Determination of Oligoelements Content of Plant Material and Assessment of Bioactive Compounds from *Calendula officinalis* Lyophilized Extract

TUNDE JURCA[1], ELEONORA MARIAN[1]*, BOGDAN TITA[2], SIMONA VICAS[3], ANNAMARIA PALLAG[1], IMRE TOTH[4], LASZLO KRUSPER[4], MIHALY BRAUN[5], LAURA VICAS[1]

[1]University of Oradea, Medicine and Pharmacy Faculty, Department of Pharmacy, 29 Nicolae Jiga Str., 410028, Oradea, Romania
[2]Vasile Goldis Western University of Arad, Pharmacy Faculty, 86 Liviu Rebreanu Str., 310045, Arad, Romania
[3]University of Oradea, Faculty of Environmental Protection, 26 General Magheru Street, Oradea, Romania
[4]University of Debrecen, Department of Inorganic and Analytical Chemistry, Egyetem ter 1 sz., Debrecen, Hungary
[5]Hungarian Academy of Science, Institute for Nuclear Research, Bem tér 18/c, Debrecen, Hungary

The marigold extracts are used in traditional medicine for its anti-inflammatory, decongestive, antiseptic, antifungal, antimicrobial and immunostimulating properties. Existing studies have partially proved these properties, but are incomplete with regard to the possibility of standardizing the extract using the best working conditions so as it to be included in dietary supplements. The purpose of this study is to investigate the chemical composition of the marigold extracts, their contents in flavonoids and polyphenolic compounds, as well as to determine the contents in oligoelements of the plant material through the ICP-OES method. Based on the results of this study we conclude that a complete extraction may be accomplished after lyophilizing the solution of hydroalcoholic extract, followed by extraction in acid medium so as to permit a better assessment of the bioactive compounds.

Keywords: Calendula officinalis, bioactive compounds, oligoelements

Marigolds (*Calendula officinalis*) originate from the Mediterranean zone and grow spontaneously or are cultivated as decorative plants, in gardens, along buildings walls, footpaths, roads or throughout parks. (Figure1). They are widely spread and known in all Europe since the Middle Ages.

Marigolds contain flavonoids, carotenoids, vitamin C, essential oils, bitter substances, triterpenoid saponins, resins, mucilages. Due to these components, marigolds present anti-inflammatory, decongestive, antiseptic, antimicrobial and immunostimulating properties, also being able to regulate menstrual cycles and biliary functions.

Marigolds are indicated in ulcerations, eczemas, gastritis, gastric ulcer, duodenal ulcer, colon inflammations, colecistitis, hepatobiliary diseases, menstrual disorders, mycosises, vaginitides, cystitides and cancer [1].

The flowers have medical purpose, being harvested under sunlight, when the corolla is open and emanates oils.

The variety of flavonoids occurring in plant materials is usually large, the components of flavonoid fractions comming from different classes of aglycone, mono- and polyglycoside, or acylated compound, and differ from each other in polarity, molecular weight, and chromatographic and spectrophotometric properties. Every plant has an original and unique flavonoid profile, which makes quantification difficult. For this reason the methods frequently used to determine the total flavonoid content of herbal materials include hydrolysis of the glycosides to reduce the variety and number of analytes. The aglycones obtained can then be quantified by UV spectrophotometric determination as aluminium chelate complexes, as described in several pharmacopoeia [2].

Fig. 1. Marigold flowers

The purpose of this study was to effectuate a determination of the oligoelements content of the dry plant material and to evaluate the bioactive compounds using modern methods of identification and quantitative determination.

In this sense, we have elected as a method to determine composition, high-performance liquid chromatography (HPLC) equipped with diodes in order to detect bioactive compounds in UV spectrometry and metals with ICP-OES.

ICP-OES (Inductively coupled plasma optical emission spectrometry) is an analytical technique used for the detection of trace metals. This type of spectrometry uses inductively coupled plasma to produce ions and excited electrons, that later emit electromagnetic radiations at wavelengths that are characteristic of a certain element [3-5]. The intensity of these emissions reveals the concentration of an element in the analyzed sample. The method presents high sensitivity, varying from one element to another, from 1 ppm to 10 ppb.

One objective of the study was to assess the antioxidant capacity of the marigold extract.

The HPLC method represents the best suited option for flavonoid identification inasmuch as the separation methods are already established and PDA (diode array

* email: marian_eleonora@yahoo.com; Phone: +40 729159838

detector) and MS (mass spectrometer) coupling is easy to accomplish.

Experimental part

Materials and methods

Assessment of content in bioactive compounds

The identification of flavonoidic and polyphenolic compounds in the marigold extract was achieved though HPLC analysis using a HPLC Shimadzu SPD-M10A system equipped with a diode array detector (PDA). A Kinetex 5 µm C18 100A column was used.

