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Comparative Fingerprint and Extraction Yield of Medicinal Herb Phenolics with Hepatoprotective Potential, as Determined by UV-Vis and FT-MIR Spectroscopy

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Abstract

The present study was aimed to compare the polyphenolic composition of six medicinal herbs, from wild flora of Romania. The plants investigated, *Cynara scolimus* (artichoke), *Taraxacum officinalis* (dandelion), *Chelidonium majus* (celandine), *Hypericum perforatum* (St. John's wort), *Silybum marianum* (Mary thistle) and *Lycopodium clavatum* (Wolf's claw) are known, to have hepatoprotective action. Using in parallel glycerol-water, ethanol-water and methanol, the solvent-dependence of the extract fingerprint and composition in bioactive molecules was studied by UV-Vis and Infrared (FT-MIR) spectrometry. The extraction yields, calculated as an extraction factor (EF) were superior in acidic methanol comparative to glycerin and ethanol, favorising the increase in phenolic acids against flavonoid derivatives . Based on the differences of polarity between the three solvents used, higher EF values were obtained for dandelion, artichoke, celandine and St. John wort, more rich in phenolic acids than flavonoids. Mary thistle and Wolf's claw had lower concentrations of phenolics, but higher content of lignans and terpenoids. Based on the FT-MIR peaks from 8 regions, for each plant extract, has been determined the fingerprint region between 900 and 1500 cm^{-1} and identified the specific functional groups. A good, significant correlation was found between the concentration of total phenolics calculated by UV-Vis spectrometry and FTIR methods, after calibration with gallic acid. The value of the MIR signal at 1743 cm^{-1} may be considered a good indicator of phenolics concentration in such extracts. Combined UV-Vis and FTIR spectroscopy are recommended as rapid and reliable tools to investigate the fingerprint and to predict the composition of medicinal plants or to evaluate the quality and authenticity of different standardized formulas.

Keywords: FT-MIR, hepatoprotection, medicinal herbs, quality and authenticity, UV spectrometry

Introduction

The traditional herb medicines showed, since centuries, beneficial effects on health promotion, out of side effects, as compared with synthetic drugs. It is also known that their composition is dependent on many ontogenic or genotypic factors influenced by their environment, age, time of harvesting, drying and storage, as well the solvent used to obtain extracts. Due to their natural heterogeneity, the quality of herbs from wild environments shows great fluctuations, so their standardization has been extensively promoted during the last years, the following three attributes being verified: authenticity, purity and assay of their action (Hussain *et al.*, 2009; Yadav and Dixit, 2008). The identification of phytochemicals' fingerprint by chromatography and spectroscopy may provide effective information about qualitative and quantitative composition of herbal medicines and their pattern recognition can be achieved by chemometry (Bender, 2005; Maloney, 2004) and used to discriminate among different herbs and extracts, with noticeable role in the development of standardized formulas, like other pharmaceuticals to make these remedies evidence-based medicines, (Giri *et al.*, 2010; Liang *et al.*, 2004; McGuffin *et al.*, 1997; Yadav and Dixit, 2008), according to WHO requirements (WHO, 2003)

The evaluation of a herbal product by its metabolomic fingerprinting can be accomplished by appropriate methods, including HPLC with UV (DAD), ELSD, MS detection or GC-MS, HPTLC-densitometry, FT-MIR, NIR, NMR or a combination of these techniques (Fan *et al.*, 2006; Giri *et al.*, 2010; Gong *et al.*, 2006; 2009; Hashimoto and Kameoka, 2008; Li *et al.*, 2008; Mattoli *et al.*, 2006).

The UV-Vis spectroscopy offer a simple, cheap and easy-to-use technique to identify and quantify the main phytochemicals, discriminating between the lipophilic and hydrophilic phytochemicals, in relation to the polarity of the extraction solvent.

Fourier transform infrared spectroscopy (FTIR) offers a rapid and non-destructive investigation, easy to

use to fingerprint herbal extracts or powders, is relatively uncommon compared with chromatographic and classical methods (Hussain *et al.*, 2009a; Li *et al.*, 2004b; Liu *et al.*, 2006). The use of attenuated total reflectance (ATR) device evolved rapid FTIR measurements of liquids such as oils and plant extracts, allowing the identification and quantification of valuable plant biomarkers (Schultz and Baranska, 2007).

