This is the final version (unedited) of the paper published in Journal of Planar Chromatography 25 (2012) 3, 220-224.
DOI: 10.1556/JPC.25.2012.3.6
http://akademiai.com/content/e78575m6112552n2/?p=81962db92f50439293dc6942b7e8035c&pi=6

Application of direct bioautography and SPME-GC-MS for the study of antibacterial chamomile ingredients

Ágnes M. Móricz*, Szabolcs Szarka, Péter G. Ott, Dóra Kertesy, Éva B. Héthelyi, Éva Szőke, Ernő Tyihák

Á.M. Móricz, P.G. Ott, and E. Tyihák, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman O. Str. 15, H-1022 Budapest, Hungary; *E-mail: moricz.agnes@agrar.mta.hu*

Sz. Szarka, D. Kertesy, É.B. Héthelyi, and É. Szőke, Department of Pharmacognosy, Faculty of Pharmacy, Semmelweis University, Üllői Str. 26, H-1085 Budapest, Hungary

Corresponding author:

Ágnes M. Móricz, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman O. Str. 15, H-1022 Budapest, Hungary; Tel.: 0036-48-77-515; Fax.: 0036-48-77-555; *E-mail: moricz.agnes@agrar.mta.hu*

Key Words: antibacterial activity, TLC/OPLC-direct bioautography, chamomile (*Matricaria recutita* L.), SPME-GC-MS, spiroethers, herniarin, (-)-alpha-bisabolol

Summary

The isolation and characterization of antibacterial chamomile components were performed by the use of direct bioautography and solid phase microextraction (SPME)-GC-MS. Four ingredients, active against *Vibrio fischeri*, were identified as the polyacetylene geometric isomers cis- and trans-spiroethers, the coumarin related herniarin, and the sesquiterpene alcohol (-)-alpha-bisabolol.

Introduction

Chamomile (*Matricaria recutita* L.), with its aromatic white flowers and grown as a field crop in many countries, is one of the most popular and widely known herbs in traditional as well as modern folk medicine in Hungary. Chamomile flower is employed as ingredients of herbal teas and as a component of many tinctures and extracts for the treatment of e.g. indigestion, inflammations, and cramps. The beneficial effects of chamomile, such as anti-inflammatory, sedative, analgesic, spasmolytic, antioxidant and antimicrobial properties [1-4], are related to different classes of therapeutically interesting ingredients, like essential oil components, flavonoids, and coumarin derivatives [1, 5, 6].

The antimicrobial activity of volatile oils and crude extracts of chamomile has been widely studied. The chamomile oil, extracted by steam distillation, is composed mainly of sesquiterpenoids ((-)-alpha-bisabolol, bisabolol-oxides, and chamazulene (the artefact of matricine by distillation)) and polyines (cis- and trans-spiroethers) [7]. The essential oils showed antimicrobial activity against various species of bacteria, fungi and viruses [8-11], however, Gram-positive bacteria were found more susceptible than Gram-negative ones [9]. Generally the aqueous chamomile extracts were more effective against molds and yeast than bacteria, while the alcoholic ones showed higher activity against the growth of bacteria [12, 13]. The hydroalcoholic chamomile extracts contain also coumarins (herniarin,

umbelliferone), which are phytoalexins [14], exhibiting a very weak antibacterial and a stronger antifungal activity [15, 16].

Direct bioautography (DB) [17-22] can be used to visualise the activity of different matrix components separated by planar layer chromatography against cells in the adsorbent bed. Therefore, it is suitable for guiding the isolation of effective compounds e.g. from plant extracts. Our earlier DB results showed that alcoholic chamomile extracts contain ingredients with antibacterial activity against *Pseudomonas savastanoi* pv. *phaseolicola* [23], luminescence gene tagged *Pseudomonas syringae* pv. *maculicola* and *Bacillus subtilis* [19]. In our most recent studies the isolation and identification of some components of 50% aqueous ethanol chamomile flower extract, working antibacterially against *P. maculicola* and *B. subtilis*, have been achieved using overpressured layer chromatography (OPLC) [24-27] with on-line detection and fraction collection and subsequent GC-MS analyses [22]. In the most active fractions cis-, trans-spiroethers, and the coumarins herniarin and umbelliferone were identified.

