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Characterization of biological active compounds from *Verbascum phlomoides* by chromatography techniques. I. Gas chromatography

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Abstract

The extracts, decoctions and infusions of Verbascum, commonly known as "mullein", have been used in traditional medicines for centuries in almost all parts of the world. In this paper, a screening of two different extracts of Verbascum phlomoides flowers originating from the Southeast region of Romania is presented. The content of total phenolics and flavonoids from extracts were determined. Also, the GC-MS investigation was performed.

Key words: *Verbascum phlomoides*, total phenolics, flavonoids, GC-MS

Introduction

Plant-derived polyphenols receive considerable interest because of their potential antioxidant and antimicrobial properties. That is why the identification and separation of antioxidants from natural sources in order to replace the synthetic ones has received much attention and efforts. The incorporation of local knowledge concerning ecological relations into biological and ecological studies strengthens the links between man and the environment, leading to the global conservation of biodiversity.

Mullein (*Verbascum*) flowers are highly valued herbal drugs used in the treatment of inflammation, asthma, spasmodic coughs and other respiratory tract diseases. Their phenolic constituents are considered to be responsible for the anti-inflammatory and antimicrobial activity of the herb. This biannual *Scrophulariaceae* plant, flowering from April to May, is known for its diuretic, analgesic, expectorant and antiseptic properties.

Verbascum species are used for different purposes in traditional medicine around the world; therefore, researchers have tested them for different types of biological activities. *Verbascum* species contain biologically active compounds, such as flavonoids, phenylethanoid and neolignan glycosides, saponins, iridoid and monoterpene glycosides.

The traditional use of mullein flowers has been thoroughly documented in several handbooks and scientific literature. Traditional medicinal use of mullein flower connected to catarrh of the upper respiratory tract, cough and colds has been documented in handbooks [1-3]. Although relatively few pharmacological studies on mullein preparations have been reported, the pharmacological activities of certain constituents, notably the iridoid aucubin and the phenylethanoid glycoside verbascoside (acteoside), have been extensively studied and

may explain some of the effects of mullein flower [1]. An iridoid ester glycoside acylated with *p*-coumaric acid was isolated from the flowers of *Verbascum phlomoides*, together with the known one, specioside. Caffeic acid esters, verbascoside and forsythoside B were found as minor constituents. A saponin was also obtained and identified as desrhamnosyl-verbascosaponin [4].

Verbascum phlomoides L.

Mullein flower consists of the dried flowers, reduced to the corolla and the androecium, of *Verbascum phlomoides*, and their main components [1] are presented in Table 1.

Table 1. Main constitutents of *Verbascum phlomoides* flowers.

iridoid glycosides	aucubin; catalpol; 6-xylosylaucubin; 6-xylosylcatalpol; 6-(4”-p-coumaroyl)-xylosylaucubin (named phlomoide); an iridoidester glycoside- namely specioside
flavonoids	tamarixetin 7-rutinoside (predominant); tamarixetin 7-glucoside; apigenin and luteolin and their 7-glucosides; diosmin; chrysoeriol; eriodictyol; kaempferol; quercetin; rutin
phenylethanoid glycosides	verbascoside (acteoside); traces of forsythoside B (verbascoside 6’-apioside)
triterpene saponins	verbascosaponin; verbascosaponin A; verbascosaponin B; desrhamnosyl verbascosaponin
polysaccharides	arabinogalactan with a β-1,6-linked galactan backbone; arabinogalactan; xyloglucan
phenolic acids	vanillic, *p*-hydroxybenzoic, p-coumaric, ferulic, protocatechuic and *p*-hydroxycinnamic acids; *p*-coumaric acid glucoside
other constituents	phytosterol glycosides; digiprolactone; fatty acids (palmitic and linolenic acids)

The efficacy of mullein as a mild expectorant, demulcent and emollient results from the presence of saponins and mucilage [4, 5], whereas its anti-inflammatory, antimicrobial and diuretic activity are believed to be connected with the phenolic compounds, flavonoids and phenylethanoids [6, 7]. However, current knowledge about the content of phenolics in *Verbascum* is limited and no accurate analytical method for their analysis is available.