The herbs, known traditionally, to have hepatoprotective action are generally rich in polyphenols with high antioxidant potential (Mary thistle, artichoke, dandelion, greater celandine, St. John's wort, etc.) since major liver diseases are related to oxidative stress and cellular necrosis (Negi *et al.*, 2008;Utrilla, 1996).

Dandelion *(Taraxacum officinalis)* is an old remedy for grandular unbalances, used in the therapy of liver diseases for the biliary stimulation, normalization of blood circulation, dieresis and toxins release. Its bitter substances (eudesmanolides and germacranolides) named generically "taraxacin", are anti-vomitives and antioxidants (Jeon *et al.*, 2008). Dandelion contains as well flavonoids (7-D glucosides of apigenol and luteoline) and steroids (stigmasterol, sitosterol, ergosterol) which block the *de novo* synthesis of cholesterol and are competitors of cholesterol deposition (Williams *et al.*, 1996). It contains also inulin with protective action against diabetes and liver diseases (Schütz *et al.*, 2006), similarly to artichoke.

Since many years it was reported that artichoke (*Cynara scolimus*) induces regeneration of rat liver (Maros *et al.*, 1966), mainly due to cynarin, a phenolic derivative which stimulates liver cell excretion (Li *et al.*, 2004a; Wang *et al.*, 2003). Luteolin, a flavone which inhibit the *de novo* cholesterol synthesis as well sesquiterpene lactones has coleretic and cholagogue effects (Saenz Rodriguez *et al.*, 2002). The leaf extract of artichoke reduces mild dispepsia (Marakis *et al.*, 2002) and have antioxidant potential, as demonstrated on leukocytes (Perez-Garcia *et al.*, 2000).

Greater celandine (*Chelidonium majus*) is a medicinal poppy (Bone, 1996) with beneficial antihepatotoxic and meanwhile controversial effects on liver (Duke, 1985; Mitra *et al.*, 1992; 1996). It is rich in alkaloids (Gu *et al.*, 2010), mainly chelidonin, a naphtofenantridin derivative which stimulates the enzymatic production in liver and pancreas, it is colecystokinetic and antispastic (Gilca *et al.*, 2010; Hriscu *et al.*, 1980). It was demonstrated experimentally to be antiinflamator, anticancerigenic şi antimicrobian (Vavreckova *et al.*, 1996) being recommended in cyrosis and chronic hepatitis therapy. Using a standardized extract (4 mg chelidonin for 6 weeks) for pacients with digestive syndroms, a significant reduction of symptoms was reported (Ritter and Schatton, 1993). Sanguinarine is another alkaloid found in celandine which acts as a colchicine-like cytostatic (Lopus and Panda, 2006; Malikova *et al.*, 2006) with antiviral, antiinflamatory and antibacterial potential (Zdařilová *et al.*, 2006). Meanwhile, overdoses are recommended to avoid, due to its alkaloid charge and hepatotoxicity (Benninger *et al.*, 1999).

Mary thistle (*Silybum marianum*) is known to have hepatoprotective action and to be a hepatocyte activator (Ferenci *et al.*, 1989; Salmi and Sarna, 1982), being used not only as tea but also in some standardized drugs such as Silimarine (generic name of lignan flavonoids, a mixture of silibine, silibinine, silicristine and silidianine). Silibine is the most active derivative, the extracts being standardized at 70-80% silibine. The mechanisms involved in its hepatoprotective action are diverse and include antioxidant and antiperoxidation effects (Basaga *et al.*,1997; Bosisio *et al.*, 1992), detoxifying effect (Baer-Dubowska *et al.*, 1998; Miguez *et al.*, 1994), antiviral action (McPartland, 1996) and glutathion protection (Cabrera, 1996). Silimarine protects hepatocytes agaist toxic effects of acetaminophen, ethanol, carbon tetraclorine (Bosisio *et al.*, 1992; Favari and Perez-Alvarez, 1997; Muriel *et al.*, 1992), decrease the fibrosis by III-peptide procolagen inhibition (Feher *et al.*, 1989) and inhibit cytochrome P 450 (Baer-Dubowska *et al.*, 1998).

St. John's wort (*Hypericum perforatum*) contains several compounds with hepatoprotective properties, by synergistic action of hypericins and phenolics. Hypericins are reddish-violet condensed anthraquinones found (0,1-0,2%) in leaves and flowers. Due to their UV fluorescence, are photoactivated in sun, being good candidates for tumor photodynamic therapy. It has also anti-depressive and antiviral, has cholagogue and choleretic properties (Ali and Olivo, 2002; Kurth and Spreeman, 1998). Flavonoid glucosides (rutoside) protects the liver against oxidative stress, especially by its action on superoxid dismutase (Istudor, 2003). The condensed tannins and hyperforin, have antibiotic effects, inhibits cytochrom P450 CYP3A4 şi CYP2C9 (Barnes *et al.*, 2001; Gitea *et al.*, 2007; Kurth and Spreeman, 1998; Nahrstedt and Butterweck, 1997).