Solid phase microextraction (SPME) is a recently developed technique, which was introduced in the early 1990s. The target analytes can be adsorbed on the SPME fiber by immersing it in the sample or by exposing it to the sample headspace, in which case the matrix interferences can be drastically reduced [28-30]. The main advantages of this technique are simplicity of operation, speed, its solventless nature, analyte selectivity and preconcentration, and the possibility of the easy coupling to a powerful analytical tool, the gas chromatography-mass spectrometry [31].

In this paper the characterisation of antibiotic chamomile ingredients is presented using TLC/OPLC-DB with luminescent *Vibrio fischeri* bacteria and SPME-GC-MS.

Experimental

1. Materials

Aluminum foil-backed normal particle silica gel $60F_{254}$ 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 at all four edges (OPLC-NIT, Budapest, Hungary). All used solvents were of analytical grade from Reanal (Budapest, Hungary). Test substances (-)-alpha-bisabolol, and herniarin were from Sigma–Aldrich (Budapest, Hungary).

2. Preparation of extracts

Chamomile (*Matricaria recutita* L.) flowers were obtained from Hungarian drug store chains (JuvaPharma and Herbaria) as well as collected in the end of May 2011, in Harta (in the Great Plain, Hungary); the latter sample is dried at 25 ^{o}C, in the dark. 1500 mg of flowers were extracted with 10 mL of 50% ethanol in a 20 mL screw-capped glass bottle by maceration and shaking (Dragon Lab SK-L330-Pro digital linear shaker) for three-times 2h within 24 h. After filtration (filter paper MN 616 from Reanal) with the help of vacuum the solutions were stored at 5 ^{o}C and applied for TLC/OPLC-DB and isolation.

3. TLC and OPLC separations

TLC separation of the components of 50% aqueous ethanol chamomile extract was performed with chloroform-acetone 99:1 (v/v) as a mobile phase in an unsaturated chamber on preconditioned (130 ^{o}C, 3 h) normal particle size silica gel layers with 10 cm height. The samples were applied at 12 mm height with Linomat IV sample applicator (CAMAG, Muttenz, Switzerland) or a microsyringe. In 2D-TLC the sample was applied in a spot, 12 mm far from the edges. The first dimension was developed as described above and afterwards the dried plate was chromatographed in the 2nd dimension with chloroform-acetone 95:5 (v/v).

Personal OPLC BS50 system (OPLC-NIT, Budapest, Hungary) [24-26] was used for infusion OPLC [27]. The infusion operating mode means that we don't let the mobile phase flow out of the layer, therefore, the separated components remain on it, which can be achieved by closing of the mobile phase outlet. Conditions of the separation were as follows: 20 cm x 20 cm sealed TLC chromatoplate, sample application in bands at 3 cm from the edge, 50 bar external pressure, 400 μL rapid mobile-phase flush, 400 μL min^{-1} mobile phase flow rate, 4414 μL mobile phase, 672 s development time. The mobile-phase was pure chloroform.

The chromatoplates, developed by TLC or infusion OPLC, were dried by cold air stream using a hair-dryer (5 min). The chromatographic spots were visualised using a UV lamp (λ = 365 nm) (CAMAG), vanillin-sulphuric acid reagent (40 mg vanillin + 10 mL ethanol + 200 μL concentrated sulphuric acid; the dipped plate was heated to 110 ^{o}C for 5 min), a Shimadzu CS-930 dual-wavelength TLC scanner (Shimadzu Co., Kyoto, Japan) at 350 nm, and in direct bioautographic systems.

For isolation of active ingredients, 0.5 mL extract was applied in a 17 cm wide band. After development, the appropriate chromatographic bands marked under UV light were scraped off and extracted with acetone. The antibacterial activity of the concentrated (by cold air stream) extracts was checked with TLC-DB. The identification was performed with SPME-GC-MS analyses of the extracts and/or by the comparison of R_f value of active components with test substances.

