

# Separation and Identification of Antibacterial Chamomile Components Using OPLC, Bioautography and GC-MS

Ágnes M. Móricz<sup>1\*</sup>, Szabolcs Szarka<sup>2</sup>, Péter G. Ott<sup>1</sup>, Éva B. Héthelyi<sup>2</sup>, Éva Szóke<sup>2</sup> and Ernő Tyihák<sup>1</sup>

<sup>1</sup>Plant Protection Institute, Hungarian Academy of Sciences, Herman O. Str. 15, 1022 Budapest, Hungary

<sup>2</sup>Department of Pharmacognosy, Faculty of Pharmacy, Semmelweis University, Üllői Str. 26, 1085 Budapest, Hungary

**Abstract:** Components of 50% aqueous ethanol chamomile (*Matricaria recutita* L.) flower extract, previously found antibacterial in a TLC-bioautographic study, were separated and isolated by the use of on-line overpressured layer chromatography (OPLC). This system consisted of an OPLC 50 BS system, an on-line coupled flow-through UV detector, and a manual fraction collector. The collected fractions were investigated by GC-MS analysis and by TLC re-chromatography with subsequent visualization, performed after use of the vanillin-sulphuric acid reagent, or under UV illumination, or applying bioautographic detection. The main compounds of the collected 11 fractions were identified by GC-MS. The results showed that the antibacterial effect of 50% aqueous ethanol extract of chamomile is ascribable to cis-, trans-spiroethers, and the coumarins like herniarin and umbelliferone.

**Keywords:** Antibacterial activity, bioautography, chamomile, coumarins, GC-MS, *Matricaria recutita* L., OPLC, spiroethers.

## INTRODUCTION

Infectious agents are the most changeable risk factors for human diseases [1]. Because of the increasing global problem of emerging resistance of pathogens against antibiotics [2], there is an increased demand to discover new, effective, easy-to-obtain natural medicinal substances. Chamomile (*Matricaria recutita* L.) is a very popular and widely used herb in traditional folk medicine. The chamomile flowers are commonly used as ingredients of herbal teas because they contain substances which have anti-inflammatory, sedative, analgesic, anti-spasmodic, antioxidant and antimicrobial properties [3-6]. The beneficial effects are related to different classes of therapeutically interesting ingredients such as essential oil components, flavonoids, and coumarin derivatives [3, 7, and 8].

The chamomile essential oils, which are of the greatest importance among the bioactive constituents [9], consist of many active compounds such as (-)-alpha-bisabolol, chamazulene (artefact of matricine by steam distillation), (-)-alpha-bisabolol oxide A and B, (-)-alpha-bisabolone oxide A, spiroethers (cis- and trans- en-yn-dicycloether), and sesquiterpenes [10].

The chamomile volatile oils showed antimicrobial activity against certain species of bacteria, fungi and viruses *in vitro* [11], however, Gram-positive bacteria were found more susceptible than Gram-negative ones [12]. The chamomile oils were effective e.g. against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Streptococcus salivarius* bacteria as well as against *Candida albicans*, *As*

*pergillus niger*, *A. ochraceus*, *A. flavus*, *A. parasiticus*, *Fusarium culmorum* and *F. moniliforme* fungi [11-14].

It has been reported that generally the aqueous chamomile extracts were more effective against moulds and yeast than bacteria, while the alcoholic ones characteristically inhibited the growth of bacteria [15]. The inhibiting activity of hydro-alcoholic extract of chamomile was demonstrated against the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, group B *Streptococcus* and *Streptococcus salivarius*, *Streptococcus faecalis*, *Bacillus megatherium* and *Leptospira icterohaemorrhagiae* [16]. Moreover, olive oil extract of chamomile has been proved active against *Helicobacter pylori* [17].

Coumarin-related compounds, ingredients in hydro-alcoholic extracts of *M. recutita*, also represent an important group of secondary metabolites of chamomile. The coumarins are phytoalexins of many species [18], exhibiting a very weak antibacterial and a stronger antifungal activity [19, 20]. Herniarin, the main coumarin component in chamomile, is a more effective antimicrobial agent than umbelliferone [21]. Umbelliferone has been described as a stress metabolite in *M. recutita*. The amount of umbelliferone characteristically, while that of herniarin mildly rose in the plant after biotic and abiotic stress influences [8, 22]. It was also observed that chamomile oil component cis-spiroether was lowered after abiotic stress, but increased in the case of biotic stress [8].

Searching for bioactive natural products requires appropriate bioassays, focused on the desired activity (e.g. anti-fungal, antibacterial). Planar layer chromatography is an ideal technical solution for the screening of natural extracts' ingredients, because of high-throughput, low cost, easy maintenance and selectivity of detection reagents [23]. Direct bioautography [24-28], linking the separation with biological detection, potentiates the fast and easy sorting of ef-

\*Address correspondence to this author at the Plant Protection Institute, Hungarian Academy of Sciences, Herman O. Str. 15, 1022 Budapest, Hungary; Tel: 0036-48-77-515; Fax: 0036-48-77-555; E-mail: moricz\_am@nki.hu

fective components in matrixes like plant extracts. In direct bioautography, the developed chromatoplates containing separated components in spots are dipped into an indicator cell suspension and, after a required incubation time, the inhibition zones are visualized using a vital dye, or suitable luminescent indicator cells [25-27]. So the evaluation of the activity is performed directly on the adsorbent layer, at the chromatographic spots.

