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Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 285–292
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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The influence of *Calendulae officinalis flos* extracts on cell cultures, and the chromatographic analysis of extracts

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Received 14 June 2004; received in revised form 10 September 2004; accepted 6 December 2004
Available online 2 March 2005

Abstract

Three extracts of *Calendulae officinalis flos* (Asteraceae): heptane, ethyl acetate and methanol were introduced to a human skin fibroblast (HSF) cells culture and a culture of human breast cancer cells (T47D), cell culture collection ECACC number 85102201. The ethyl acetate but not the heptane and methanol extracts in concentrations above 25 μg/mL, can stimulate cell proliferation and cellular metabolism by increase of mitochondrial dehydrogenase activity. However, concentrations exceeding 75 μg/mL are toxic for cells.

The second part of the study concerned elaborating of optimal chromatographic systems for quantitative analysis of these extracts by the use of HPTLC with densitometry. Oleanolic acid, β-amyrin, β-amyrin acetate, rutin, narcissin, 3-glucoside of isorhamnetin, quercetin, isoquercitrin, vanillic acid, caffeic acid, chlorogenic acid, protokatechuic acid, *p*-coumaric acid and syringic acid were all identified.

Keywords: Extracts of *Calendulae officinalis flos*; Cell cultures; Triterpenes; Phenolic acids; Flavonoids; HPTLC with densitometry

1. Introduction

With the trend of 'coming back to nature' in the last years, the more frequent use of plant medicines, the number of studies on the therapeutic use of *Calendulae officinalis flos* has increased as well. *C. officinalis flos* is a safe drug applied in skin inflammation, regardless of the fact whether the inflammation is caused by infection or laceration [1,2]. The *C. officinalis flos* has a healing effect on external bleeding, cuts and bruises as well as strained muscles. It is also recommended for difficult wounds and varicose ulcerations of the lower legs. *C. officinalis flos* is a constituent of many medical preparations such as unguents, tinctures, dental gels, etc. [3,4]. There has been a great increase in the domain of identification of biologically active compounds occurring in this plant over the last years [5–8].

According to the literature data the plant contains triterpenes, phenolic acids, flavonoids and carotenoids. In screening of the natural marigold products one can also rely on cell cultures. They allow conducting experiments, the realisation of which on the whole organism would be impossible. In classic monolayer cultures (for instance on glass or plastic), multiplying cells form a uniform layer. Normal cells, distinct from cancerous cells, cannot grow in a multilayer because of the interactions between the membranes of the neighboring cells, which initiate the so-called contact growth inhibition, examples of such cells are human skin fibroblasts (strain of HSF cells) taken from the fragment of skin of the forearm from a healthy donor with no dermatological problems (with the agreement of the patient).

Individual variation of donors due to genetic and environmental factors will be a subject of the next paper.

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doi:10.1016/j.jpba.2004.12.034

2. Material and methods

Methanol, ethyl acetate and heptane extracts of *C. officinalis flos* (Herbapol S.A., Lublin, Poland) were prepared by heating 20 g of plant material with 300 mL of solvent for 5 h at temperature 60 °C under reflux. Each of these extracts was concentrated under reduced pressure at 30 °C to 100 mL. Methanol, ethyl acetate and heptane extracts were used for determination of flavonoids and pentacyclic triterpenes. For phenolic acids analysis 100 mL of the extract examined was evaporated to dryness, washed with hot water (50 mL) and cooled under refrigeration for 24 h. The tar-like precipitates, containing ballasts were filtered and washed with distilled water. The filtrates obtained in this way were defatted by double shaking with petroleum ether (30 mL samples) and next were extracted 10 times with diethyl ether (20 mL). The combined ether extracts were evaporated to dryness under reduced pressure and the dry residue was dissolved in 5 mL of methanol.

For cell culture, 10 mL each of the extracts were evaporated to dryness. The dry residue was dissolved in dimethyl sulfoxide (DMSO) [9–11].

The used cell line (T47D) was obtained from cell culture collection ECACC number 85102201 human breast cell carcinoma epithelial.

2.1. The trypan blue exclusion test

The use of viability stain trypan blue (0.4%, Sigma) enables to perform a quantitative analysis of the condition of the culture. Trypan blue is a stain that enters only across the membranes of non-viable/dead cells. After detaching cells from the tissue culture flask by trypsinization, a cell suspension was mixed with trypan blue solution, 3–5 min later colored (non-viable) and dye-excluding (viable) cells were counted and the percentage of viable cells was determined.