Wolf's claw *(Lycopodium clavatum*) is a less studied herb, which contains more than 35 alkaloids and other biomolecules, whose structure and pharmacologic effects are not yet elucidated The bioactive molecules are lycopodine, clavatoxin, clavolonin, clavatin, liclanitin, annapodin, izolzcopodin, as well nicotine, triterpenoids and sterols, flavonoids, radium (known as an antitumor mineral). Triterpenoids and sterols are stimulators of digestive system, antialergic, prevent the cholesterol and fats deposition, may have good effects on liver tumor inhibition, anti-inflamatory and antimicrobial action (Betancor-Fernandez *et al.*, 2003).

The present study aimed to compare the fingerprint of different extracts of the above-mentioned medicinal herbs, collected from wild flora of Romania. The dependence of the extract composition on the solvent polarity (glycerol-water *vs* ethanol-water *vs* acidic methanol) was studied by UV-Vis spectrometry and Infrared (FT-MIR) fingerprints.

Materials and methods

Medicinal plants and preparation of extracts

Six types of medicinal plants, from wild flora of different areas of Transylvania, Romania were investigated. The plants were numbered as follows: 1-*Cynara scolimus* (artichoke), 2-*Taraxacum officinalis* (dandelion), 3-*Chelidonium majus* (celandine), 4-*Hypericum perforatum* (St. John's wort), 5-*Silybum marianum* (Mary thistle), 6-*Lycopodium clavatum* (Wolf's claw). Aliquots of 15 g from each dried and grounded plant (selected from 100 g mix of leaves, stems and flowers) were extracted in 85 ml solvent: methanol 90% in water, acidulated with 1% hydrochloric acid (M) or ethanol 93% in water (E) or 30% glycerin in water (G). After sonication 30 min, centrifugation and filtration, the clear extracts were kept in the deep freezer until analysis.

UV-Vis spectra and calculation of extraction factors

The UV-Vis spectra were recorded (700-200 nm) for each extract (M, E or G) using a Jasco V 530 Spectrofotometer. There were identified the maxima wavelenghts specific for phenolics (280 and 330 nm), carotenoids (420-470 nm) and/or chlorophylls (663 nm).

To compare the yields of extraction in different solvents it has been calculated the Extraction Factors (EF) of bioactive molecules from each extract, considering the absorption values ($A_{\lambda max}$) recorded for each λ_{max} , multiplied with the dilution factor (d). The formula applied was:

$$EF = A(\lambda_{max}) \times d$$

The results were expressed as mean values of four samples per plant and in duplicated extracts from each plant. The content of total phenolics was determined by Vis spectrometry, using the standard method, Folin Ciocalteu (Singleton, 1999)

Tab. 1. The absorption maxima λ_{max} (nm) of each plant extract from UV-Vis spectra and the mean values calculated (X ± SD) for extraction factors (EF)

Plant (nr.)	$\lambda_{max.}$ (nm)	EFE Ethanol	EFG Glycerin	EFM Methanol
Dandelion (1)	279	7.69 ± 0.55	231.64 ± 10.50	240 ± 0.55
	320	7.96 ± 0.58	201.24 ± 9.20	280 ± 10.50
	396	2.07 ± 0.12	106.09 ± 7.50	0
Artichoke (2)	247	83.08 ± 3.08	43.85 ± 1.50	0
	295	82.94 ± 3.88	41.19 ± 1.35	91.71 ± 6.50
	327	97.05 ± 5.02	42.86 ± 1.65	73.31 ± 2.80
	663	0	0	6.03
Celandine (3)	212	0	255.36 ± 11.34	0
	275	67.79 ± 2.05	103.96 ± 6.90	288 ± 12.10
	330	48.94 ± 1.86	72.96 ± 2.50	219 ± 10.13
	396	13.14 ± 0.90	42.86 ± 1.98	0
	663	0	0	9.81 ± 0.40
St. John's wort (4)	270	33.89 ± 1.80	137.71 ± 8.98	422 ± 21.05
	330	19.03 ± 1.10	82.68 ± 3.98	238 ± 11.08
Mary thistle (5)	280	19.76 ± 1.06	34.88 ± 1.98	142 ± 9.58
	325	14.63± 1.01	29.26 ± 1.27	77 ± 3.10
	395	19.76± 1.12	18.01 ± 1.18	0
Wolf's claw (6)	233	57.91± 2.90	0	108.31 ± 9.80
	284	31.92 ± 1.75	25.81 ± 1.39	0
	324	29.94 ± 1.50	24.96 ± 1.35	59.4 ± 2.80
	397	12.46 ± 1.80	3.99 ± 0.84	0
	652	0	0	2.69 ± 0.54