Overpressured-layer chromatography (OPLC), as an efficient forced flow planar layer liquid chromatographic technique, results in more compact chromatographic spots and better separation efficiency than TLC-HPTLC. OPLC is also well suited for fractionation in semi-preparative ranges using analytical chromatoplates providing on-line detection and subsequent fraction collection [29, 30]. This system can be applied for efficient isolation of various substance types, among others the antimicrobial components from plant extracts.

In our earlier bioautographic experiments, the components of 50% aqueous ethanol chamomile flower extract showed the strongest antimicrobial effect against the plant pathogenic bacterium *Pseudomonas syringae* pv. *maculicola*, when compared with hexane, acetone and methanol extracts. The 50% ethanol extract ingredients (with the same  $R_f$  values) also inhibited, although with different strength the soil bacterium *Bacillus subtilis* [26].

In this paper the OPLC separation and isolation of antibacterial components of 50% aqueous ethanol chamomile extract, their re-chromatography, identification by means of GC-MS as well as bioautographic studies of concentrated, collected fractions are demonstrated.

## EXPERIMENTAL

### 1. Materials

Aluminum foil-backed normal particle silica gel 60F<sub>254</sub> plates (TLC, #5554 from Merck, Darmstadt, Germany) were used for TLC separation, as well as for OPLC but in this case the layers were sealed on all four edges (OPLC-NIT, Budapest, Hungary). All used solvents were analytical grade purchased from Reanal (Budapest, Hungary).

Triton X-100, dye reagent MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), alpha-bisabolol, umbelliferone, and alkane standard mixture (C<sub>10</sub>-C<sub>40</sub>; all even) were from Sigma-Aldrich (Budapest, Hungary).

### 2. Preparation of Extracts

Chamomile (*Matricaria recutita* L.) was collected in the end of May 2010, in Harta (in the Great Plain, Hungary) and dried at 25 °C, in the dark. 150 mg of roots, leaves and flowers were macerated for 48 h with 1 mL of 50% ethanol in 2 mL Eppendorf tubes. Samples were vortexed for 30 s, centrifuged (20 min, RCF of 10000 g, room temperature), and 10 µL of each supernatants was applied to a TLC plate that had previously been heat-treated for 3 h at 130°C. For fractionation and isolation 4.5 g of chamomile flowers (from the market; JuvaPharma) were macerated at room temperature in 30 mL 50 % aqueous ethanol for 48 h in a screw-capped glass bottle.

### 3. OPLC and TLC Separations

50% aqueous ethanol extracts of chamomile were fractionated by the use of a Personal OPLC BS50 system (OPLC-NIT, Budapest, Hungary) in the on-line operating mode [29, 30] on a TLC layer previously heated for 3 h at 130 °C. Conditions of the preparative on-line OPLC separation were as follows: off-line application of 0.5 mL extract in a 16 cm wide band at 3 cm from the edge, 50 bar external pressure, 450 µL rapid mobile-phase flush, 1 mL min<sup>-1</sup> mobile phase flow rate. The mobile-phase was 100% chloroform (0-30 min) and chloroform-acetone 95:5 (v/v) (from 30 min) and the detection was achieved using an on-line coupled flow-through UV detector at the wavelength of 350 and 320 nm, respectively. The fractions collected were concentrated by cold air stream.

To determine whether some compounds originated from chamomile or the adsorbent layer, the fractionation was performed under the same conditions but without sample application (blank). In this blank development 4 fractions were obtained. b1: 7.30-15 (in 1<sup>st</sup> gradient step), b2: 37.30-40 (parallel with chamomile fraction 5), b3: 40-45 (similar as the chamomile fraction 6), and b4: 45-55 min.

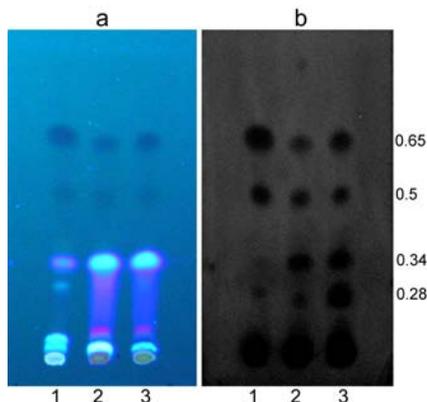
The fractions were re-chromatographed on a TLC layer; No. 1-4 were developed with chloroform-acetone 99:1 (v/v), and the No. 5-11 with chloroform-acetone 95:5 (v/v) as the mobile phase. Developed plates were dried by a cold air stream using a hair-drier (5 min). Visualization and detection were performed under UV illumination at 254 and 365 nm, after use of vanillin-sulphuric acid reagent (0.4 g vanillin, 100 mL ethanol, and 2.2 mL concentrated sulphuric acid) and heating (110°C, 3 min) and in bioautographic system.

### 4. Biological Studies of OPLC Fractions

The antibacterial effect of separated chamomile components was evaluated *in vitro* with direct bioautography [24-28] against a Gram-positive soil bacterium *Bacillus subtilis* (Bs) (strain F1276 [31]) and a Gram-negative luminescence-tagged plant pathogenic bacterium *Pseudomonas syringae* pv. *maculicola* (Psmlux) [32]. Bs was grown in Spizizen minimal broth [33] and Psmlux in King's B broth [34] at 28.5 °C and both cultures were well aerated by shaking to reach late exponential phase, exhibiting an optical density of 1.2 at 600 nm. The dried, developed chromatoplates with separated spots were immersed for 10 s into these bacterial cell suspensions.