2.2. MTT assay

Sensitivity of cells on marigold extracts activity was determined by a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [4]. Cells grown in 96-well multiplates were incubated for 3 h with MTT solution (5 mg/mL) (Sigma). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in a mixture consisting of 10% sodium dodecyl sulfate (SDS) in 0.01 M L^{-1} HCl. The product was quantified spectrophotometrically by absorbance measurement at 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

2.3. Establishment of HSF cell culture

Freshly excised skin fragments were washed two times using RPMI (1640) medium (Roswell Park Memorial Institute); Gibco BRL (UK) supplemented with antibiotics (penicillin, streptomycin, amphotericin B) and then placed into wells of 24-well plate. The explants were then overlaid with a warm 1:1 (v/v) mixture of 1% agarose and RPMI 1640 medium. The culture was performed by adding culture medium RPMI 1640 supplemented with 10% foetal bovine serum FBS on top of agarose gel and incubated at 37 °C in a humidified 5% CO_2/95% air incubator. Outgrowths of skin fibroblasts were separated and cultured.

The biological experiments were repeated six times. Data were analysed using Student's *t*-test. Significance was reported as $p < 0.05$.

2.4. HPTLC

HPTLC separation of the components of extracts (abbreviation: heptane, ethyl acetate and methanol) was performed on 10 cm × 10 cm or 10 cm × 20 cm glass HPTLC plates coated with silica gel Si60 F_{254} HPTLC plates (E. Merck, Darmstadt, Germany). Before use the plates were washed with methanol and dried for 20 min at room temperature. The standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-Amyrin (0.01%) and β-amyrin acetate (0.01%) were dissolved in ethyl acetate; oleanolic acid (0.01%), flavonoids and phenolic acids were dissolved in methanol (0.01%). Ten or thirty microlitre samples of standards and extracts were spotted by means of an AS 30 automatic applicator (Desaga, Heidelberg, Germany) under nitrogen at 2.5 atm. Chromatograms were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland) on a distance of 8.5 cm.

Mixed phases were used; for triterpenes: hexane, dichloromethane, methanol, water (4:5:0.9:0.1, v/v); for flavonoids: toluene, ethyl acetate, formic acid, water (1:9:2.5:2, v/v); for phenolic acids: heptane, dichloromethane, diisopropyl ether, formic acid, water (Table 1).

Table 1
The programs used for multiple gradient elution for phenolic acids

Mobile phase	Step no.					
	1	2	3	4	5	6
Heptane/dichloromethane (7:3) containing 0.1 mL of formic acid (% v/v)	60	60	60	60	80	80
Diisopropyl ether (% v/v)	40	40	40	40	20	20

The location of the spots was determined: for phenolic acids under UV light (λ = 366 and 254 nm); for flavonoids after spraying with 1% methanolic diphenylboric acid-β-ethylamino ester (diphenylboryloxyethylamine, NP) and followed by 5% ethanolic polyethylene glycol-4000 (PEG) under UV light λ = 366 nm; for triterpenes: after spraying with anisaldehyde-sulphuric acid reagent (AS) and heating at 100 °C for 5 min in daylight or UV, λ = 366 nm [12].

Videodocumentation was obtained by use of videoscaning equipment Desaga VD-40 comprising an Hitachi CCD video camera. The documentation was processed with 'ProViDoc' software, running under Microsoft Windows 98/NT/2000, adapted to work with the camera.

Densitograms were obtained by use of a Desaga CD 60 densitometer controlled by a Pentium computer. For qualitative purposes linear scans were obtained with slit dimension of 0.1 mm × 2 mm at λ = 254 and 366 nm for phenolic acids and flavonoids, λ = 520 nm for triterpenes.

The chromatographic experiments were repeated three times.

3. Results and discussion

The viability and proliferation activity of normal human skin fibroblasts (HSF) and human breast carcinoma cell line (T47D) in the presence of different concentrations of extracts (25–250 μg/mL) of *C. officinalis flos* was investigated. Samples of the herbal extracts were applied for standard concentration ranges tested in biological in vitro investigation on human cells. Preliminary investigations show that concentrations of the herbal extracts in the 25–250 μg/mL range were optimal for assessing the stimulation of proliferation of cells and toxicity analysis.

