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CHARACTERIZATION OF SOME PLANT EXTRACTS BY ULTRASOUND-ASSISTED EXTRACTION IN SUNFLOWER OIL USING THIN LAYER CHROMATOGRAPHY AND SPECTROPHOTOMETRY UV-VIS

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ABSTRACT. The purpose of this work was to prove the enrichment of sunflower oil with active principles (polyphenolic acids, flavones, flavonoids) from plants, using the ultrasounds-assisted extraction (UAE) process. The analytical technique used for qualitative characterization of the UAE samples is thin layer chromatography (TLC), a relatively simple technique, which under certain separation and detection conditions highlights the bioactive components of the plants studied in our study. UV-VIS spectrophotometry was used to evaluate the level of flavonoids. The UAE samples in sunflower oil were also characterized by determining the alpha-tocopherol content through a gas-cromatographic method (GC-MS), and by evaluating the antioxidant activity through an indirect DPPH spectrophotometric method. The oily plants extracts were obtained to be used as raw material in the formulation of a dermato-cosmetic product, and thus the characterisation performed herein is very useful in supporting the effects of the above-mentioned products.

Keywords*: sunflower oil, ultrasound-assisted extraction, TLC, UV-VIS, GC-MS*

INTRODUCTION

In recent years, people have begun to appreciate the gifts of nature again, taking advantage of the beneficial content of plants: flavones and their glycosides, polyphenolic compounds, carotenoids, triterpenes, chlorophylls, essential oils.

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All these compounds can be extracted by maceration in solvents (ethanol) or edible oils (sunflower oil, olive oil) [1]. Over time, studies have been carried out on the effectiveness of sunflower oil on the skin. It was chosen for its emollient action and to restore the stratum corneum, creating a protective barrier and maintaining hydration of the epidermis. In its composition, sunflower oil has an important amount of alpha-tocopherol, a fat-soluble antioxidant, responsible for reducing the oil autooxidation and for protecting the cell membranes against peroxidation [2].

Most of the studies were carried out in disadvantaged areas, on newborns and on children suffering from severe acute malnutrition, demonstrating the fact that, although it is a cheap product, topical therapy with sunflower oil was beneficial as an adjuvant therapy, improving the skin's barrier function, reducing the risk of skin infections [3-5].

Over time, numerous products based on sunflower oil have been reported: emulsions, soap, creams [6, 7]. It has been proven that these products have an anti-inflammatory, anti-acne, moisturizing and protective effect.

Several procedures are known for extracting active principles from plants: maceration (hot maceration, cold maceration), ultrasounds-assisted extraction, microwave-assisted extraction, liquid CO_2 extraction, solvent extraction [8]. Ultrasounds-assisted extraction is a fast process (minutes to several hours), compared to conventional maceration (7-15 days) [9-11].

The aim of the current study is to evaluate some bioactive compounds extracted to sunflower oil by ultrasound-assisted procedure from Calendula officinalis, Hypericum perforatum, Galium verum, Taraxacum officinale, Achillea millefolium, Equisetum arvense and Rubus idaeus. These plants were chosen because they are an easy to find raw material, inexpensive and for their anti-inflammatory, wound-healing, antioxidant properties, and for the effect of skin regeneration [12-15]. They are all well characterized regarding their effects by traditional medicine use in rural zones of Romania. Also, this study includes a short characterization of a dermatocosmetic cream formulation containing the above plant extracts.

RESULTS AND DISCUSSION

TLC Identification

Figures 1a and 1b represent the images obtained after the TLC analysis. Each UAE sample to sunflower oil is characterized by a fingerprint similar to that of the plant with which the oil was enriched, under the proposed TLC conditions. The bioactive compounds were identified based on the

comparison of retention factors (Rf) values with those for the standards separated in the same chromatographic conditions. To eliminate possible interferences, a sample of sunflower oil used in UAE process was also applied.

