| Methyl sulfonyl ethane | 0.1 | 0.1 |
| 1,2-Benzisothiazole | 0.2 | 0.1 |
| Cycloctanoic sulfur | 5.8 | 1.4 |
| ***Others*** | | |
| 1,1,2,2-Tetrachloroethane | 3.4 | 1.0 |
| Erucyclamide | 8.4 | 6.6 |

* The ion current generated depends on the characteristics of the compound and is not a true quantitation.

cinogenic agent (Zheng *et al.*, 1992) and found to exhibit cytotoxic activity against several solid tumor cell lines (Kubo *et al.*, 1996).

Aromatic compounds were also found. Phenolics usually possess antimicrobial and antifungal activities and consequently defensive functions.

Aldehydes and ketones often act as allelochemicals. Such activities were proven for the three identified by us aldehydes, decanal is attractant for some insects (Mattiacci *et al.*, 2001; Wang *et al.*, 1999), nonanal is a repellent (Huber and Borden, 2001; Wang *et al.*, 1999), dodecanal has some pheromone-like activity (Cosse *et al.*, 2002).

A few halogen-containing compounds were found in the investigated volatiles, but we succeeded to identify only 1,1,2,2-tetrachloroethane. Chlorinated ethanes were found recently in some algae and higher plants, but their functions are not clear. Some of them have a high carcinogenic potential (Greim and Wolf, 1984). The identified by us perchloroethane was shown to be cancerogenic in mice, but not in rats and also is a weak mutagene.

The volatile fraction contained some sulfur compounds including cyclooctanoic sulfur. Dimethyl disulfide and analogous compounds are found in many biologically active extracts. Dimethyl disulfide attracts some insects (Reddy *et al.*, 2002) and is used to improve the fragrance and taste of some foods (Ren *et al.*, 2001).

### *Polar constituents*

After separation of the lipophylic compounds with trichloromethane from the methanol extract the aqueous part was extracted with *n*-butanol. The concentrated butanol extract was silylated and the polar constituents possessing hydroxyl, amine and carboxyl groups transformed into volatile TMS-ethers were analyzed by GC/MS. In this complex mixture were identified 29 compounds (about 25% of the mixture) belonging to different groups as alcohols, acids, hydroxy acids, polyhydroxy acids, esters, sugars, amino acids and terpenoids.

Free fatty acids and oxidized acids are important constituents of butanol fractions from higher plants, algae and invertebrates. The main fatty acids of the plant were found in free state. Such free acids possess defensive functions in plants, be-

cause of their insecticidal and antimicrobial activity (Kanias *et al.*, 1992). Caffeic acid possesses antimicrobial, antifungal and antiviral activities. Benzenepropionic acid and its α-hydroxy derivative are relatively rare in higher plants and can be used as a characteristic feature of *C. lanatus*. 2-Hydroxy propanoic acid is regularly identified in butanol fractions of higher plants and algae, whereas malic acid and butanedioic acid are characteristic for some higher plants. 3-Methoxy-4-hydroxy benzoic acid is a defensive compound in plants, due to its antibacterial and antifungal activities. Similar activity might possess the phosphoric acid methyl ester.

The presence of the amino acid prolin was also established.

As it was expected carbohydrates were found in this fraction. Significant concentrations of glucose and fructose were identified, followed by lower concentrations of sucrose. *myo*-Inositol and an unidentified isomer were also present.

Sulfates often have valuable biological activities. Two terpenoid sulfur derivatives, menthyl *t*-butyl sulfinate and dimenthyl sulfite, were identified in the butanol fraction, which could contribute to the biological activities of the investigated plant.

Unexpected, sitosterol and stigmasterol were found in the polar fraction. According to the extraction procedure the sterols should be completely extracted with trichloromethane from the total extract, where were identified as mentioned above. Sitosterol and stigmasterol, which were found in the butanol fraction, might be explained with presence of unstable complexes of sterols with polar compounds like sugars, amino acids, etc. not soluble in trichloromethane. Such complexes should remain in the polar fractions. Probably through the silylation procedure they undergo destruction and set free the sterols, which form TMS ethers. Recently, in two marine sponges (*Chondrosia reniformis* and *Verongia aerophoba*) significant amount of sterols were found in the butanol fraction by the same procedure (Nechev *et al.*, 2002).

### *Cytotoxic activity*

Six fractions from *C. lanatus* were screened for cytotoxic activity by using Brine shrimp (*Artemia salina*) assay and as active reference substance, caffeic acid phenetyl ester (CAPE), ($LD_{50}$ = 0.45 ± 0.05 μg/ml). The water/methanol fraction of the dichloromethane extract showed a significant higher activity ($LD_{50}$ 47.99 ± 17.49) than the butanol fraction of the methanol extract ($LD_{50} > 1000$). After separation of the former three sub-fractions were obtained and two of them, fraction B ($LD_{50}$ 1.57 ± 1.25) and the sterol fraction ($LD_{50}$ 13.99 ± 9.10), showed even higher activities. The volatile fraction exhibited also a strong cytotoxic activity ($LD_{50}$ 4.37 ± 1.14).

It could be concluded that *C. lanatus* contains various bioactive compounds including such with strong cytotoxic activity and could be recommended as a plant of phytopharmaceutical importance.

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