The chromatoplates dipped into Bs suspension were incubated for 2 h in a humidity chamber at 30 °C, then immersed for 5 s in an aqueous solution of MTT (100 mg Triton X-100 and 80 mg MTT in 100 mL water). The incubation of the treated chromatoplates was continued for 1 h until inhibition zones appeared as clear regions against a darker background where the viable, metabolically active cells could reduce the yellow MTT to the bluish MTT-formazan. The image of the bioautogram was recorded by use of a scanner (Mustek 1200 ED Plus).

For detection of the bioluminescent light emission, the chromatoplates immersed into Psmlux cell suspension were put into a transparent glass cage ensuring an air phase above the adsorbent layer, and protecting against drying. The



**Fig. (1).** Antibacterial effect of 50% aqueous ethanol extracts of chamomile roots (1), leaves (2) and flowers (3) against *Pseudomonas savastanoi* pv. *maculicola*; Each extract (10  $\mu$ L) was applied and developed with chloroform-acetone 99:1 (v/v) as the mobile phase; The  $R_f$  values of the active spots are presented on the right side;

a - the developed plate seen under UV light (365 nm) before biological detection;

b - the bioautogram obtained by use of luminescent *Psmlux* bacteria. Their emitted light was collected with a cooled camera (dark areas mean inhibition zones).

bioautograms were documented immediately after inoculation by use of a computer-controlled cooled CCD camera (IS-4000, Alpha Innotech, San Leandro, USA) with the exposure time of 15 min. The light emitted by the bacterial cells is closely dependent on reductive metabolic activity (which in turn depends on viability), so the darker areas on the images indicate lack of metabolic activity.

### 5. Apparatus and GC-MS Conditions

The analyses of the fractions collected were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) coupled with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler. Separations were performed using an SLB-5ms (Sigma-Aldrich, St Louis, MO, USA) capillary column (30 m  $\times$  250  $\mu$ m  $\times$  0.25  $\mu$ m). The GC oven temperature was programmed from 60  $^{\circ}$ C (1 min isothermal) to 280  $^{\circ}$ C at 10  $^{\circ}$ C  $\text{min}^{-1}$  and finally held for 7 min. Helium was the carrier gas at 1.0 mL  $\text{min}^{-1}$  in constant flow mode. Injection volume was 1.0  $\mu$ L. The injector was programmed from 120  $^{\circ}$ C to 300  $^{\circ}$ C at 720  $^{\circ}$ C  $\text{min}^{-1}$  using a 40 psi pressure pulse for 1.0 min while splitless time was set to 0.8 min.

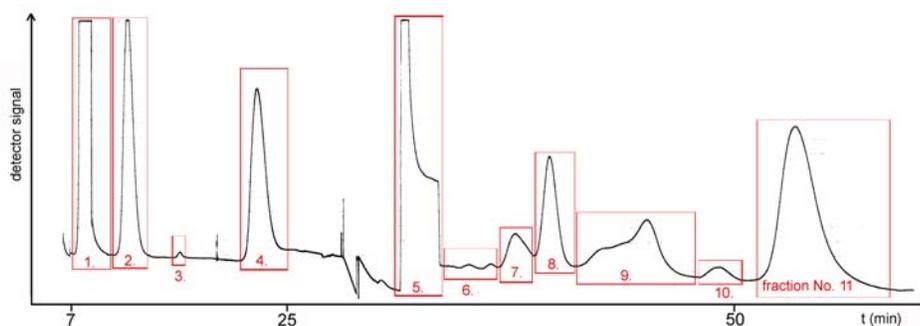
The mass selective detector was equipped with a quadrupole mass analyzer and was operated in electron ionization mode at 70 eV. Full scan analyses were performed in the mass range of 40-500 amu at 3.2 scan  $\text{s}^{-1}$ . Data were evaluated by MSD ChemStation D.02.00.275 software (Agilent).

The identification of the compounds was carried out by comparing retention times, retention indices and recorded spectra with data of authentic standards. Mass spectral and retention data known from the literature [10, 35-37] and the NIST 05 library were also consulted. The temperature programmed retention indices ( $I^T$ ) were calculated according to the equation given by Van den Dool and Kratz [38].