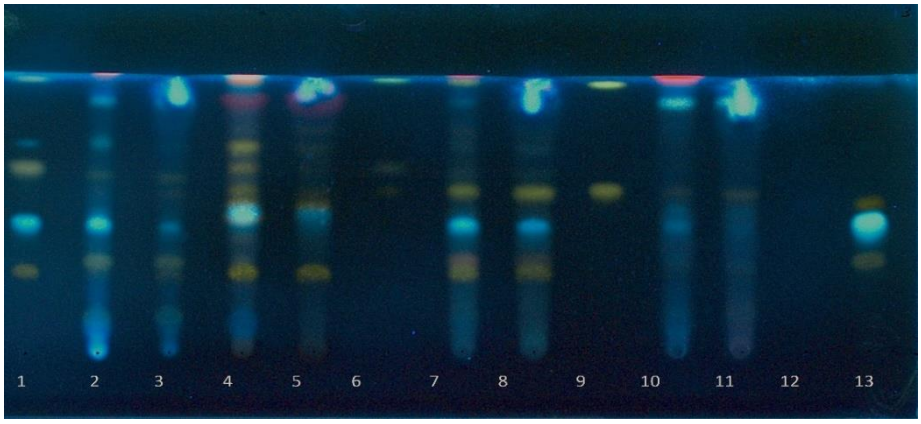

Figure 1a: Standard 1 - Rutin, Chlorogenic acid, Apigenin-7-glucoside, Apigenin (1); Calendula officinalis plant - Calendula officinalis UAE sample(2,3); Hypericum perforatum plant - Hypericum perforatum UAE sample (4,5); Standard 2 - Isoquercetin, Kaempferol, Kaempferol-3-glucoside (6); Galium verum plant –Galium verum UAE sample (7,8); Standard 3 - Luteolin, Luteolin-7-glucoside (9); Taraxacum officinale plant - Taraxacum officinale UAE sample (10,11); Sunflower oil (12); Standard 4-Izoramnetin-3-rutinoside, Chlorogenic acid, Hyperoside (13)

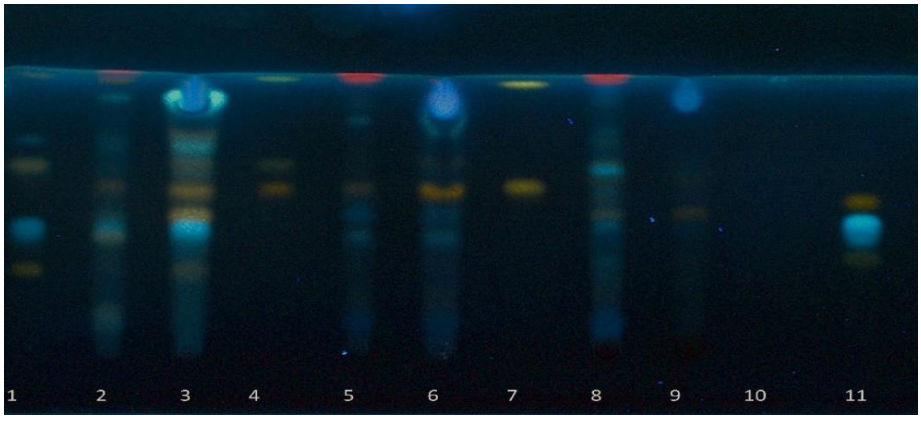

Figure 1b: Standard 1 - Rutin, Chlorogenic acid, Apigenin-7-glucoside, Apigenin (1); *Achillea millefolium* plant - *Achillea millefolium* UAE sample (2,3); Standard 2 - Isoquercetin, Kaempferol, Kaempferol-3-glucoside (4); *Equisetum arvense* plant - *Equisetum arvense* UAE sample (5,6); Standard 3 - Luteolin, Luteolin-7-glucoside (7); *Rubus idaeus* plant –*Rubus idaeus* UAE sample (8,9); *Sunflower* oil (10); Standard 4-Izoramnetin-3-rutinoside, Chlorogenic acid, Hyperoside (11).

The results of the TLC analysis show the polyphenolic acid composition of all the UAE samples, but also the presence of various flavones and their glycosides: rutin in *Calendula officinalis* and *Achillea millefolium* UAE samples; luteolin in *Hypericum perforatum*, *Galium verum, Achillea millefolium* and *Equisetum arvense* UAE samples; hyperoside in *Calendula officinalis*, *Taraxacum officinale* and *Rubus idaeus* UAE samples; apigenin glycoside or kaempferol in *Achillea millefolium* and *Equisetum arvense* UAE samples.

Quantitative determination of total flavonoids content (TFC) by spectrophotometry in UAE samples

Method Validation for UAE sample from Calendula officinalis to sunflower oil

The method validation was carried out according to the requirements of ICH Q2(R1) [16] for a new product by evaluation the typical validation characteristics as linearity, accuracy, precision and robustness.

Linearity: The calibration curve for rutin, based on which the flavonoid content was determined from the UAE samples, is represented in figure 2 and was plotted in the concentration range 2.2µg/mL-176.6µg/mL.