4. Bioassay

The antibacterial effect of separated chamomile components was evaluated *in vitro* with direct bioautography (DB) [17-22] against the Gram-negative, naturally luminescent, marine bacterium *Vibrio fischeri*. The *V. fischeri* strain (Lumistox test strain, Hach-Lange Ltd.) was grown at 28.3 ^{o}C in the dark with good aeration until it reached an optical density of 2.3 - 2.6,

i.e. for ca. 4-5 h, in the following liquid medium (slightly modified recipe of NCAIM, Corvinus University, Budapest; ingredients are expressed in g dm^{-3}): pepton 5, yeast extract 5, meat extract 6, NaCl 24, $MgSO_4$ 3.4, $MgCl_2$ *$6H_2O$ 5.3, KCl 0.7, $CaCl_2$ 0.1.

For detection of the bioluminescent light emission, the dried, developed chromatoplates were immersed into a cell suspension of *Vibrio fischeri* (for 5 s), put into a transparent glass cage ensuring an air phase above the adsorbent layer and protecting against drying. The bioautograms were documented immediately after inoculation by use of a computer-controlled cooled CCD camera (IS-4000, Alpha Innotech, San Leandro, USA) with an exposure time of 2-4 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. Solid phase microextraction (SPME)

The air-dried and powdered chamomile inflorescence was put into 20 mL headspace vials sealed with a silicone/Polytetrafluoroethylene (PTFE) septum. Acetone extracts of appropriate chromatographic bands were carefully evaporated to dryness at room temperature in similar vials prior to SPME-GC-MS analyses. The static headspace solid phase microextraction sample preparation was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 µM StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100 ºC, the extractions were performed by exposing the fiber for 20 min at 100 ºC to the headspace of a 20 mL vial containing the plant material. Then the fiber was transferred immediately to the heated injector port of the GC-MS, and desorbed for 1 min at 250 ºC. The SPME fiber was cleaned and conditioned in the Fiber Bake-out Station in pure nitrogen atmosphere at 250 ºC for 15 min after desorption.

6. Apparatus and GC-MS Conditions

The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent HP-5MS capillary column (30 m × 250 µm × 0.25 µm). The GC oven temperature was programmed from 60 ºC (3 min isothermal) to 200 ºC at 8 ºC/min (2 min isothermal), 200-230 ºC at 10 ºC/min (5 min isothermal) and finally 230-250 ºC at 10 ºC/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. Injector temperature was 250 °C and the split ratio was 1:20.

The mass selective detector was equipped with a quadrupole mass analyzer and was operated in electron ionization mode at 70 eV in full scan mode (40-500 amu at 3.2 scan/s). Data were evaluated by MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with data of authentic standards. Mass spectral and retention data known from the literature [7, 32-34] and the NIST 05 library were also consulted.

Results and discussion

The antibacterial components of 50 % aqueous ethanol extracts of chamomile from different sources were screened by the high-throughput, fast, and cheap TLC-DB using *Vibrio fischeri* as a test microorganism. According to the result (**Figure 1**), all samples were rich in compounds having an inhibiting/killing effect against these bacteria (see black areas). The bands of the active ingredients, which were visible under UV light (3 components at R_f 0.65, 0.5, and 0.34), were scraped off and extracted with acetone. The TLC-DB evaluation of all 3 extracts confirmed their antibiotic effect against *V. fischeri* (**Figure 2**). However, it seemed

that the active ingredients 1 and 2 could transform to each other. These transformations could have had occurred on the adsorbent layer, so they were also observed by a 2D-TLC (**Figure 3**). Therefore, it was thought that those two components are geometric isomers, which suggestion was further examined by SPME-GC-MS analyses. The SPME-GC-MS total ion current (TIC) chromatogram of air-dried and powdered chamomile inflorescence demonstrates that the volatile part of chamomile flower is a complex mixture comprising principally sesquiterpenes ((-)-alpha-bisabolol, bisabolol-oxides A and B, farnesene, spathulenol), acetylene-derivatives (spiroethers), and the coumarin-related herniarin (**Figure 4a**). In spots 1 and 2 (at R_f 0.65 and 0.5) (Figure 1) both cis- and trans-spiroether isomers (Figure 4b) whereas in spot 3 (at R_f 0.34) herniarin were detected (not shown). According to the ratio of isomers, the cis isomer was determined as the component at R_f 0.65, while the other component with lower R_f (0.5) was identified as trans-spiroether.