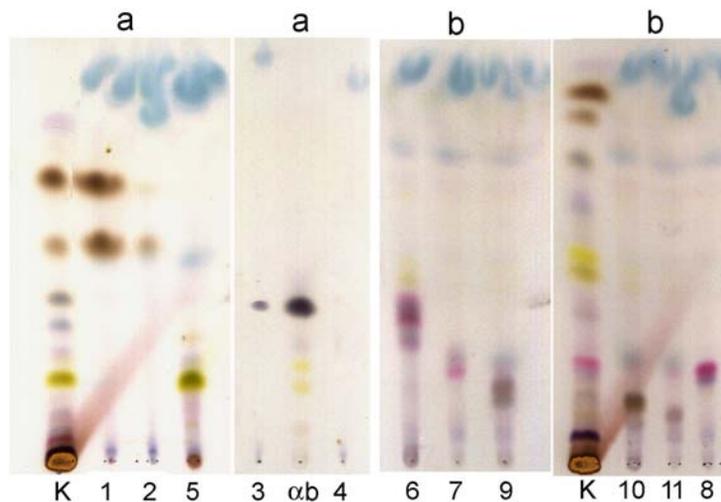
### RESULTS & DISCUSSION

50 % aqueous ethanol extracts of different chamomile organs were tested for richness in antibacterial components with the help of bioautography, a cheap and fast *in vitro* biological screening method. Fig. (1) shows that roots, leaves and flowers all contain compounds having an inhibitory and/or killing effect against luminescent gene-tagged *Pseudomonas syringae* pv. *maculicola* (see black areas on Fig. 1b). Although the root contains higher amounts of the two active components with  $R_f$  0.5 and 0.65, the other ones with  $R_f$  values 0.34 and 0.28 are almost totally missing. There are inhibition zones in chromatographic spots with the same  $R_f$  values in leaf and flower extracts, but in the flower extract they are stronger, therefore, flowers were selected for further investigation. We found that the yellow tubular florets contained a more diverse set of antibacterial molecules than the white ligular florets (not shown).

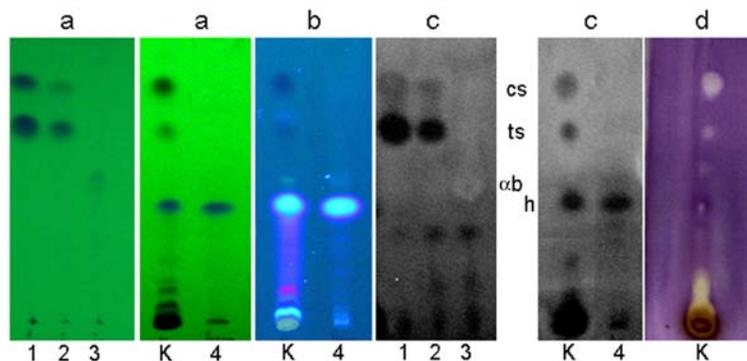
The exploitation of OPLC in the on-line operating mode seemed promising for the efficient separation and collection of ingredients of chamomile extracts (Fig. 2). The TLC rechromatography and development with vanillin-sulphuric reagent of the fractions 1-4, collected in the first gradient step, showed that the fractions were dominated by one or two main constituents (Fig. 3). The purity of these fractions was also showed by their photos taken under UV light (Figs. 4a, 4b). Fractions 1 and 2 contained the same two compounds in different proportion, and there was only one detectable component in fractions 3 and 4. Fractions 5-9 collected during the second gradient step looked like containing more than one or two main components (Figs. 3, 5a, 5b, 6a, and 6b).



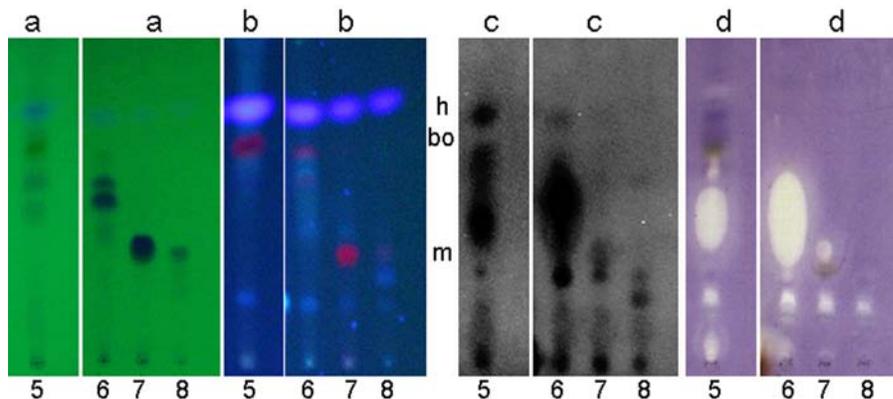
**Fig. (2).** OPLC separation of 0.5 mL chamomile flower extract and the 11 collected fractions; OPLC conditions were as follows: 50 bar external pressure, 450  $\mu$ L rapid mobile-phase flush, 1 mL  $\text{min}^{-1}$  mobile phase flow rate; The mobile-phase was: 0-30 min 100% chloroform (first step), from 30 min chloroform-acetone 95:5 (v/v) (second step) and the UV detection was achieved at 350 and 320 nm, respectively.



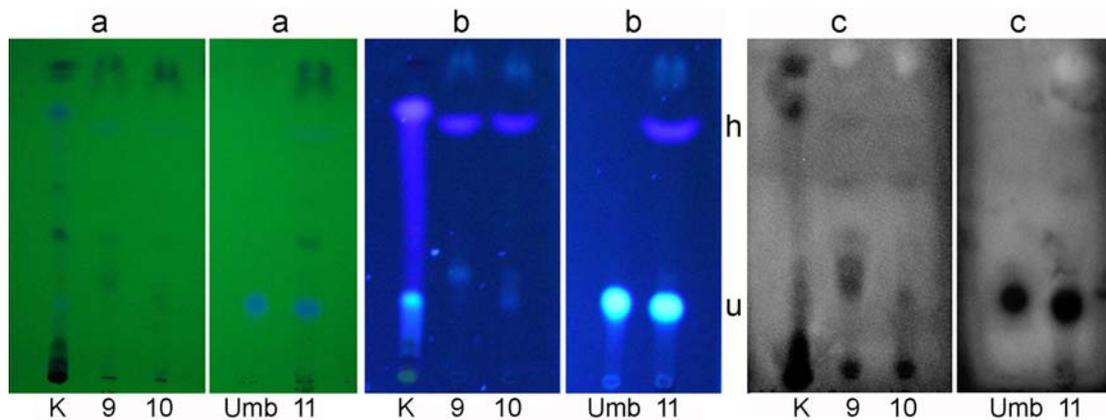
**Fig. (3).** TLC study of chamomile flower extract and the collected fractions visualized by vanillin-sulphuric reagent; K- 10  $\mu$ L of chamomile extract, 1-11- the fractions,  $\alpha$ b- 5  $\mu$ g of alpha-bisabolol; a – layers were developed with chloroform-acetone 99:1 (v/v); b – layers were developed with chloroform-acetone 95:5 (v/v).