The mobile phase for elution was constituted of acetonitrile (A) and distilled water – formic acid (99.9:0.1) (v/v) (B). The concentration gradient contained as following: min. 1:2% A, min. 25: 50% A, min. 35: 98% A. The elected debit was of 1.5 mL/min and the detection was conducted at wavelengths of 270, 310 and 360 nm.

By comparing the retention time (t_R) with those of standard flavonoids and polyphenol solutions (100 µg/mL), flavonoid compounds such as flavanols (epicatechin) and flavonols (rutin, quercetin and myricetin) were found, as well as phenolic acids (gallic acid, caffeic acid and p-coumaric acid).

The following products of chemical and pharmaceutical purity were used as reagents: ethanol (Scharlab Ungaria Kft. a.r.), methanol (Scharlan ME0310 a.r.), acetic acid (Molar Chemicals Kft. a.r.), hydrochloric acid (J.T.Baker reagent grade), ethyl acetate (Molar Chemicals Kft. a.r.), dimethylsulfoxide (Molar Chemicals Kft a.r.), acetonitrile (Sigma Aldrich gradient grade for HPLC). The hydrogen peroxide and nitric acid used for digestion was procured from Merck, Germany.

The used standars are as following: Naringenin (Sigma-Aldrich), Caffeic acid (Sigma, HPLC), Vanillic acid (Sigma-Aldrich, HPLC), Gallic acid (Sigma), Rutin (Sigma, HPLC), Myricetin (Fluka), Quercetin (Sigma, HPLC), Epicatechin (Fluka), Ferulic acid (Sigma-Aldrich), Luteolin (Fluka), p-Coumaric acid (Fluka), Sinapic acid (Aldrich), Catechin (Sigma-Aldrich, HPLC).

The used equipment presents the following characteristics: Rotary evaporator (Scanvac Buchi Labortechnik AG), Sonicator (Elma S40 H Elmasonic), Shimadzu LC10A HPLC, Pump (LC 10 AD VP), Detector (SPD-M10A VP), Lyophilizer (Scanac Cool Safe Vacuumbrand).

Sample preparation

The dried marigold flowers were grinded and submitted to extraction in ethanol, 70%. The extract was stored at room temperature, away from light for 24h, after which it was filtered using a 7.0-7.5 µm Rundfilter Nylon filter. The filtered extract was placed into a rotary evaporator to clear away the ethanol and was further lyophilized. The lyophilized powder was submitted to two types of extraction: neutral and acid extraction. Three series of samples were prepared and naringenin was elected as an internal standard.

ICP-OES spectrometry

The digestion of the plant samples was performed with the help of a Microwave Milestone MLS-1200. The ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) measurements were performed with a sequential ICP spectrometer IRIS Intrepid II.

As some heavy metals are found in small quantities in the plant material – incompatible with the conventional detection limits of the ICP-OES, an ultrasonic nebulizer system was used. The coupling of the nebulizer and the ICP-OES improved the detection limits up to 5-50 times.

In order to defragment the organic plant material, 0.1500-0.2500 g of the marigold samples were dried for 12h at approximately 80°C temperature, were grinded and further placed in PTFE (polytetrafluorethylene) spheres along with 4.5 mL concentrated nitric acid and 0.5 mL hydrogen peroxide. Digestion was carried out in accordance with the temperature programs for plant materials, first stage: 300 W power - 5 minutes, second stage: 600 W power – 5 min. The resulted solutions were stored in volumetric flasks with ultrapure water added up to 50 mL. Three marigold test samples and a control sample were made, all in the same working conditions. They were analyzed with the aid of ICP-OES [6, 7].