The activity of three (methanol, ethyl acetate and heptane) extracts of *C. officinalis flos* on the proliferation and viability of normal human skin fibroblasts (HSF) and human breast carcinoma (T47D) cell lines were compared. HSF and T47D cells were seeded at a density of 2 and 4×10^4 cells/mL, respectively. The experiments were carried out during 72 h with results read every 24 h. Parallel, a 24 h experiment aimed at toxicity analysis of the tested biologi-

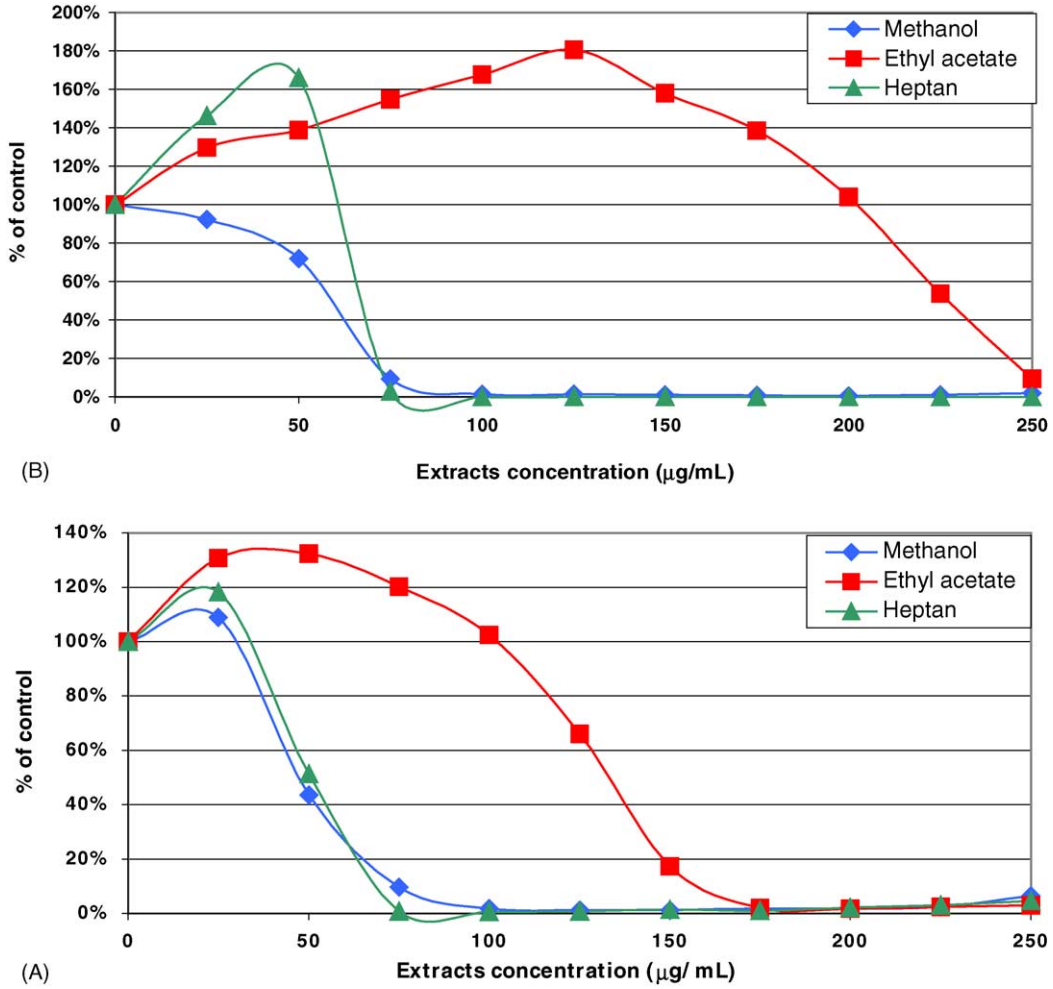

Fig. 1. The effect of 24 h treatment with extracts of *C. officinalis flos* on: (A) T47D cells (4×10^5 cells/mL), (B) HSF cells (1×10^5 cells/mL). The MTT assay.

Table 2
The effect of 24 h treatment with marigold extracts on HSF cells (1×10^5 cells/mL) and T47D cells (4×10^5 cells/mL), the trypan blue exclusion test

Kind of extract	Extract concentration (μg/mL)	Percentage of viable HSF cells, confidence level 95%	Standard deviation of the mean, $S = s/\sqrt{n}$	Percentage of viable T47D cells, confidence level 95%	Standard deviation of the mean, $S = s/\sqrt{n}$
Methanol extract	25	90.4 ± 3.1	1.206	90 ± 3.5	1.361
	75	0.6 ± 0.5	0.194	14.2 ± 7.8	3.035
	125	0	0	0.4 ± 0.6	0.233
Ethyl acetate extract	25	89.8 ± 1.8	0.700	89.4 ± 3.5	1.361
	75	70.7 ± 6.4	2.490	58.4 ± 3	1.167
	125	26.7 ± 10.4	4.046	10.4 ± 3.8	1.478
Heptane extract	25	88.8 ± 2.4	0.933	87.8 ± 2.2	0.856
	75	0	0	18.8 ± 4.4	1.712
	125	0	0	0	0
Control		95 ± 3	1.167	96.2 ± 1.2	0.466

cally active compounds on HSF (1×10^5 cells/mL) and T47D (4×10^5 cells/mL) monolayers were carried out.