**Fig. (4).** Bioautographic detection of chamomile flower extract (K, 10  $\mu$ L) and the collected fractions (1-4) against *Pseudomonas savastanoi* pv. *maculicola* and *Bacillus subtilis*; a/b- the developed layers under UV light 254/365 nm; c- bioautogram using luminescent *P. maculicola* (dark spot = inhibition zone); d- bioautogram using *B. subtilis* (light spot = inhibition zone); The layers were developed with chloroform-acetone 99:1 (v/v) as mobile phase; cs – cis-spiroether ( $R_f=0.65$ ), ts – trans-spiroether ( $R_f=0.5$ ),  $\alpha$ b – alpha-bisabolol ( $R_f=0.4$ ), h – herniarin ( $R_f=0.34$ ).



**Fig. (5).** Bioautographic detection of the collected fractions (5-8) against *Pseudomonas savastanoi* pv. *maculicola* and *Bacillus subtilis*; a/b- the developed layers under UV light 254/365 nm; c- bioautogram using luminescent *P. maculicola* (dark spot = inhibition zone); d- bioautogram using *B. subtilis* (light spot = inhibition zone); The layers were developed with chloroform-acetone 95:5 (v/v) as mobile phase; h – herniarin ( $R_f=0.72$ ), bo – bisabolol oxides ( $R_f=0.6$ ), m – matricarin ( $R_f=0.3$ ).

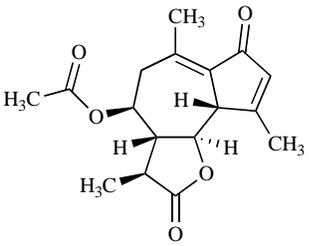
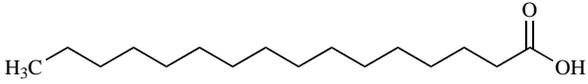
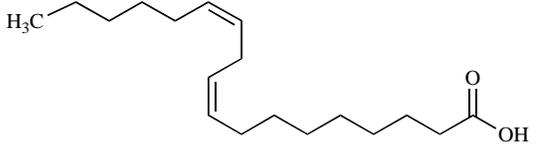
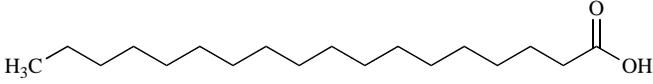
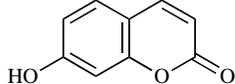


**Fig. (6).** Bioautographic detection of chamomile flower extract (K, 10  $\mu$ L), the collected fractions (9-11) and umbelliferone (u, 3  $\mu$ g) against *Pseudomonas savastanoi* pv. *maculicola*;  
 a/b- the developed layers under UV light 254/365 nm;  
 c- bioautogram using luminescent *P. maculicola* (dark spot = inhibition zone);  
 The layers were developed with chloroform-acetone 95:5 (v/v) as mobile phase;  
 h – herniarin ( $R_f=0.72$ ), u – umbelliferone ( $R_f=0.19$ ).

**Table 1. The Chamomile Flower Components, in the Collected 11 OPLC Fractions Identified by GC-MS**

| Fractions | Components Detected by GC-MS | $I^r$ | Chemical Structures of Identified Components |
|-----------|------------------------------|-------|--|
| 1-2       | cis-Spiroether               | 1898  |  |
|           | trans-Spiroether             | 1909  |  |
| 3         | alpha-Bisabolol              | 1697  |  |
| 4         | Herniarin                    | 1750  |  |
| 5         | Bisabolol oxide A            | 1767  |  |
|           | Bisabolol oxide B            | 1670  |  |
| 6         | Achillin                     | 2186  |  |

Table 1. contd....

| Fractions | Components Detected by GC-MS | $I^T$ | Chemical Structures of Identified Components   |
|-----------|------------------------------|-------|--|
| 7-8       | Matricarin                   | 2412  |    |
| 9         | Palmitic acid                | 1965  |    |
|           | Linoleic acid                | 2140  |    |
|           | Stearic acid                 | 2165  |    |
| 10        | no Determined Compound       |       |  |
| 11        | Umbelliferone                | 1873  |  |

$I^T$ : temperature programmed retention index

pounds in different proportion, and there was only one detectable component in fractions 3 and 4. Fractions 5-9 collected during the second gradient step looked like containing more than one or two main components (Figs. 3, 5a, 5b, 6a, and 6b).