FT-MIR measurements

The Fourier Transform Infrared spectrum (FTIR) of each extract was recorded in the MIR region, from 4000 to 900 cm^{-1}, and 64 scans were accumulated for each spectrum using the Horizontal Attenuated Total Reflection (HATR) device, using a Shimadzu Prestige 2 FTIR spectrometer (with apodization Happ-Genzel). The spectral data were processed using the IR solution Software Overview (Shimadzu) and OriginR 7SR1 Software (OriginLab Corporation, Northampton, USA). The spectra were registered as fluid (M, E, G) but also evaporated extracts (these data are not shown). Total phenolics were determined also by FTIR method, either using the intensity of the peak at 1742 cm^{-1} or from the area of the region 950-1900 cm^{-1}, considering the calibration curve with pure gallic acid (range of concentrations 5 to 30 mg/ml methanol).

Results and discussions

Extraction factors of bioactive molecules, based on UV-Vis spectra

The comparative UV-Vis spectra of the ethanol (E) and glycerin (G) extracts of the six medicinal plants were recorded (data not shown), as well in methanolic extracts (M) (Fig. 1), methanol being considered a "reference" solvent known to extract phenolics and terpenoids from these plants.

Based on their specific spectra, the absorption maxima ($\lambda_{max.}$) of each plant extract and the mean values of extraction factors (EF) were calculated for each solvent (E, G and M) (Tab. 1).

To have an integrated image of the differences between plants, solvent type and concentrations of bioactive molecules extracted, the EF mean values at 270-290 nm (for phenolic acid derivatives extracted in E, G, M) (EFE1, EFG1, EFM1) and at 317-340 nm (for flavonoid derivatives) (EFE2, EFG2, EFM2) for each of the 6 plants were represented (Fig. 2).

According to Tab. 1 and Fig. 2 data, it has been noticed that extraction factors in acidic methanol were superior to glycerin and ethanol, especially for phenolic acids (EFE1, EFG1, EFM1) comparing to flavonoid derivatives (EFE2, EFG2, EFM2). Based on the differences of polarity between the three solvents used (glycerin the most polar, followed by methanol and ethanol) it has been noticed high-