The antibacterial effect of (-)-alpha-bisabolol, one of the main volatile components of chamomile, was examined against *V. fischeri* using OPLC-DB. The forced flow OPLC provides faster, better separation, more compact chromatographic spots (more sensitive detection) than conventional TLC/HPTLC; therefore, the application of this technique can be very attractive for the separation of components of complex matrices. The presence of the not UV active (-)-alpha-bisabolol in the 50% aqueous ethanol chamomile extract was recorded by making it visible with vanillin-sulphuric acid reagent (**Figure 5a-5c**) as well as by GC-MS measurements [22]. Figure 5 shows that besides cis- and trans-spiroethers as well as herniarin, also (-)-alpha-bisabolol inhibited/killed *V. fischeri* bacteria cells. Though, the extract did not contain (-)-alpha-bisabolol in appropriate concentration that could appear as inhibiting zone. The confirmation of the observable chromatographic spots obtained from various visualisation methods also can be seen in Figure 5. In fact, among the components of the 50%

aqueous ethanol extract the cis and trans spiroethers (these are also components of the chamomile essential oil) generate significant antibacterial activity to *V. fischeri*.

Conclusions

These results show that planar layer chromatography, especially forced flow OPLC coupled with DB is a very useful tool for guiding the detection and directed isolation of antibacterial compounds. This high throughput and relatively cheap technical solution enables simple as well as fast chemical and biological screening of complex matrices such as plant extracts, aiding the discovery of new, efficient antimicrobials for which there is a high demand because of the growing resistance of pathogens to the majority of presently known and used antibiotics [35].

Although components of chamomile are mainly used for medical purposes, their use can be extended e.g. for food or cosmetic applications as well. The volatile components are chemically sensitive molecules; therefore, they in pure form need to be stored in airtight containers in the dark in order to prevent chemical changes.

Acknowledgement

This work was supported by OTKA grant No. PD83487.

References

1. *N.G. Bisset* and *M. Wichtl*, Herbal Drugs and Phytopharmaceuticals: A Handbook for Practice on a Scientific Basis, 2nd ed.; CRC Press: Boca Raton, FL, 2001.

2. World Health Organization. Monographs on Selected Medicinal Plants, vol. 1: Flos Chamomillae. WHO, Geneva, 1999.
3. *J.K. Srivastava*, and *S. Gupta*, in *R.R. Watson* (ed.) Complementary and Alternative Therapies in the Aging Population, Elsevier Inc., Academic Press. 2009.
4. *D.L. McKay*, and *J.B. Blumberg*, Phytother. Res. **20** (2006) 519-530.
5. *É. Szőke, E. Máday, S.A. Kiss, L. Sonnewend,* and *É. Lemberkovics*, J. Am. Coll. Nutr. **23** (2004) 763S-767S.
6. *M. Repcák, J. Imrich,* and *M. Franeková*, J. Plant Physiol. **158** (2001) 1085–1087.
7. *É. Szőke, E. Máday, E. Tyihák, I.N. Kuzovkina,* and *É. Lemberkovics*, J. Chromatogr. B. **800** (2004) 231-238.
8. *M. Lis-Balchin, S.G. Deans,* and *E. Eaglesham*, Flavour Fragr. J. **13** (1998) 98-104.
9. *M.E. Aggag*, and *R.T. Yousef*, Planta Med. **22** (1972) 140-144.
10. M. Berry, Pharm. J. **254** (1995) 191-193.
11. *K.M. Soliman*, and *R.I. Badeaa*, Food Chem. Toxicol. **40** (2002) 1669-1675.
12. *K.M. Al-Ismail*, and *A. Talal*, Agric. Sci. **30** (2003) 330-337.
13. *M. Cinco*, *E. Banfi, A. Tubaro*, and *R.D. Loggia*, Int. J. Drug. Res. **21** (1983) 145-151.
14. *M. Daniel*, and *R.P. Purkayastha*, Handbook of Phytoalexin Metabolism and Action. Dekker Inc: New York, 1995.
15. *T. Ojala, S. Remes, P. Haansuu, H. Vuorela, R. Hiltunen, K. Haahtela,* and *P. Vuorela*, J. Ethnopharmacol. **73** (2000) 299-305.
16. *U. Afek, J. Orenstein, S. Carmeli, V. Rodov*, and *J.M. Bar*, Phytochemistry. **50** (1999) 1129-1132.
17. *L. Botz, S. Nagy,* and *B. Kocsis*, in *Sz. Nyiredy* (ed.) Planar Chromatography – A Retrospective View for the Third Millennium, Springer, Budapest, 2001.
18. *C. Weins*, and *H. Jork*, J. Chromatogr. A, **750** (1996) 403-407.