GC-MS analysis determined the following compounds in the fractions (numbered): **1**- cis- and trans-spiroethers; **2**- cis- and trans-spiroethers with trans dominance; **3**- alpha-bisabolol; **4**- herniarin; **5**- bisabolol oxide A and B; **6**- achillin; **7**- matricarin; **8**- matricarin; **9**- small amount of palmitic, linoleic and stearic acids; **10**- no determined compound, **11**- umbelliferone (Table 1). They are already known compounds having been detected in chamomile [7, 35, and 39]. Figs. (7-9) demonstrate a GC-MS TIC (Total Ion Current) chromatogram of some proper chamomile fractions and the characteristic fragmentation of their main components used for identification by spectra library. After the GC-MS study of chamomile fractions, further investigations were performed to prove the presence of biologically active molecules.

The antibacterial effect of the 11 fractions was examined by bioautography against Psmlux and Bs (Figs. 4-6). Although both cis and trans spiroether isomers were detected in fractions 1 and 2, it is supposed from their respective cis:trans area ratio (1:1.46 and 1:2.82) that the first fraction contained the cis, while the second the trans variant. These

isomers showed stability in concentrated solutions (examined by GC-MS), but were not stable on the TLC layer, as checked by the use of 2D-TLC with the same mobile phase in both directions. The transformation could also have come off during the UV detection as well as the concentration process made by cold air stream. Despite of the fact that the trans-spiroether is more stable than the cis one, usually the cis isomer is in a higher amount than the trans in chamomile [40], and so was it in our sample, too. As to many polyacetylenes, antifungal and antibacterial activity is also attributed to chamomile spiroethers [41]. According to our results (Fig. 4), both isomers showed inhibiting/killing effect against Psmlux (Fig. 4c) as well as against Bs (Fig. 4d), however, with different strength. Comparing their activity (the correlation between their amount and the size of the inhibition zone), trans-spiroether had a bigger effect on Psmlux, than cis, while they similarly inhibited Bs.

Alpha-bisabolol, found in fraction 3, is considered to be the main chamomile ingredient contributing to a mild anti-inflammatory effect [42] and it is also mildly antibacterial [43]. Alpha-bisabolol did not exert any inhibition on Psmlux (Fig. 4c), even when higher amount of test compound was used (not showed). It had only a slight antibacterial effect against Bs (Fig. 4d).

Fractions 5 and 6 were collected at the front and right after the front of the stronger mobile phase used in the second gradient step, therefore they contain many substances not

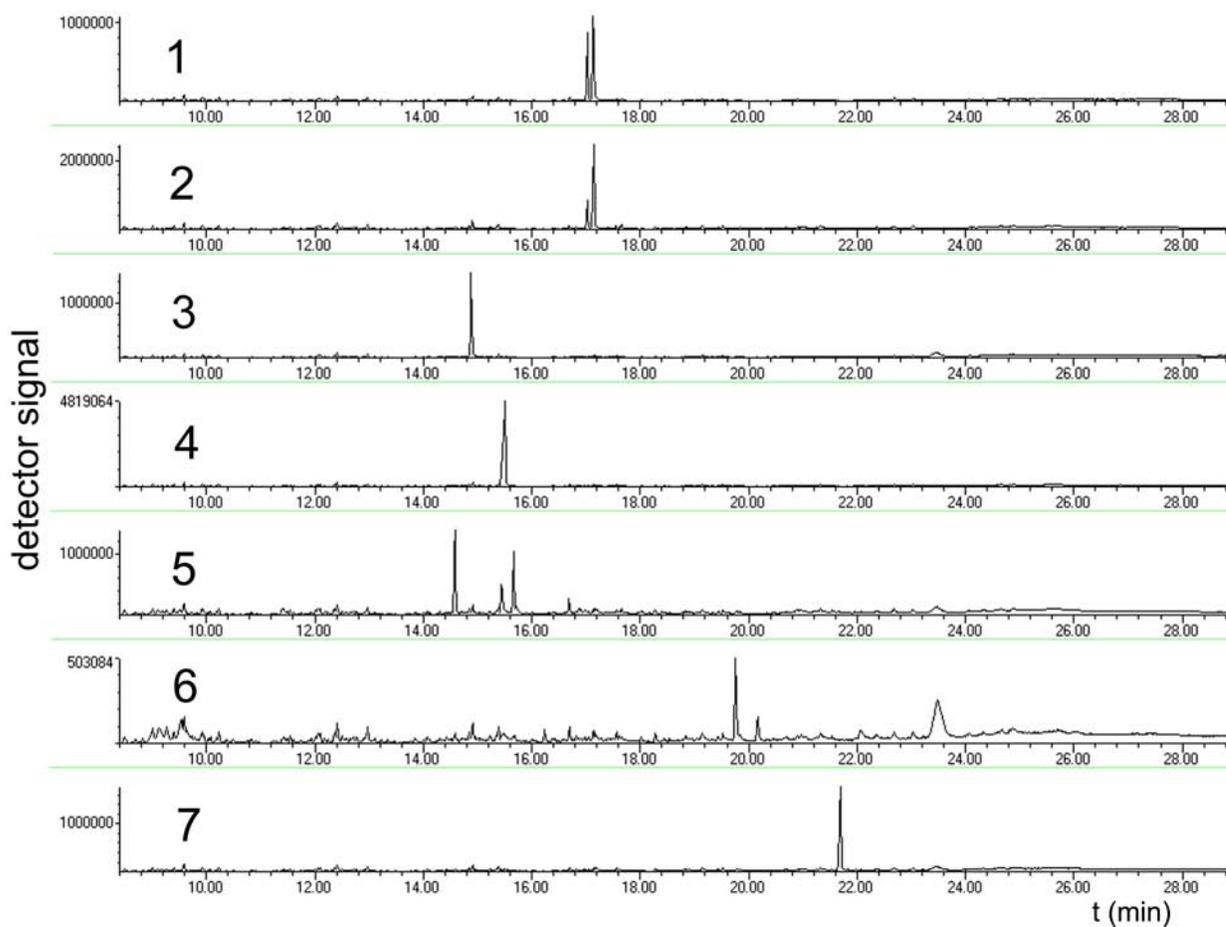


Fig. (7). TIC chromatograms of the fractions 1-7 obtained by GC-MS analysis.