According to our data, the ethyl acetate extract had the lowest toxicity (trypan blue exclusion test) both to the HSF and T47D cell monolayers (Table 2). Just at the concentration 125 μg/mL, the percent of viable HSF cells was 26.7 and for T47D cells was 10.4. That result was also confirmed by mitochondrial dehydrogenase activity test (MTT) (Fig. 1). The optimal concentration, that both stimulated enzyme activity and had no toxicity for the cells was about 25 μg/mL.

Table 3
The effect of 24–72 h treatment with marigold extracts on HSF cells (2×10^4cells/mL) and T47D cells (4×10^4 cells/mL), the trypan blue exclusion test

Kind of extract	Time of culture (h)	Extract concentration (μg/mL)	Percentage of viable HSF cells, confidence level 95%	Standard deviation of the mean, $S = s/\sqrt{n}$	Percentage of viable T47D cells, confidence level 95%	Standard deviation of the mean, $S = s/\sqrt{n}$
Methanol extract	24	25	92 ± 1.6	0.620	89 ± 2.8	1.089
		50	1.5 ± 0.6	0.233	72 ± 10.2	3.891
		75	0	0	0	0
	48	25	91 ± 2.6	1.011	70 ± 5.7	2.217
		50	0.2 ± 0.4	0.155	48.7 ± 9	3.501
		75	0	0	0	0
	72	25	78 ± 2.6	1.011	78.8 ± 6.7	2.607
		50	0.2 ± 0.4	0.155	36.5 ± 3	1.167
		75	0	0	0	0
Ethyl acetate extract	24	25	94.5 ± 2.4	0.933	93.4 ± 1.5	0.583
		75	84.7 ± 4.2	1.634	79.3 ± 7	2.723
		125	0.2 ± 0.4	0.155	66 ± 5.2	2.023
	48	25	87.8 ± 3.7	1.439	96 ± 1.6	0.622
		75	85.3 ± 3.5	1.361	44 ± 3.5	1.361
		125	0	0	0	0
	72	25	80 ± 1.7	0.661	93.6 ± 1.8	0.700
		75	81.3 ± 3.1	1.206	39.3 ± 4	1.556
		125	0	0	0	0
Heptane extract	24	25	96 ± 2.4	0.933	93.2 ± 5.6	2.178
		50	0.2 ± 0.4	0.155	12.6 ± 5.3	2.062
		75	0	0	0	0
	48	25	88 ± 1	0.389	83.8 ± 1.7	0.661
		50	0	0	0	0
		75	0	0	0	0
	72	25	70.3 ± 2.5	0.972	73 ± 4.6	1.789
		50	0	0	0	0
		75	0	0	0	0
Control	24		97 ± 1.2	0.466	95.5 ± 1.6	0.620
	48		94.3 ± 1.5	0.583	96.5 ± 1.4	0.544
	72		95.8 ± 2	0.778	96.7 ± 1.2	0.466

Heptane and methanol extracts in concentrations exceeding 25 μg/mL were toxic after 24 h incubation for both analysed cell cultures. Next, the viability and proliferation activity of cells (density of 2 and 4×10^4 cells/mL) during 72 h incubation with the tested extracts was analysed. Ethyl acetate extract was less toxic for cells and stimulated proliferation better than the heptane and methanol ones (Table 3). For ethyl acetate extract at concentration of 75 μg/mL the viability after 24, 48 and 72 h was 84.7, 85.3 and 81.3%, respectively, for HSF cells and 79.3, 44 and 39.3% for T47D cells. Moreover, mitochondrial dehydrogenase activity of normal fibroblasts (HSF) was more resistant to the inhibitory influence of ethyl acetate extract than tumor cells (T47D). The results showed that concentrations up to 75 μg/mL had no significant influence on dehydrogenase activity or the viability of HSF cells. On the other hand, there was a gradual, time and concentration dependent decrease of the analysed parameters on T47D cells after incubation with these extract