19. *Á.M. Móricz, E. Tyihák*, and *P.G. Ott*, J. Planar Chromatogr. **23** (2010) 180-183.

20. *Á.M. Móricz, Gy. Horváth, P. Molnár, B. Kocsis, A. Böszörményi, É. Lemberkovics,* and *P.G. Ott*, J. Planar Chromatogr. **23** (2010) 406-410.

21. *I.M. Choma*, and *E.M. Grzelak*, J. Chromatogr. A **1218** (2011) 2684-2691.

22. *Á.M. Móricz, Sz. Szarka, P.G. Ott, É.B. Héthelyi, É. Szőke*, and *E. Tyihák*, Med. Chem. **8** (2012) 85-94.

23. *E. Tyihák, E. Mincsovics, Gy. Kátay, Zs. Király-Véghely, Á.M. Móricz,* and *P.G. Ott*, J. Planar Chromatogr. **21** (2008) 15–20.

24. *E. Mincsovics, M. Garami, L. Kecskés, B. Tapa, Z. Végh, Gy. Kátay*, and *E. Tyihák*, **82** (1999) J AOAC Int. 587–598.

25. *E. Tyihák, E. Mincsovics*, and *Á.M. Móricz*, J. Chromatogr. A **1232** (2012) 3-18.

26. *E. Mincsovics*, and *E. Tyihák*, Nat. Prod. Commun. **6** (2011) 719-732.

27. *E. Mincsovics, É. Sárdi, I. Velich, Gy. Kátay*, and *E. Tyihák* J. Planar Chromatogr. **15** (2002) 280–285.

28. *C.L. Arthur*, and *J. Pawliszyn*, Anal. Chem. **62** (1990) 2145-2148.

29. *H. Lord*, and *J. Pawliszyn*, J. Chromatogr. A **885** (2000) 153-193.

30. *M. Riu-Aumatell, M. Castellari, E. López-Tamames, S. Galassi,* and *S. Buxaderas*, Food Chem. **87** (2004) 627-637.

31. *P. Arias Abrodo, D. Díaz Llorente, S. Junco Corujedo, E. Dapena de la Fuente, M.D. Gutiérrez Álvarez,* and *D. Blanco Gomis,* Food Chem. **121** (2010) 1312-1318.

32. *R.P. Adams*, Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured Publ. Corp.: Carol Stream, USA, 2001.

33. *A. Iordache, M. Culea, C. Gherman*, and *O. Cozar*, Nucl. Instrum. Methods Phys. Res. B **267** (2009) 338-342.

34. *É. Szőke, E. Máday, J. Gershenzon, J.L. Allen*, and *É. Lemberkovics*, J. Chromatogr. Sci. **42** (2004) 229-233.

35. *J.N. Eloff*, S. Afr. J. Sci. **96** (2000) 116- 118.