A HPLC method established for the simultaneous determination of eight flavonoids and two phenylethanoids in the flowers of *Verbascum phlomoides* and *Verbascum*

densiflorum samples [8] was appropriate for the quality assurance and the differentiation of these samples.

The aim of our study was to investigate by gas chromatography technique the plants of *Verbascum phlomoides* L., originating from Southeast region of Romania. The objectives were to obtain and characterize two different extracts obtained from *Verbascum phlomoides* L.

Materials and Methods

Materials

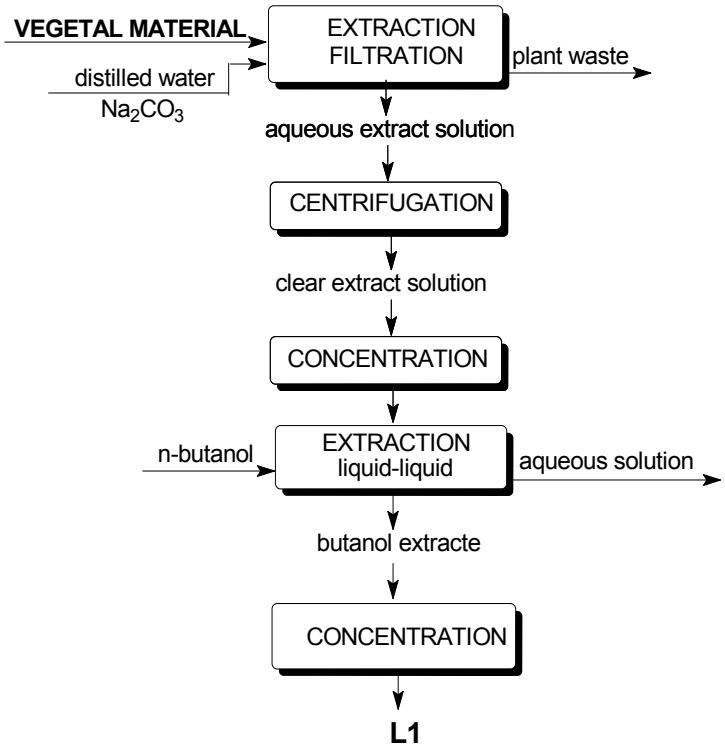
The flower samples were collected from plants of *Verbascum phlomoides* L. growing in the Southeast region of Romania (Ilfov County).

Analytical grade n-butanol, ethanol, methanol and distilled water were used for extraction procedures and for analysis of extracts. Gallic acid, rutin, aluminium chloride, and Folin-Ciocalteu reagent were purchased from Fluka.

Sample preparation

a) *Verbascum phlomoides* butanol extract (Scheme 1)