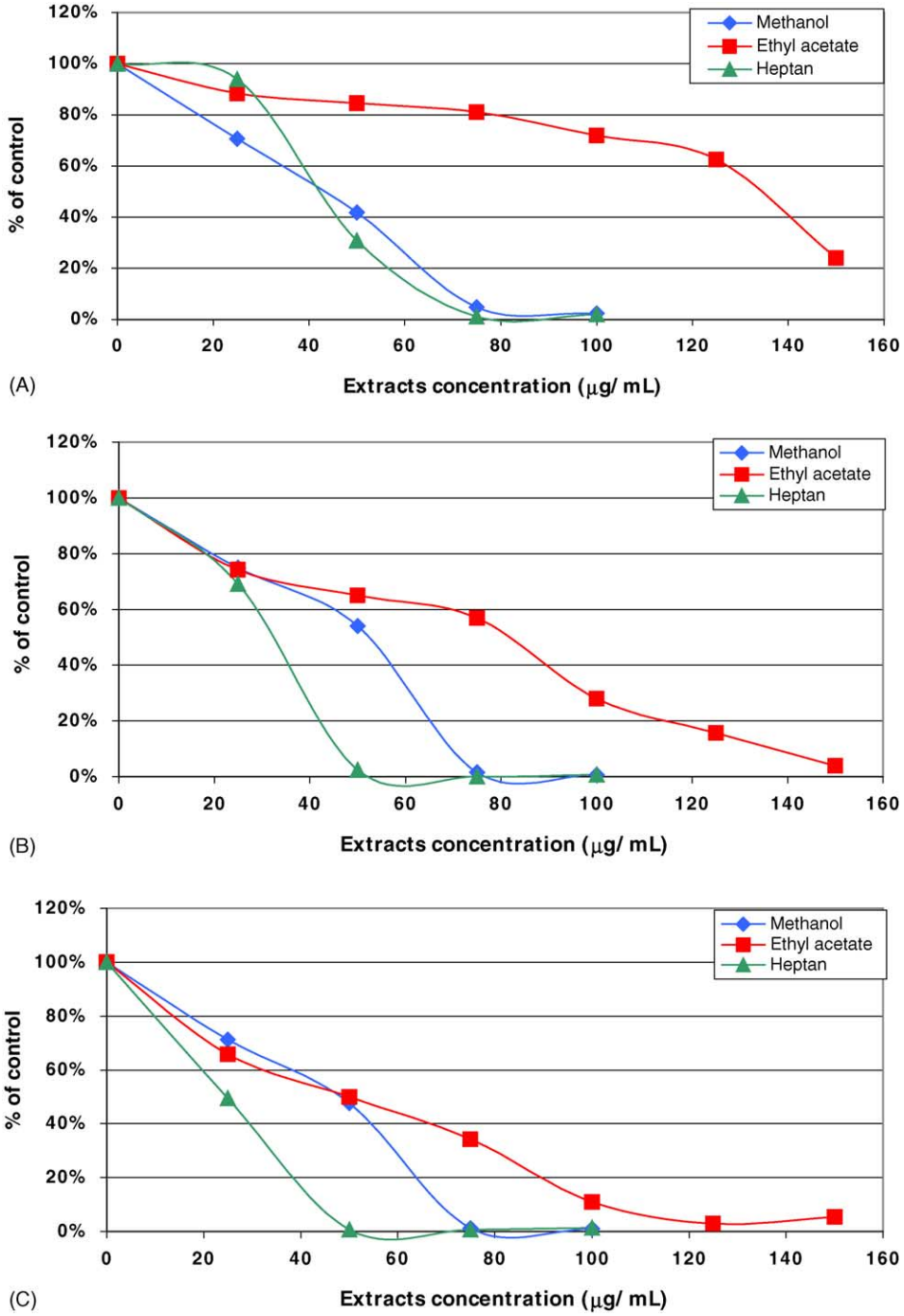

Fig. 2. The effect of 24–72 h treatment with extracts of *C. officinalis flos* on T47D cells (4×10^4 cells/mL). The MTT assay. (A) 24 h, (B) 48 h, (C) 72 h.

concentrations (Figs. 2 and 3). The differences in extract concentration activity that were observed may be due to at least two reasons. First, inhibition of mitochondrial dehydrogenase activity by marigold extracts came far after destruction of cellular membrane that could be also more sensitive to its action. Second, inhibition of the enzyme activity could be a result of cellular membrane destruction and death of the cell, but not caused by influence of extract components on mitochondrial functions. To resolve this problem further investigations are needed. However, the results of investigations of human skin fibroblast (HSF) indicate, that high densities of cells culture (1×10^5 cells/mL) are better adapted to concentrations of ethyl acetate extract than low densities of cell culture (2×10^4 cells/mL) (Tables 2 and 3). Therefore, it is suggested that substances that are present in ethyl acetate extracts may be responsible for flesh-wound healing.