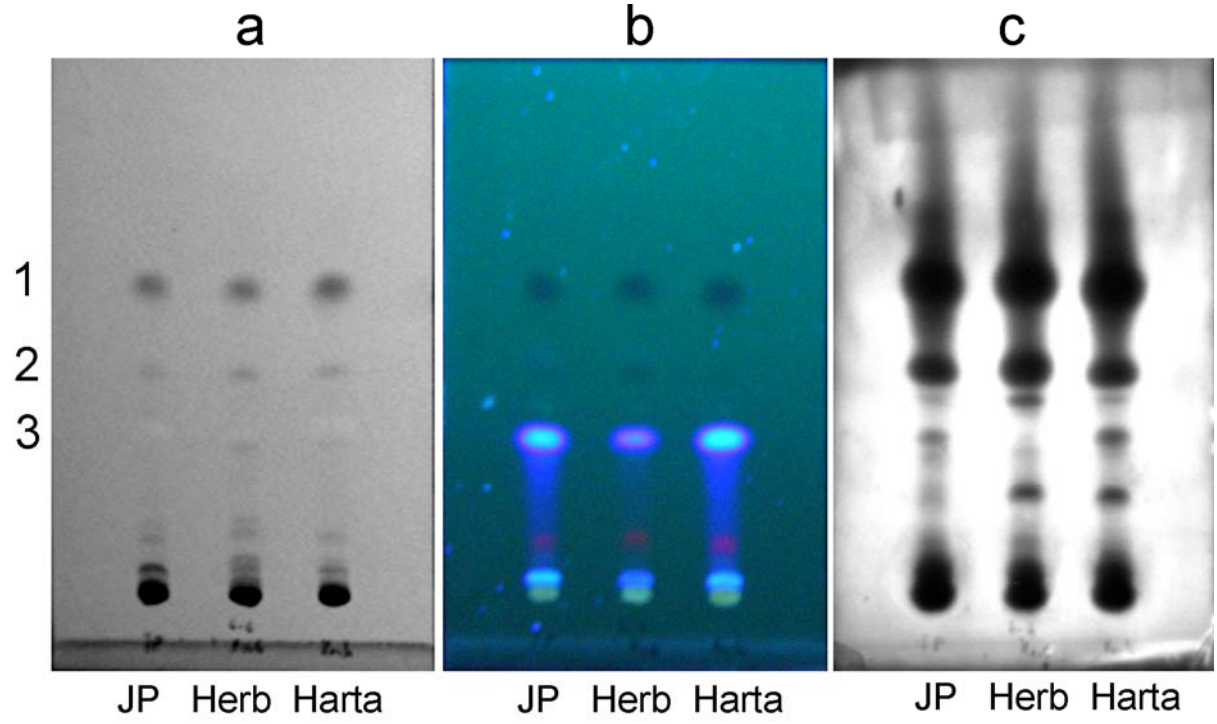

Figure 1

Detection of ingredients of 50% aqueous ethanol extracts (4 µL of each) of chamomile obtained from JuvaPharma (JP) and Herbaria (Herb) as well as collected in Hungary in the Great Plain (Harta). The isolated antibacterial UV active components (1, 2 and 3) were separated by TLC.

a/b – the developed layers under UV light λ=254/365 nm

c – bioautogram using *Vibrio fischeri* (dark spot = inhibition zone)

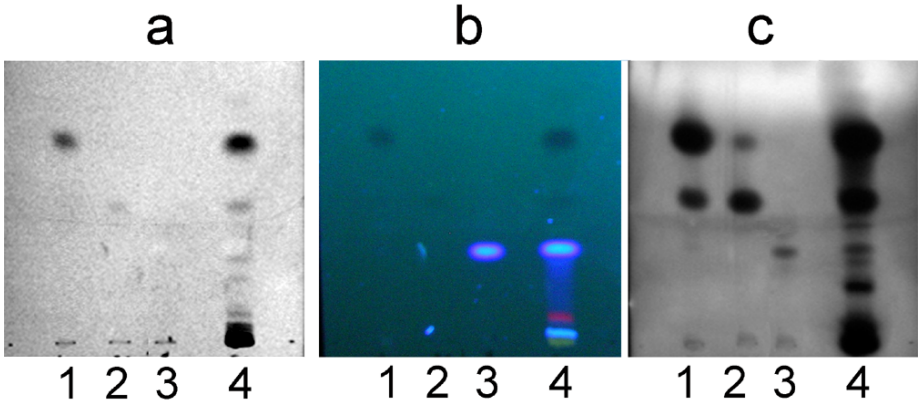

Figure 2

Detection of ingredients of whole chamomile extract and the isolated antibacterial UV active components separated by TLC; 1, 2, 3 – isolated chamomile components extracted from the chromatographic bands at R_f 0.65, 0.5, and 0.34, respectively (see Figure 1), 4 – 6 μL of 50% aqueous ethanol extract of chamomile collected in Harta

a/b – the developed layers under UV light λ=254/365 nm

c – bioautogram using *Vibrio fischeri* (dark spot = inhibition zone)