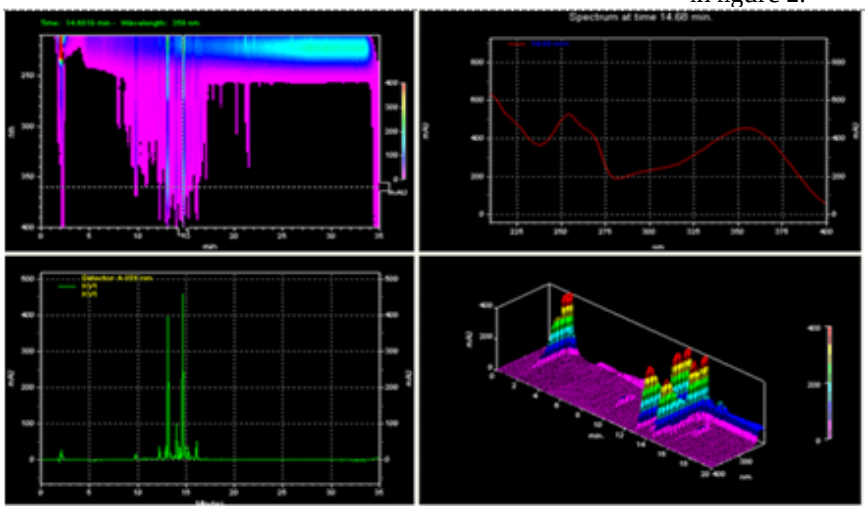
Results and discussions

Assessment of bioactive compounds content

The data was processed in a three-dimensional system at three wavelengths: 270, 310 and 360 nm, respectively. Flavonols were detected at 360 nm, hydroxycinnamic acids at 310 nm and hydroxybenzoic acids and flavanols at 270 nm. Identification of the components was based on the spectrum and the retention time (t_R) in comparison to the standards. The qualitative and quantitative composition of the extracts is presented in tables 1 and 2.

Extraction in neutral medium

50 mg lyophilized powder were measured, to which a mixture of 2 mL 90% methanol, 0.5% acetic acid and 10 mL internal standard were added. It was maintained for 90 seconds in an ultrasonic bath, then placed in a rotary evaporator. The perfectly dried sample was dissolved in 1000µL dimethylsulfoxide. The three-dimensional chromatogram of the neutral marigold extract is illustrated in figure 2.



Fi. 2. Three-dimensional chromatogram of the neutral marigold extract

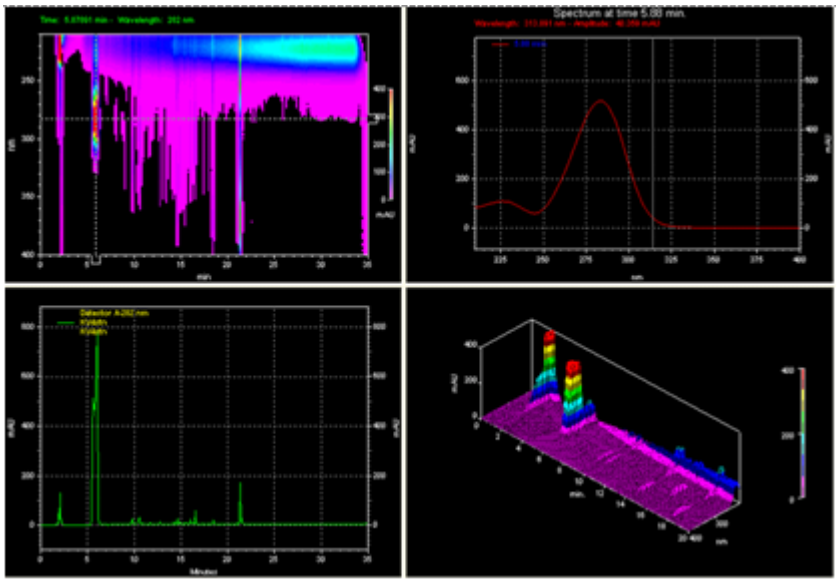
Table 1

QUALITATIVE AND QUANTITATIVE COMPOSITION OF THE NEUTRAL (UNHYDROLIZED) MARIGOLD EXTRACT

Flavonoids mg/kg	Epicatechin	Quercetin	Rutin	Myricetin
Unhydrolyzed extract	79.82±0.12^{a,b}	0.00	272.35±21.15^{a,a}	13.63±0.02^{b,b}

Phenolic acids mg/kg	P-coumaric acid	Caffeic Acid	Gallic acid
Unhydrolyzed extract	4985.27±10.25^{a,b}	1695.25±2.03^{a,b}	0.00

*Different letters along the rows describe statistical significant differences.



Fi. 3. Chromatogram of the acid marigold extract

By observing the chromatogram resulted at 360 nm, the identified flavonoids are: rutin (t_R -13.141 min), myricetin (t_R - 15.25 min), epicatechin (t_R -14.677 min). Quercetin was not highlighted in the neutral extract.

At 310 nm phenolic acids were observed: caffeic acid (t_R–10.805 min) and p-coumaric acid (t_R - 12.92 min). Gallic acid was not highlighted in the neutral extract.

At 270 nm epicatechin was identified at t_R – 14.677 min., corresponding to 79.82 mg/kg.

Extraction in acid medium

50 mg lyophilized powder were measured, to which a mixture of 4 mL 25% methanol, 1mL hydrochloric acid 6M and 10 µL internal standard were added. It was maintained for 90 s in an ultrasonic bath, further placed in a heated bath at 85-90°C for 2h and lastly, 2.5 mL ethyl acetate were added, finally being submitted to a rotary evaporator. The perfectly dried sample was dissolved in 1000µL dimethylsulfoxide. The three-dimensional chromatogram of the acid marigold extract is illustrated in figure 3.