Interesting conclusions on proliferation and viability of normal human skin fibroblasts (HSF) and human breast carcinoma (T47D) cell lines encouraged us to carry out a quantitative study of three extracts of *C. officinalis flos* by the use of HPTLC combined with densitometry. This part of the study was concerned with the elaboration of the optimal chromatographic systems for the separation of the components of the plant extracts. The objective was to acquire the greatest number of well-developed zones. In the study, chromatographic systems elaborated earlier (e.g., for

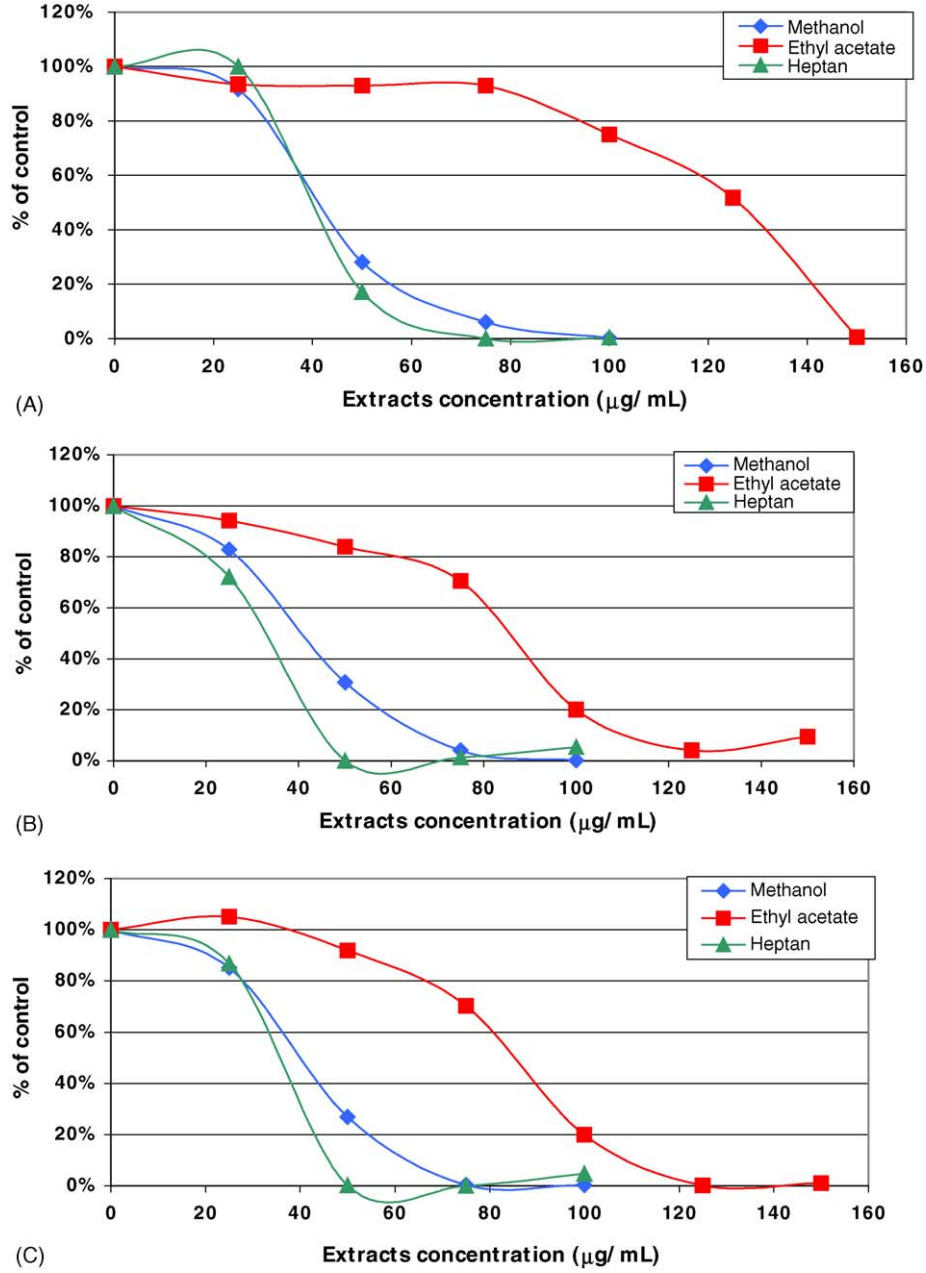

Fig. 3. The effect of 24–72 h treatment with extracts of *C. officinalis flos* on HSF cells (2×10^4 cells/mL). The MTT assay. (A) 24 h, (B) 48 h, (C) 72 h.