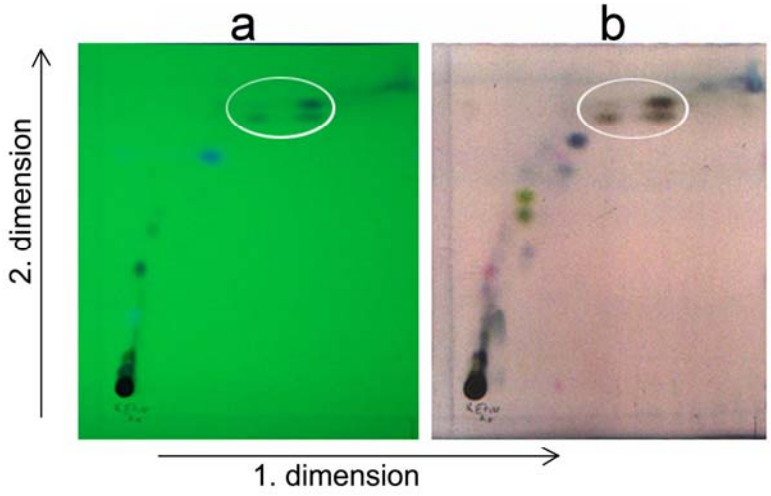

Figure 3

2D-TLC separation of 10 µL of 50% aqueous ethanol extract of chamomile obtained from JuvaPharma. The circled area shows the spontaneous transformation of the 2 components to each other.

a – the developed layers under UV light (254 nm)

b – visualisation with vanillin-sulphuric acid reagent

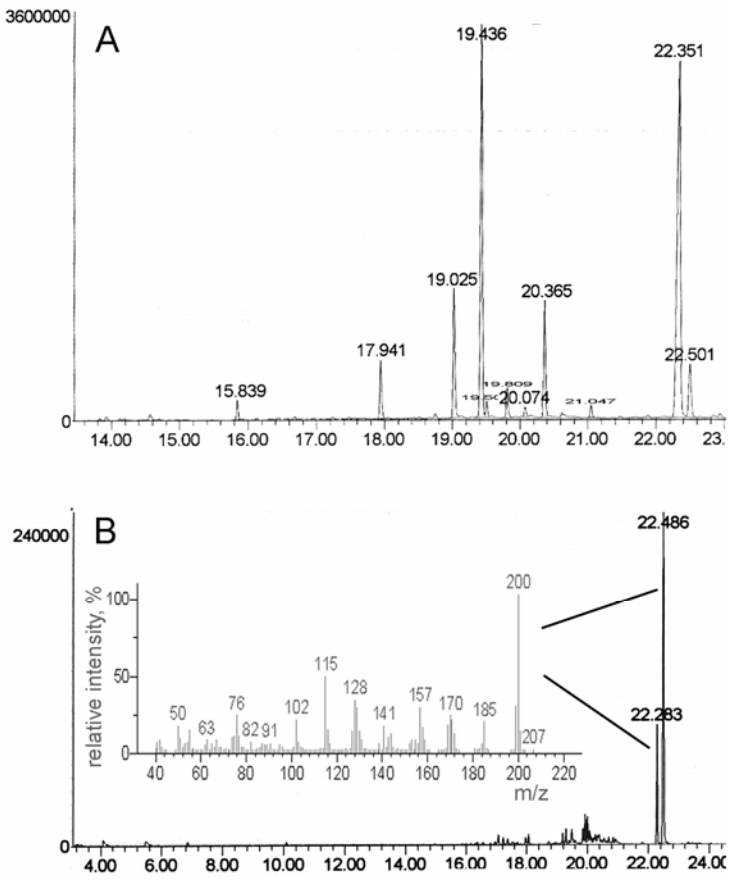

Figure 4

TIC chromatograms of (a) 5g of dried powered chamomile flower (JuvaPharma) and (b) isolated chamomile components extracted from the chromatographic bands at R_f 0.5 with their mass spectra obtained by SPME-GC-MS analysis;

Peaks at different t_R were identified as: 15.8 min – trans-beta-farnesene, 17.9 min – spathulenol, 19.0 min – bisabolol-oxide B, 19.4 min – (-)-alpha-bisabolol, 20.0 min – herniarin, 20.3 min – bisabolol-oxide A, 22.3 min – cis-spiroether, 22.5 min – trans-spiroether