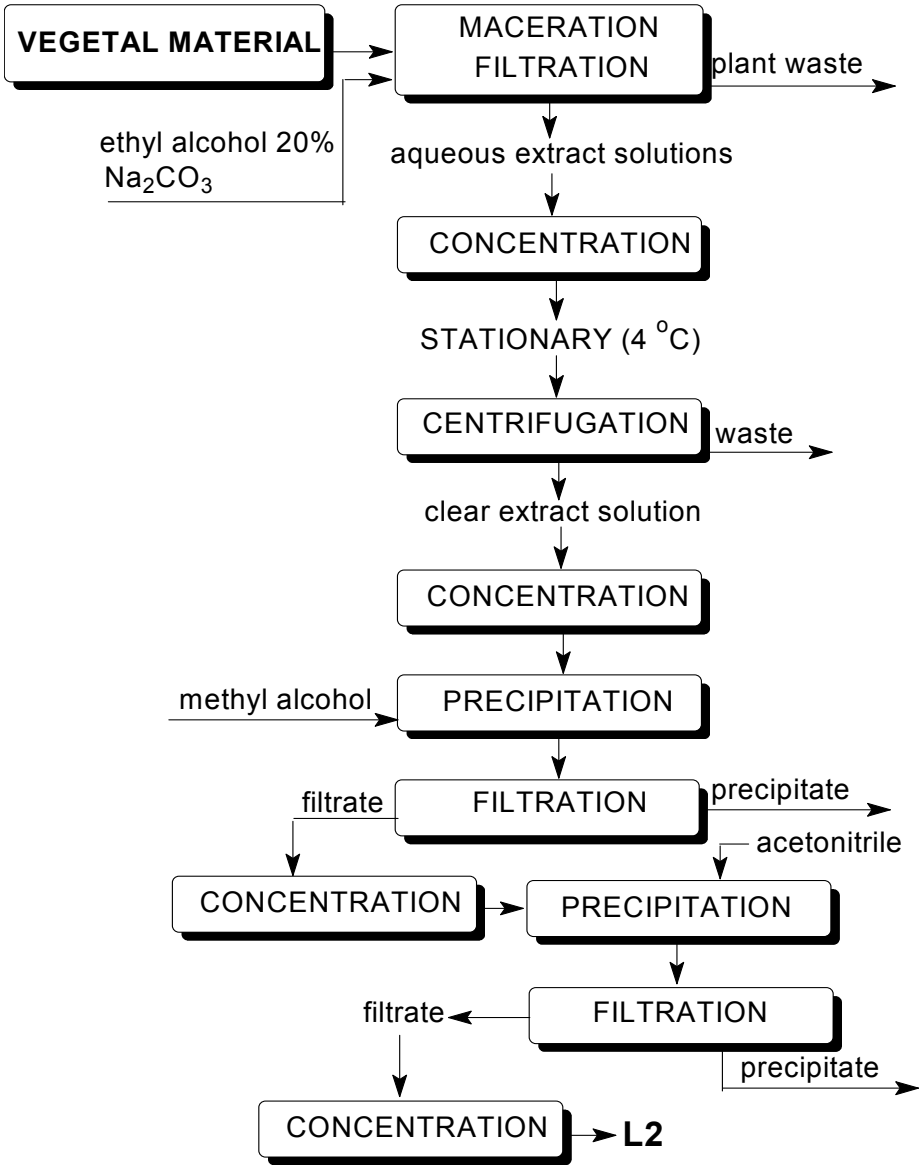
Plant material (200 g) was extracted with 2000 ml distilled water (plant material/solvent ratio=1/10, m/v), made alkaline by adding 15 g $CaCO_3$, at 50ºC, under continuous stirring, for 2h. After filtration, an opalescent brown extract resulted (1420 ml) which was further centrifuged for 30 min at 3000 rpm. The clear aqueous extract obtained after centrifugation (approx. 1355 ml) was concentrated at 50ºC under low pressure (72-74 mmHg) to reach a volume of 150 ml which was further subjected to successive liquid-liquid extraction procedure using n-butanol (4×300 ml). An aqueous extract/solvent ratio=1/2 (v/v) was used. The resulted butanol extracts (approx. 1220 ml) were mixed and concentrated at 60ºC and low pressure (72-74 mmHg) to obtain the L1 fraction as a brown solid waste.