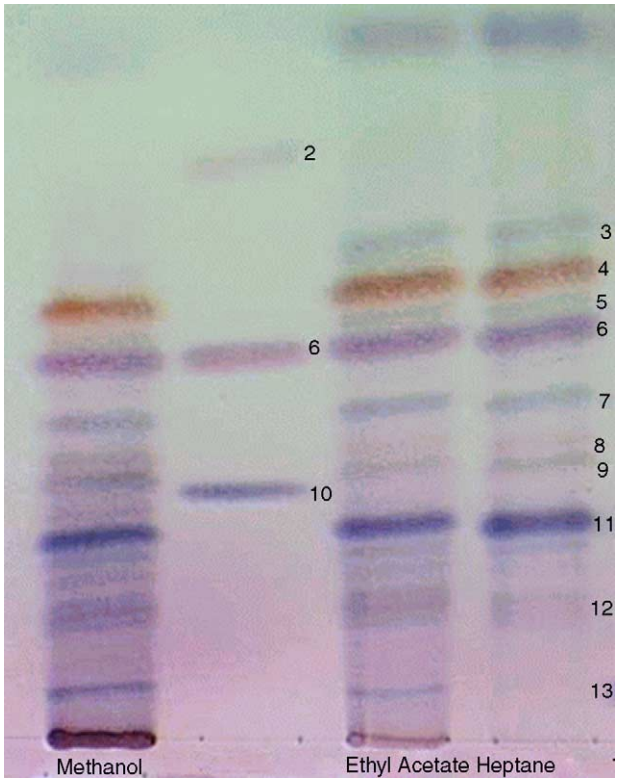

Fig. 4. Results from HPTLC of the extracts of *C. officinalis flos*—investigation of triterpenes. Chromatographic system: Si60/hexane + dichloromethane + methanol + water (4:5:0.9:0.1). Zone no.: 10, oleanolic acid; 6, β-amyrin; 2, β-amyrin acetate. Video documentation: ProViDoc-Desaga (λ = 520 nm).

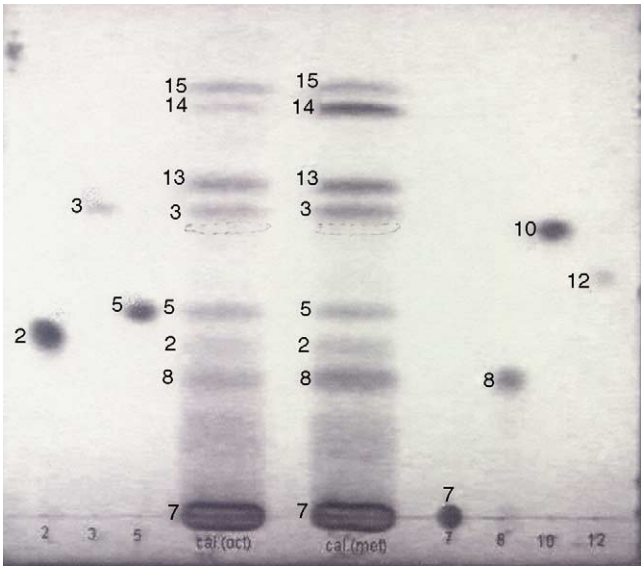

Fig. 5. Results from HPTLC of the ethyl acetate (cal.(oct)) and methanol (cal.(met)) extracts of *C. officinalis flos*—investigation of phenolic acids. Zone and standard no.: 2, protocatechuic acid; 3, vanillic acid; 5, syringic acid; 8, caffeic acid; 7, chlorogenic acid; 10, *p*-coumaric acid; 12, ferulic acid; 14, 15, not identified. Chromatographic system: Si60, mobile phase as in Table 1. Video documentation: ProViDoc-Desaga λ = 254 nm.

separating phenolic acids) were used and applied for the chromatographic analysis.

For the heptane and ethyl acetate extracts special attention was given to triterpene fraction: the standards were: oleanolic acid, β-amyrin and β-amyrin acetate.

Triterpene derivatives have strong anti-inflammatory, anti-edematous, anti-viral, anti-allergic and anti-tumor activity [13–15].

In ethyl acetate and methanol extracts, flavonoids and phenolic acids were analysed. The reference substances were: rutin, narcissin, 3-glucoside of isorhamnetin, isoquercitrin, quercetin and the following acids: caffeic, vanillic, chlorogenic, protokatechuic, *p*-coumaric and syringic.