After acid extraction, rutin appears only in very small quantities (t_R – 13.109 min), though quercetin is highlighted (t_R – 18.364 min). The myricetin quantum is more increased (t_R – 13.109 min) than the neutral extract (13.63 mg/kg) (table 2).

By detecting at 310 nm phenolic acids were identified as well as caffeic acid (t_R – 10.805 min) and p-coumaric acid (t_R – 12.92 min), but in reduced quantities in the acid medium versus the neutral one. After the extraction in acid medium, the quantity of epicatechin increases (t_R – 14.677 min), but rutin is decreased (t_R – 13.109 min.) and myricetin is increased (t_R – 13.109 min). Quercetin appears due to the acid hydrolysis of rutin.

As for the phenolic acids, gallic acid (t_R – 4.45 min) appears alongside p-coumaric acid (t_R – 12.92 min) and caffeic acid (t_R – 10.805 min).

The most complete extraction is the acid one therefore, based on the results from the HPLC method, we specify that the acid lyophilized marigold extract is the most appropriate so that the future phytocomplex may present therapeutic efficiency superior to common extracts.

Assessment of oligoelements content

Concentrations of ten elements (major and trace components) from the marigolds were determined using ICP-OES.

The found elements are expressed in mg metal/kg plant material. In large quantities were found: aluminium (20.642±0.006 mg/kg) and iron (17.931±0.010 mg/kg). Large quantities of barium (3.966±0.007 mg/kg), manganese (3.562±0.005 mg/kg), strontium (2.537± 0.011 mg/kg) were also found. Small quantities of other metals were determined: copper, lithium, nickel, zinc (<0.100 mg/kg) [8].

Assessment of therapeutic properties of the marigold extract

Once components of the flanovoid class and polyphenolic compounds were determined, the antioxidant capacity of the marigold extract was studied using three methods (2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid - ABTS), 1,1-diphenyl-2-pycrylhydrazyl (DPPH), and 2,4,6-tri(2-pyridyl)-s-triazine (FRAP). With a total polyphenol content of 112.42±0.01 expressed in gallic acid (GAE, determined with Folin-Ciocalteu's method) and

Table 2
QUALITATIVE AND QUANTITATIVE COMPOSITION OF THE ACID (HYDROLIZATED) MARIGOLD EXTRACT

Flavonoids mg/kg	Epicatechin	Quercetin	Rutin	Myricetin
Hydrolyzated extract	$381.30\pm2.05^{a,b}$	$628.04\pm0.15^{a,a}$	$17.12\pm0.65^{a,a}$	$226.02\pm0.12^{b,b}$

Phenolic acids mg/kg	P-coumaric acid	Caffeic Acid	Gallic acid
Hydrolyzed extract	$121.21\pm3.08^{a,b}$	$552.14\pm1.15^{a,b}$	$156.45\pm1.02^{a,b}$

*Different letters along the rows describe statistical significant differences.

Table 3
ANTIOXIDANT CAPACITY OF THE MARIGOLD EXTRACT

Sample	Total polyphenolic content (mg GAE/100g DW	Total flavonoid content mg QE/100g DW	DPPH%	ABTS (mmol Trolox equivalent/g DW)	FRAP (mmol Trolox equivalent/g DW)
Alcoholic marigold extract	112.42 ± 0.01^{h}	42.12 ± 2.41^{f}	4.17 ± 1.31^{e}	6.68 ± 1.14^{e}	15.01 ± 0.03^{h}

*DW- dry weight; *Different letters along the rows describe statistical significant differences.

a total flavonoid content of 42.12±2.41 expressed in quercetin (QE), the marigold extract presents a very potent antioxidant capacity according to the values presented in table 3 [9-11].

The antioxidant capacity proven through these in vitro methods certifies the use of the extract in miscellaneous phytochemical products, having positive effects for population health, plant materials being easily procured, from the Romanian flora.

Conclusions

In the interest of efficient extraction we lyophilized the herbal extracts using a neutral and an acid extraction method. We measured the samples and the standard solutions in complete spectrum, but carried out the evaluation in 270 nm, 310 nm and 360 nm wavelengths. Comparing the results from the neutral and the acid extraction we found discrepant components in the plant. The identification of some components lasts equal amounts of retention time and was carried out using complete spectral analysis. Some flavonoid type compounds appeared in the neutral mixture, whilst they disappeared in the acid one. This can be explained through the hydrolysis of the sugar part (quercetin can be measured, instead of rutin). Based on this data we can state that for a better extraction of bioactive principles, preliminary studies are necessary to highlight the best working conditions so that their extraction is complete.

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