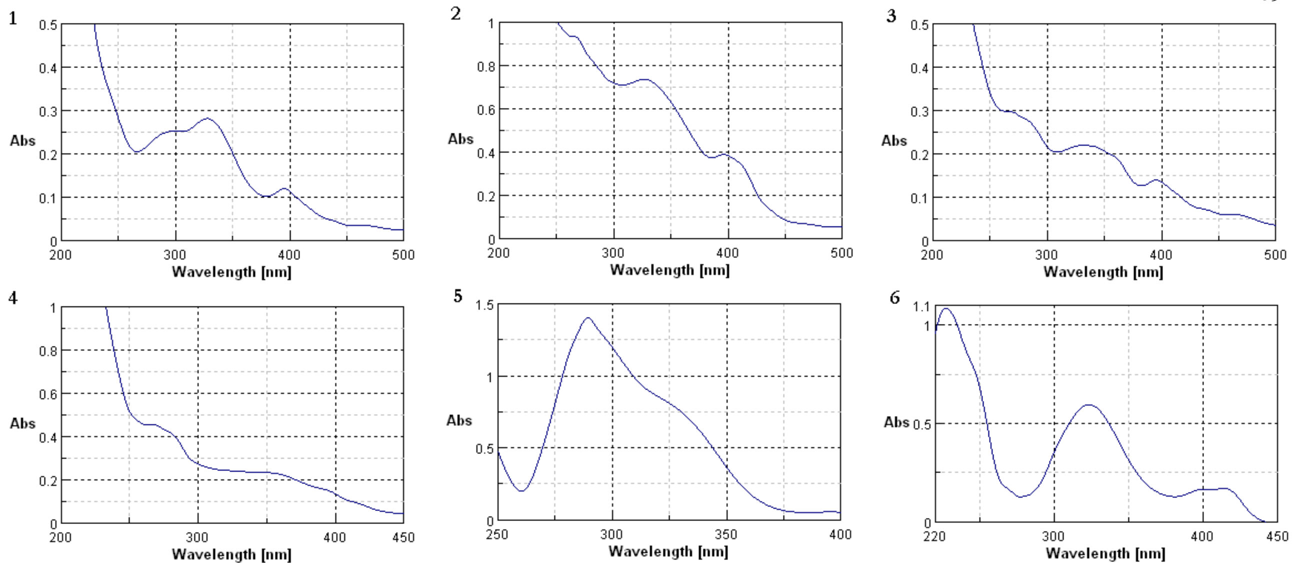


Fig. 1. Comparative UV-Vis spectra of methanolic (M) extracts of *Taraxacum officinalis* (1); *Cynara scolimus* (2); *Chelidonium majus* (3); *Hypericum perforatum* (4); *Silybum marianum* (5); *Lycopodium clavatum* (6)

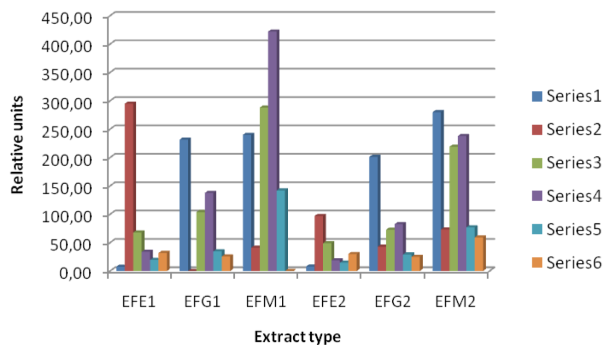


Fig. 2. Comparative mean values of extraction factors (EF) in different solvents (ethanol-E, glycerin-G, metahnol-M) at 270-290 nm (EFE1, EFG1, EFM1) and 317-340 nm (EFE2, EFG2, EFM2) for each of the studied plants (series 1-6)

er EF values for dandelion (1), celandine (3) and St. John wort (4), more rich in polar molecules, such as phenolic acids and flavonoids. Plants 1 and 3 had similar EF in M and G, plant 2 (artichoke) was better extracted in ethanol and was more rich in phenolic acid derivatives. St.John's wort (4) components were two times better extracted in methanol than glycerin, and low EF values in ethanol, an indication of polar active molecules. Mary thistle (5) and Wolf's claw (6) contained reduced concentrations of phenolics, but high absorptions in methanol at 280 and 233 nm, respectively, which might be attributed to higher concentrations of lignans and terpenoids.

For therapeutic reasons it has been consider that ethanol extracts or evaporated methanolic extracts can provide higher concentrations of bioactive molecules from these plants. Anyway, as it was reported recently, methanolic extracts showed hepatoprotective activity against Carbon Tetrachloride-Induced Hepatotoxicity in Rats (Ahsan *et al.*, 2009). Of course for humans the total elimination of methanol (under vacuum) is a condition to have a safe standardized extract.

Tab. 2. Absorption peak areas of different regions (1-8) of FTMIR spectra recorded for methanol extracts (M) of the studied medicinal plants

Wavenumber (1/cm)	1	2	3	4	5	6
3300-3350 (8)	0.70	0.92	0.63	0.82	0.93	0.80
2800-3000 (7)	0.89	0.81	0.98	0.90	0.82	0.75
1500-1520 (5)	0.35	0.22	0.30	0.52	0.32	0.30
1300-1440 (4)	0.72	0.80	0.80	0.95	0.43	0.63
1170-1230 (3)	0.90	0.90	0.92	1.15	0.78	0.80
1000-1100 (2)	1.35	1.45	1.32	1.50	1.05	1.28
<1000 (1)	0.50	0.78	0.60	0.85	0.60	0.52
Total phenolics (by FTIR) mg GA eq./ml M	11.33	11.03	11.16	10.18	8.05	9.26
Total phenolics (by Vis spectrometry) mg/ GA eq./ml M	9.307	9.066	9.171	8.386	6.669	7.642