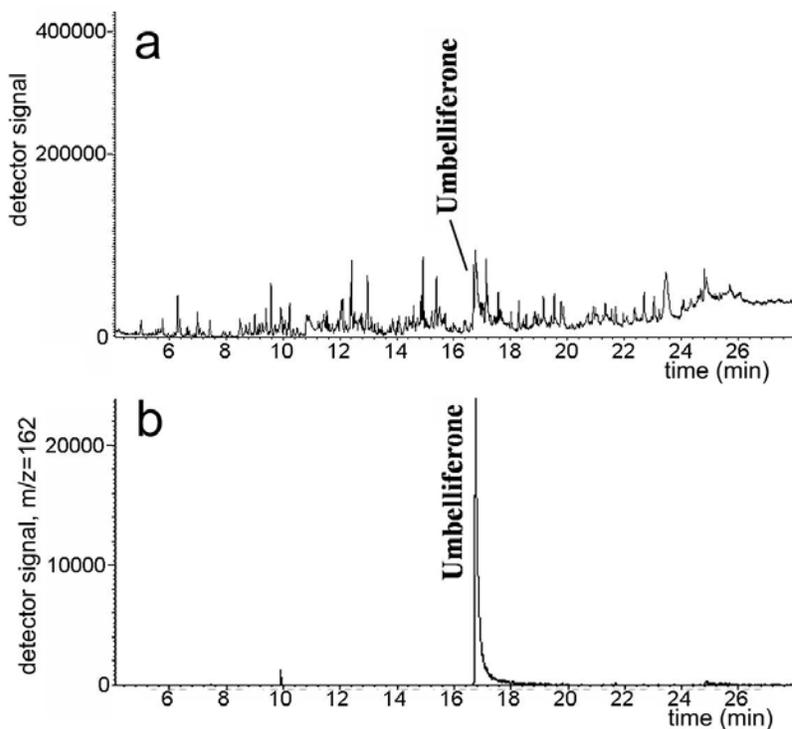
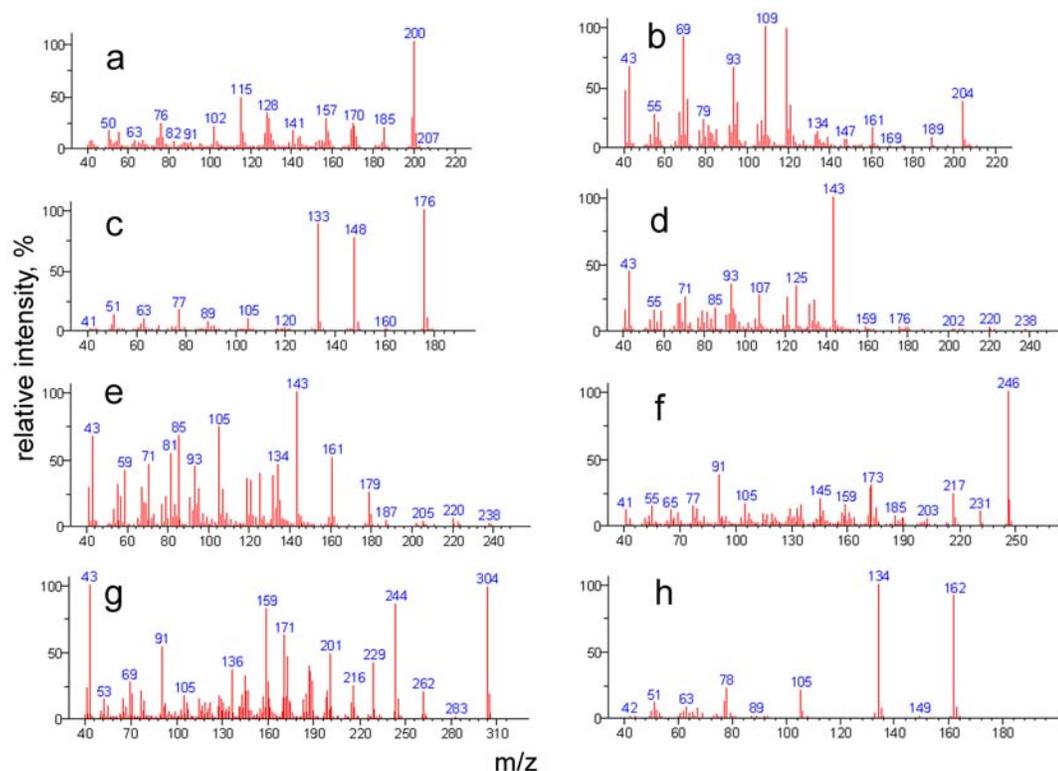