Polyphenolic compounds have a wide spectrum of biological activity, including viro- and cytostatic-anti-oxidant, anti-inflamatory and anti-fungal action [16–20].

The best chromatographic system for triterpenes was: silica gel Si60 (HPTLC) and the mixed solvent hexane/dichloromethane/methanol/water (4:5:0.9:0.1, v/v) as a mobile phase. In the heptane extract 10 zones were obtained in which β-amyrin was identified. β-Amyrin acetate and oleanolic acid were absent. In comparison with the heptane extract, the zone numbered 12 in the ethyl acetate extract occurred in higher concentration, and an additional zone numbered 13 appeared. As in case of heptane and ethyl acetate extracts, in the methanol extract (Fig. 4) the presence of β-

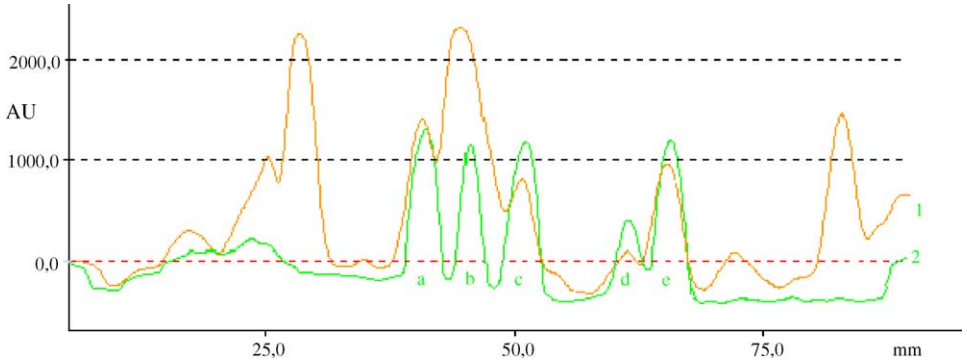

Fig. 6. Densitogram of the methanol extract of *C. officinalis flos* (λ = 366 nm). Abbreviation: a rutin, b narcissin, c chlorogenic acid, d isoquercitrin, e 3-glucoside of isorhamnetin; the densitogram "2" is the mixture of standards.

amyrin was detected, however, β-amyrin acetate, oleanolic acid and zone no. 1 and 3 were absent.

A further part of the study was concerned with the analysis of free phenolic acids in the ethyl acetate and methanol extracts. In HPTLC the simple phase system was applied, which is: a polar adsorbent—silica gel Si 60 and medium polar mobile phase. The best results were obtained by the technique of multiple development of chromatograms [21] with gradient according to the scheme given in Table 1. Phenolic acids were identified against standards in methanol solutions (0.01%) of these compounds (Fig. 5).

In HPTLC the highest hR_f values (=50) were obtained for vanillic acid (zone no. 3) and the lowest for caffeic acid (zone no. 8) and chlorogenic acid ($hR_f = 0$). In the methanol extract the free phenolic acids occurred in greater concentrations than in ethyl acetate extract. In the methanol extract of marigold, flavonoids were also examined (Fig. 6). Four compounds were identified: rutin (3), isoquercitrin (6), narcissin (4) and 3-glucoside of isorhamnetin (7). The plates with silica gel Si60 F_{254} were developed with the mobile phase consisting of toluene, ethyl acetate, formic acid and water (1:9:2.5:2).

4. Conclusions

Qualitative chromatographic investigations revealed that heptane extract contained weakly polar compounds as triterpenes, but there were no traces of phenolic acids or flavonoids. In the ethyl acetate extract there were weakly polar substances as triterpenes, among others: β-amyrin. In this extract the presence of polar compounds—phenolic acids and flavonoids were detected.

In turn, the methanol extract contained only the polar compounds, phenolic acids and flavonoid glycosides.

What result from that is that for achieving positive effects in the cell culture, one should use an extract containing compounds of a wide polarity range. β-Amyrin and the other triterpenes present in the heptane extract have probably negative influence on cells culture, similarly as flavonoids glycosides: rutin, narcissin, 3-glucoside of isorhamnetin, isoquercitrin and phenolic acids: vanillic, protocatechuic, syringic, *p*-coumaric, caffeic and chlorogenic acid in the methanol extract.

The compounds present in ethyl acetate extract have positive influence on cells culture (Figs. 4 and 5) and therefore may help in flesh-wound healing in patients in vivo.

Their mutual action and the possibility of synergism could be controlled by microbiological methods in vitro, which would be the subject of future investigations.

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