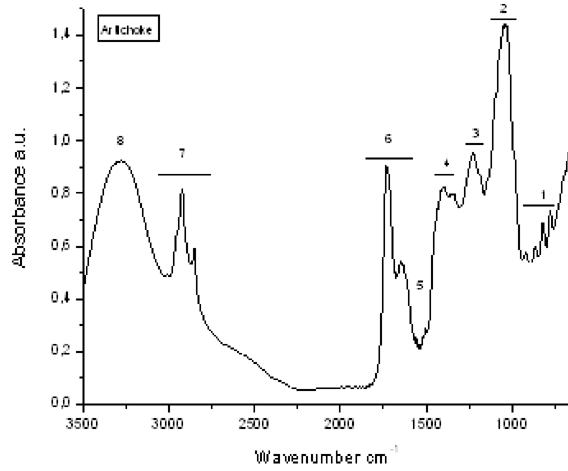
FT-MIR fingerprint

The FT-MIR spectra (4000-900 cm^{-1}) of E and G extracts of each plant were registered and the specific wavenumbers and intensities were considered (data not shown). Fig. 3 presents the FT-MIR spectra of M extracts and Tab. 2 includes the corresponding absorption peak areas for specific regions (1-8). In Tab. 2, is included the total phenolics concentration in methanol (M) extracts determined by FTIR and by Vis spectrometry.

The functional groups identification was based on the FTIR peaks attributed to stretching and bending vibrations. Eight areas (marked from 1 to 8) (Fig. 3) were identified in the MIR domain and the fingerprint region was localized between 900 and 1500 cm^{-1} (areas 1-4).

Area 1 (< 1000 cm^{-1}) corresponds to C-H bending vibrations from isoprenoids, area 2 (997-1130 cm^{-1}) to stretching vibrations C-O of mono-, oligo- and carbohydrates, with signals at 1030, 1054, 1104, and 1130 cm^{-1}, while area 3 (1150-1270 cm^{-1}) corresponds to stretch-

1



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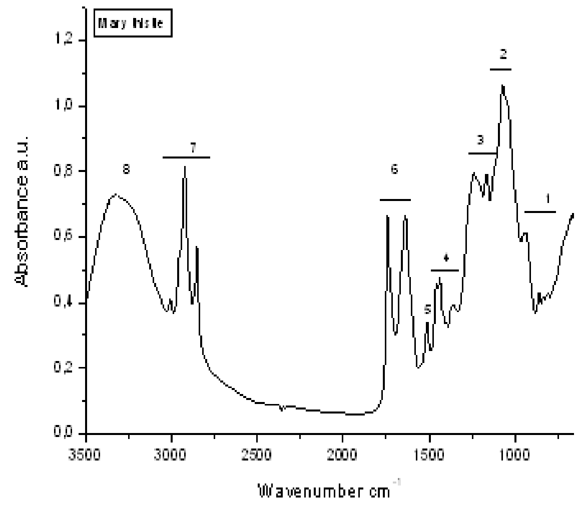

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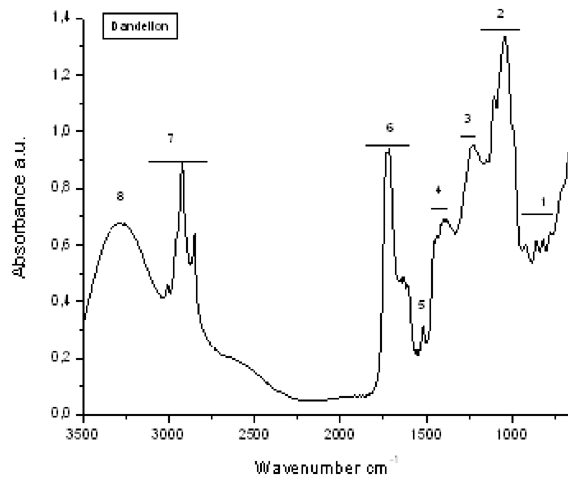

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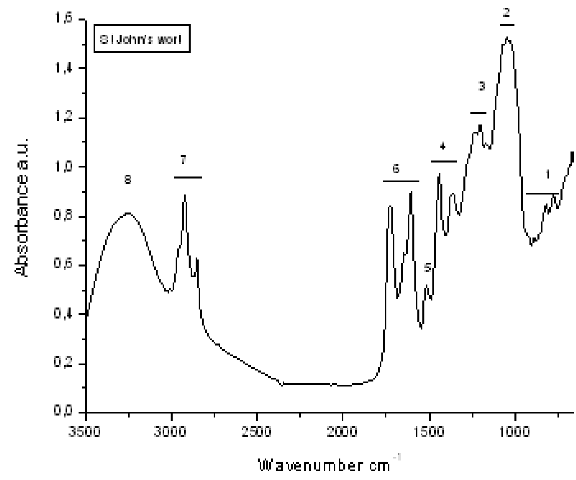