Scheme 1. Extraction of *Verbascum phlomoides* L. flowers to obtain L1 fraction.

b) *Verbascum phlomoides* ethanol extract (Scheme 2)

200 g plant material were macerated with 2000 ml 50% ethanol (plant material/solvent ratio=1/10, m/v) and 15 g $CaCO_3$ at room temperature, under occasionally stirring for 3h. After filtration, it is obtained an opalescent brown extract (1370 ml) which was further concentrated at 40ºC under low pressure (72-74 mmHg) to remove the solvent, after that being stored at 4ºC for 24h. The extract was further centrifuged for 45 min at 3000 rpm, resulting waste 1 and 325 ml aqueous extract. This was concentrated at 50ºC under low pressure (72-74 mmHg) to a volume of 100 ml and precipitated with methanol (aqueous extract/solvent ratio=1/4, v/v). The precipitate was further dried in an oven at 40ºC. The filtrate was concentrated at 40ºC under low pressure (72-74 mmHg) to obtain the L2 fraction as a slightly hygroscopic brown powder.



Scheme 2. Extraction of *Verbascum phlomoides* L. flowers to obtain L2 fraction.

Gas chromatography–mass spectrometry

The extracts were analyzed without derivatization using gas chromatography–mass spectrometry. (GC–MS) analyses were performed on a Shimadzu GC apparatus coupled with

a mass detector and a Thermo Scientific column (60 m, 0.25µm, 0.25 mm). Working conditions were: injector 220°C, oven temperature: start 35ºC hold 5 min; programmed from 35 to 150°C at 2ºC min^{-1}, maintain for 8 minutes, then increase to 220ºC at 5ºC min^{-1} and finally hold to 220ºC for 40.5 minutes; carrier gas helium (at linear velocity 24 cm s^{-1}); a split/splitless injector was used at 220°C in split mode. MS conditions: detector voltage: 1kV; ion source temperature: 250°C; mass range: 35–300 mass.

Apparatus

All absorbance measurements were carried out using a JENWAY 6405 UV–VIS spectrophotometer. A 1.0 cm optical path length glass cell was used in all measurements.

Determination of total phenolics

The total phenolic contents were estimated as gallic acid (GA) equivalents per gram of dried plant extract, according to Folin-Ciocalteu phenol reagent method [9]. To 100µl of the extract (200µg) was added 0.5 ml of Folin-Ciocalteu phenol reagent and 1 ml of sodium carbonate (4% w/v) and the volume made up to to 2 ml, contents were mixed and allowed to stand for 30 minutes. Absorbtion at 765 nm was measured. The total phenolic content was expressed as gallic acid equivalents using a standard curve generated with gallic acid. Data presented are average of four measurements.

Determination of total flavonoids

Total flavonoid contents were estimated following the aluminium chloride colorimetric method [10]. Briefly, aliquots of 2 ml (200µg/ml) of the extracts were added to 2 ml of a 3% $AlCl_3$ solution in methanol and after incubation for 10 min at room temperature, the absorbance was measured at 430 nm. Total flavonoid contents were calculated from a calibration curve of rutin analyzed under the same conditions. The flavonoids content was expressed in rutin (R) equivalents per gram of dried plant extract. The determination was conducted in triplicate and values are expressed in mean ± SD.

Results and Discussion

The total phenolics and flavonoids content values for the two different fractions of *Verbascum phlomoides* (L1 and L2) are presented in Table 2.

Table 2. Flavonoids and phenols content in L1 and L2 samples.

Samples	Flavonoids (mg rutin/g extract)	Phenols (mg GA/g extract)
L1	30.7	4.183
L2	10.1	4.933

The flavonoid content of L1 extract is higher as compared to L2 extract, which is richer in polyphenols. Further HPLC-MS studies will offer information concerns to the identification and quantification of these components.

The components identified in *Verbascum phlomoides* L. extracts by GC-MS, their retention indices and their percentage composition are summarized in Tables 3-4 where all the compounds are arranged in order of their elution on the column.

Table 3. Retention time (TR) and chromatographic area percentages of compounds identified in L1 fraction of *Verbascum phlomoides* L. extract.