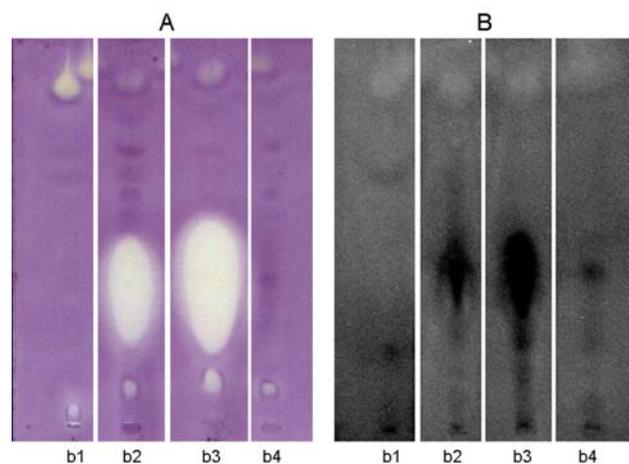
Fig. (8). GC-MS analysis of the fraction 11: (a) TIC chromatogram; (b) extracted ion chromatogram (162 amu; the molecular ion of umbelliferone).



**Fig. (9).** The MS spectra of the main components of the chamomile flower fractions; a – peaks in fraction 1 and 2 at  $t_R$  17.02 and 17.13 min, identified as cis- and trans-spiroethers; b – peak in fraction 3 at  $t_R$  14.89 min, identified as alpha-bisabolol; c – peak in fraction 4 at  $t_R$  15.46 min, identified as herniarin; d – peak in fraction 5 at  $t_R$  15.65 min, identified as bisabolol oxide A; e – peak in fraction 5 at  $t_R$  14.57 min, identified as bisabolol oxide B; f – peak in fraction 6 at  $t_R$  19.74 min, identified as achillin; g – peak in fraction 7 at  $t_R$  21.67 min, identified as matricarin; h – peak in fraction 11 at  $t_R$  16.80 min, identified as umbelliferone.

only from the chamomile (see Fig. 3), but also from the adsorbent bed (e.g. binding components). Fractions 5 and 6 strongly inhibited both bacteria (Fig. 5), however, this may not be ascribed (only) to the activity of the well-known chamomile components, the bisabolol oxides or achillin, identified in these fractions by GC-MS. Achillin can be characteristically detected in *Achillea* species [44], but its presence in *M. recutita* has already been demonstrated, too [35]. The results of blank development also proved (Fig. 10) that blank fractions collected at the front and right after the front from the adsorbent layer had strong antibacterial effect against both microorganisms.

Matricarin was detected in fractions 7 and 8, but the latter contained it only in traces. Matricarin, as a chamomile ingredient, was identified in 1959 by Cekan *et al.* In later literature we have found data only about the presence in *Achillea* and *Artemisia* species [45, 46]. In bioautographic *in vitro* experiments matricarin (in fraction 7) slightly affected PsmLux as well as Bs (Fig. 5). Other antibiotic components in compact spots with similar, low  $R_f$  can be detected in more fractions (Figs. 4 and 5). Their source, according to the blank development (Fig. 10), is the original adsorbent bed (bleeding), not the plant extract.



**Fig. (10).** Bioautographic detection of fractions collected during the blank development; b1: 7.30-15 min (in 1<sup>st</sup> gradient step), b2: 37.30-40 min (parallel with chamomile fraction 5), b3: 40-45 min (similar as that of chamomile fraction 6), and b4: 45-55 min; A – bioautogram using *B. subtilis* (light spot = inhibition zone); B – bioautogram using luminescent *P. maculicola* (dark spot = inhibition zone); Fraction b1 was developed with chloroform-acetone 99:1 (v/v) as mobile phase, b2-b4 were chromatographed with chloroform-acetone 95:5 (v/v).

Fraction 9, including low amount of fatty acids, and fraction 10 without any identified component, had no antibacterial effect against the Gram-positive Bs, while the Gram-negative Psmlux was slightly susceptible to fraction 9 (Fig. 6). However, because fatty acids usually display antibacterial activity against Gram-positive bacteria and to a much lesser extent against the Gram-negatives [47], it can be assumed that not an antibacterial effect of fatty acids was observed, they might have been in too low a concentration in the adsorbent layer.

Coumarins herniarin and umbelliferone were detected in fractions 4 and 11. We have to note that herniarin appeared first in the fourth fraction, but it was there in all further fractions in a quite low concentration because of peak tailing. These chamomile fractions and the umbelliferone test substance, at the same  $R_f$  with the main component of fraction 11, showed growth inhibiting effect in the case of Psmlux (Figs. 4 and 6). Bs was weakly sensitive to the presence of herniarin (Fig. 4), but not to umbelliferone (not showed). These results may prove that chamomile coumarins can play a role in the plant defence against not only fungal but also bacterial attack.

## CONCLUSIONS

On-line OPLC can be exploited for the efficient separation and isolation of different substances, like antimicrobial components of a complex biological matrix. As usually a TLC method can easily be adapted for OPLC, so it is obviously active in a TLC-bioautographic study. Combining this solution with a GC-MS analysis, the results here showed that the antibacterial effect of 50% aqueous ethanol extract of chamomile is ascribable to cis-, trans-spiroethers, and the coumarins like herniarin and umbelliferone.

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