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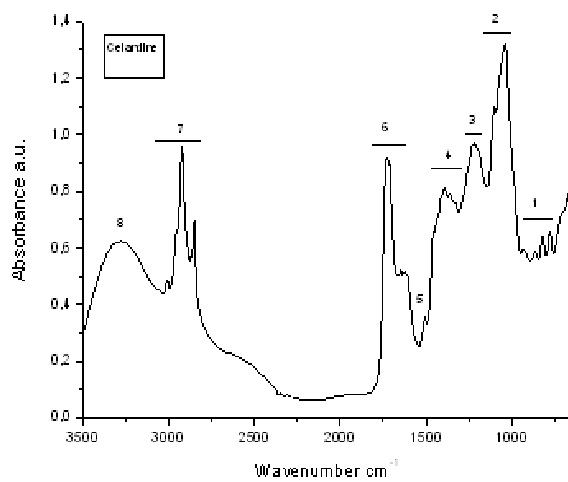

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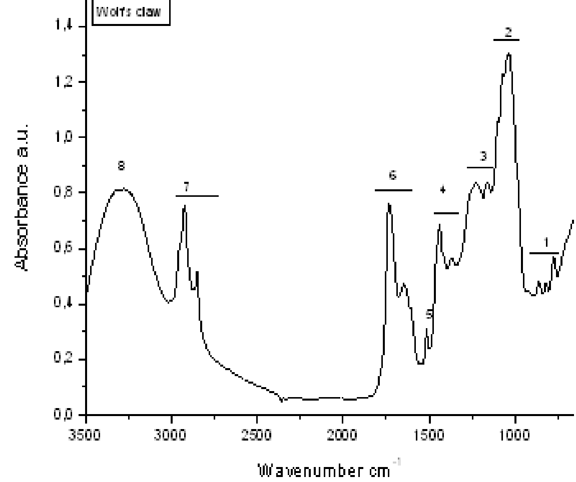

Fig. 3. The FTIR fingerprint of the metanolic (M) extracts of the studied plants: *Cynara scolimus* (1); *Taraxacum officinalis* (2); *Chelidonium majus* (3); *Silybum marianum* (4); *Hypericum perforatum* (5); *Lycopodium clavatum* (6). The specific regions are numbered 1 to 8

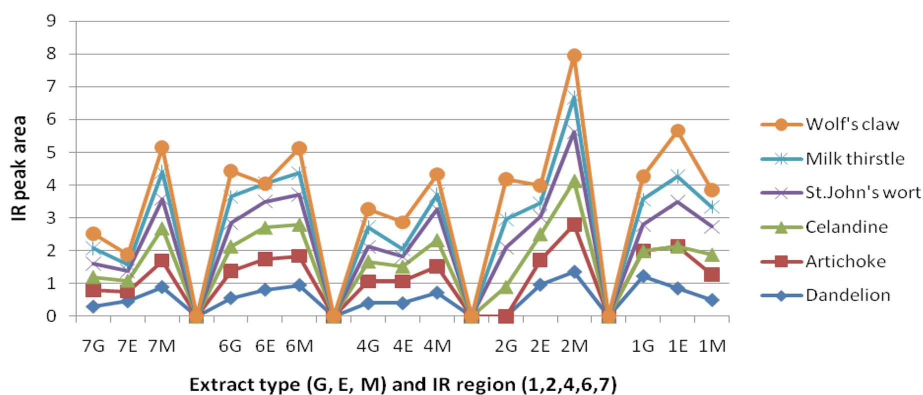

Fig. 4. Comparative representation of the differences recorded between the areas of regions 1, 2 , 4, 6, 7 corresponding to each plant extract (ethanol-E, glycerin-G or methanol-M)

ing vibrations of carbonyl C-O or O-H bendings. Area 4 (1300-1450 cm^{-1}) correspond to stretching vibrations C-O (amide) and C-C stretchings from phenyl groups, while area 5 (1500-1600 cm^{-1}) to aromatic domain and N-H bending vibrations. Area 6 is a complex one (1600-1760 cm^{-1}), corresponding to bending vibrations N-H (amino acids), C=O stretchings (aldehydes and cetones, esters) as well to free fatty acids (1710 cm^{-1}) and glycerides (1740 cm^{-1}). Area 7 (2800-2900 cm^{-1}), corresponds to C-H stretching vibrations specific to CH_3 and CH_2 from lipids, metoxy derivatives, C-H (aldehydes), including *cis* double bonds. Area 8 (3350-3600 cm^{-1}) corresponds to stretching vibrations of OH groups (from water, alcohols, phenols, carbohydrates, peroxides) as well from amides (3650 cm^{-1}).