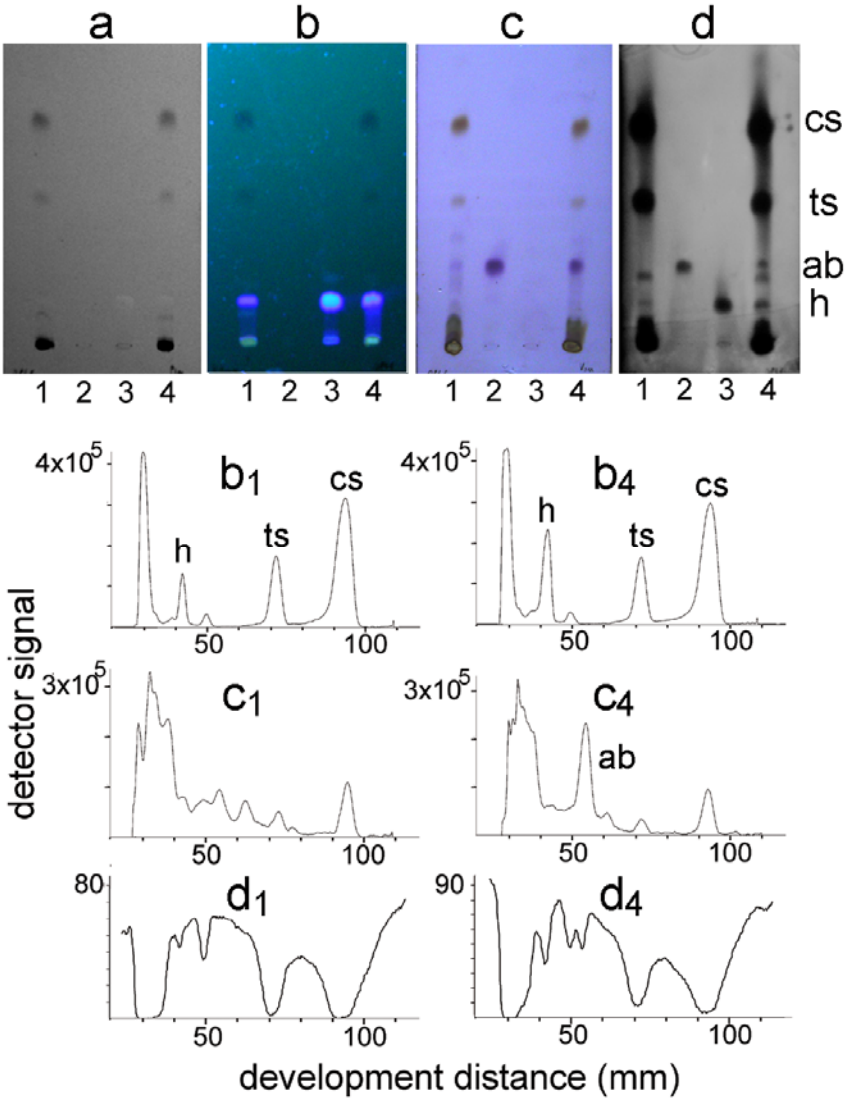

Figure 5

Detection of ingredients of 50% aqueous ethanol chamomile extract separated by infusion OPLC; 1 – 6 µL 50% aqueous ethanol chamomile (Herbaria) extract; 2 – 3 µg (-)-alpha-bisabolol; 3 – 3 µg herniarin; 4 – 6 µL 50% aqueous ethanol chamomile extract + 1.5 µg of each (-)-alpha-bisabolol and herniarin test substances

a/b – the developed layers under UV light λ=254/365 nm

c – visualisation with vanillin-sulphuric acid reagent

d – bioautogram using *Vibrio fischeri* (dark spot = inhibition zone)

b_1 and b_4 – densitometric evaluation of tracks 1 and 4 in Figure 5b at 350 nm

c_1 and c_4 – densitometric evaluation of tracks 1 and 4 in Figure 5c at 600 nm

d_1 and d_4 – the measurement of the intensity of the luminescent light in tracks 1 and 4 in Figure 5d

cs – cis-spiroether, ts – trans-spiroether, ab – (-)-alpha-bisabolol, h – herniarin