Peak	Retention time, min	Area	Height	A/H	Name
1	33.914	910559	82706	11.01	cyclohexasiloxane, dodecamethyl-
2	36.036	368025	31106	11.83	6-methyl-5-hepten-2-one
3	38.751	650992	40500	16.07	nonanal
4	41.183	736447	75006	9.82	ethyl caprylate
5	45.076	422230	40312	10.47	furfural
6	45.768	50603651	2098644	24.11	acetic acid
7	47.233	752305	51649	14.57	camphor
8	47.909	329146	38600	8.53	ethyl pelargonate
9	48.256	5699143	422909	13.48	benzaldehyde
10	52.805	1762643	128312	13.74	alpha-isophorone
11	56.039	10933445	520339	21.01	ethanone, 1-phenyl
12	56.752	2864388	188182	15.22	benzoic acid, ethyl ester
13	58.524	873053	64072	13.63	isovaleric acid
14	60.741	1755781	137610	12.76	azulene
15	62.543	1064863	69997	15.21	benzenemethanol, alpha,alpha-dimethyl-
16	69.771	1094146	104582	10.46	benzeneethanol
17	71.324	1695105	194828	8.7	1-dodecanol
18	71.806	895960	112975	7.93	furfuryl alcohol
19	72.206	5592124	554132	10.09	phenol
20	73.144	1637944	241469	6.78	ethyl myristate
21	74.74	4222372	480347	8.79	2-pentadecanone, 6,10,14-trimethyl-
22	77.412	28477899	3439593	8.28	ethyl palmitate
23	78.043	1320965	138092	9.57	ethyl 9-hexadecenoate
24	78.543	5048335	541269	9.33	2,4-di-tert-butylphenol
25	80.407	4457797	419322	10.63	diethyl phthalate

GC and GC–MS analysis of *Verbascum phlomoides* L extracts showed the presence of 25 components accounting for L1 extract comprising 3 alcohols, 4 ketones, 3 aldehydes, 3 organic acids and 7 esters (Table 3). There were 3 acids, 4 alcohols, 7 esters, 5 ketones and 3 aldehydes in L2 extract (Table 4). In both extracts, esters and ketones are the major constituents.

Table 4. Retention time (TR) and chromatographic area percentages of compounds identified in L2 fraction of *Verbascum phlomoides* L. extract.

Peak	Retention time, min	Area	Height	A/H	Name
1	33.914	910559	82706	11.01	cyclohexasiloxane, dodecamethyl-
2	36.036	368025	31106	11.83	6-methyl-5-hepten-2-one
3	38.751	650992	40500	16.07	nonanal
4	41.183	736447	75006	9.82	ethyl caprylate
5	45.076	422230	40312	10.47	furfural
6	45.768	50603651	2098644	24.11	acetic acid
7	47.233	752305	51649	14.57	camphor
8	47.909	329146	38600	8.53	ethyl pelargonate
9	48.256	5699143	422909	13.48	benzaldehyde
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12	56.752	2864388	188182	15.22	benzoic acid, ethyl ester
13	58.524	873053	64072	13.63	isovaleric acid
14	60.741	1755781	137610	12.76	azulene
15	62.543	1064863	69997	15.21	benzenemethanol, alpha,alpha.-dimethyl-
16	69.771	1094146	104582	10.46	benzeneethanol
17	71.324	1695105	194828	8.7	1-dodecanol
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22	77.412	28477899	3439593	8.28	ethyl palmitate
23	78.043	1320965	138092	9.57	ethyl 9-hexadecenoate
24	78.543	5048335	541269	9.33	phenol, 2,4-bis(1,1-dimethylethyl)-
25	80.407	4457797	419322	10.63	diethyl phthalate

Conclusions

Two extracts separated from *Verbascum phlomoides* L. in this study were evaluated. Thus, total phenolics content in the extracts was determined spectrometrically applying the Folin-Ciocalteu assay, while flavonoid content was estimated following the aluminium chloride colorimetric method. The paper also presents the first information on the characterization by chromatographic techniques of the alcoholic extracts.

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