In alcoholic extracts there are absorption peaks in the domain 1300-1800 cm^{-1}, more than in glycerin, e.g. at 1558, 1517 and1467 cm^{-1} , as well in the region 1380-1450 cm^{-1}. Such differences were noticed also by other authors, after processing the second derivative (Liu si col, 2006) in Angelica extracts, where typical signals specific to cellulose and hemicelluloses at 3413 and 1054 cm^{-1}. The signals at 1642 and 1536 cm^{-1} correspond to amide I band (carbonyl group) and amide II (stretching νCN + bending δNH) found in glycoproteins. Carbonyl groups have specific signals at 1743 cm^{-1}. Fig. 4 represents the differences recorded between the IR areas 1, 2 , 4, 6, 7 corresponding to each plant extract (E, G or M).

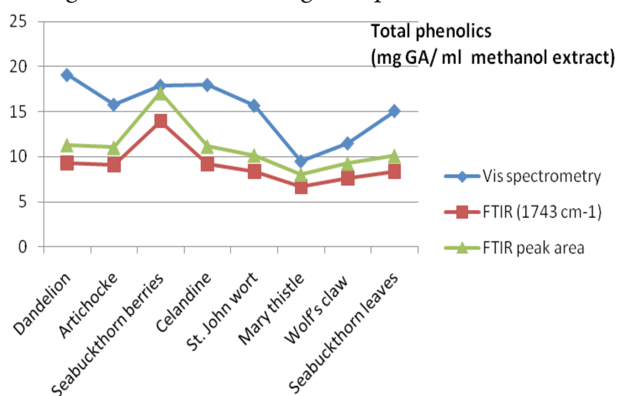

Fig. 5. Comparative representation of the total phenolics (expressed as mg GA/ml metanolic extract), calculated from Vis spectrometry, from FTIR peak intensity at 1743 cm^{-1} and from FTIR peak area

Looking to region 1 (specific to terpenoids) it has been-noticed that plants 6, 5 and 4 had higher peak areas in ethanol, similarly to results from UV-spectra. In the other IR regions (4 and 6) no significant differences between the three solvents extracts were noticed, but in regions 2 (corresponding to glucosides) and 7 (lipids), in all plant extracts, the M extract was significantly more charged in molecules that E or G extracts.

Finally it has been compared the phenolic concentrations, determined by FTIR method, based on the peak intensity at 1743 cm^{-1} and total phenolics calculated from Vis spectrometry. A significant ($p<0.05$) correlation factor was obtained, as shown in Fig. 5. It is known that the measurement by Vis spectrometry is not specific to phenolics and can overestimate concentrations, while the FTIR method using the peak area (950-1900 cm^{-1}) estimation can give also false results. It can be consider in this case that measurements based on FTIR absorption intensity at 1743 cm^{-1} offer the best evaluation of the phenolics concentration in these plants. Generally the concentrations oscillated between 15 to 20 mg GA/ml methanolic extract(15000-20000 ppm), in agreement with other determinations.

Conclusions

The data presented in this study showed that UV-Vis spectrometry and FT-MIR spectroscopy are adequate techniques to fingerprint comparatively and to evaluate the extraction yield of medicinal herbs with hepatoprotective potential. Based on UV spectrometry, the extraction yields were superior in acidic methanol comparative to glycerin and ethanol, increased in phenolic acids comparative to flavonoid derivatives. Based on the differences of polarity between the three solvents used, higher extraction yields were obtained for dandelion, artichoke, celandine and St. John wort, more rich in phenolic acids than flavonoids. Mary thistle and Wolf's claw had lower concentrations of phenolics, but higher content of lignans and terpenoids. Based on the FT-MIR spectroscopy, for each plant extract it was determined the fingerprint region

to be located between 900 and 1500 $cm^{-1}$and it has been identified the specific functional groups.

All FTIR data will be correlated and further validated with the detailed HPLC analysis of the same extracts, in order to validate the FTIR method as a good tool to investigate the fingerprint and to predict the composition of medicinal plants or to evaluate the quality and authenticity of different standardized formulas.

Acknowledgements

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