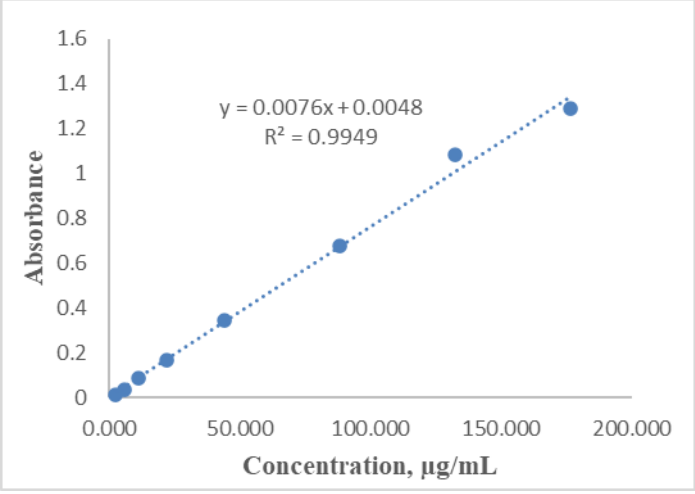

Figure 2. Calibration curve for rutin

ANOVA test showed that there was a proportionality relationship between the absorbance and concentration at 95% confidence level. The correlation coefficient of the regression line is 0.9949. The confidence interval of the intercept includes zero value. The statistical significance of the slope is checked by the Student test ($t_{calculated} > t_{critical}$), thus demonstrating the linearity

of the curve. An additional element confirming the linearity of the method is the fulfilment of the condition that the experimental value of the Fisher test is greater than the critical value. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the residual standard deviation of the calibration curve (SD) and the slope of the calibration curve (b), where LOD=3.3xSD/b and LOQ=10xSD/b [17].

The results of linearity are summarized in Table 1.

Table 1. Linearity verification by ANOVA test

Statistical characteristics of the regression	**Acceptability criteria**	**Rutin**
Regression line equation	-	y=0.0076x+0.0048
Correlation coefficient	>0.9900	0.9949
Determinations, n	-	8
Degrees of freedom, n-2	-	6
Standard error of regression	-	0.0385
Standard error of slope	-	0.00022
Student's test $t_{n-2}(1-\alpha/2)$	$t_{calculated} > t_{critical} = 2.4469$	34.12
Confidence interval for slope (95% confidence)	does not include zero value	0.00704÷ 0.00813
Standard error of intercept	-	0.01911
Confidence interval for intercept (95% confidence)	include zero value	-0.04196÷0.05158
Fisher test (95% confidence) Fcritical=$F_{1,n-2}(1-\alpha)$= 5.9874	$F_{calculated} > F_{critical}$	1164.2
LOQ (µg/mL)		11.7391
LOD (µg/mL)		3.8645

Accuracy

A known amount of standard stock solution of rutin was added at different levels in a placebo solution. The placebo solution was prepared the same way as the samples for analysis by spectrophotometry, but sunflower oil was used instead of UAE sample. Recovery values of 83-88% were achieved for rutin (table 2).

Table 2. Accuracy

Accuracy	**Theoretical concentration of rutin, µg/mL**	**Obtained concentration of rutin, µg/mL**	**Recovery, %**	**Acceptability criteria**
Level 1	11,213	9,325±0,01	83,16	R=80-110%
Level 2	44,851	39,711±0,02	88,54	
Level 3	89,702	79,250±0,10	88,35	

Precision

Precision was investigated using six UAE samples from *Calendula officinalis* to sunflower oil prepared and analysed. The result is presented as the average of the measurements and precision expressed as relative standard deviation, RSD=0.71% (Acceptability criteria: RSD ≤ 5%).

Robustness

For testing the method robustness, the reaction time was varied, starting from 5min to 60min. In the interval 40-50min, a stable absorbance value was observed.

By meeting all the validation requirements, the analytical method is suitable for quantifying the total flavonoids content in the UAE samples.

The results for TFC of the plants and of UAE samples in sunflower oil, as well as the respective extraction yields, are shown in Table 3.

Table 3. Total Flavonoid Content in the plants and UAE samples to sunflower oil

Name	**TFC determined in the ground plant, mg rutin /g average±SD, n=3**	**TFC determined in the UAE sample reported to the plant, mg rutin /g average±SD**	**Extraction Yield %**
Calendula officinalis	5.118±0.073	0.448±0.025 (n=6)	8.75
Hypericum perforatum	5.730±0.007	0.717±0.002 (n=3)	12.51
Galium verum	4.257±0.202	0.466±0.018 (n=3)	10.95
Taraxacum officinale	2.737±0.105	0.287±0.010 (n=3)	10.49
Achillea millefolium	4.638±0.062	0.373±0.020 (n=3)	8.04
Equisetum arvense	3.125±0.126	0.540±0.010 (n=3)	17.28
Rubus idaeus	18.961±0.109	1.303±0.015 (n=3)	6.87

After analyzing the individual UAE samples and observing the potential of each one, we prepared an UAE mixture that includes all 7 plants, keeping the plant:oil ratio of 1:10 (w/w). This mixture was used in the formulation of a cream along with a professional cream base which was bought from Ellemental. A mixture solution of 30%sodium benzoate-15%potassium sorbate was added as a preservative.

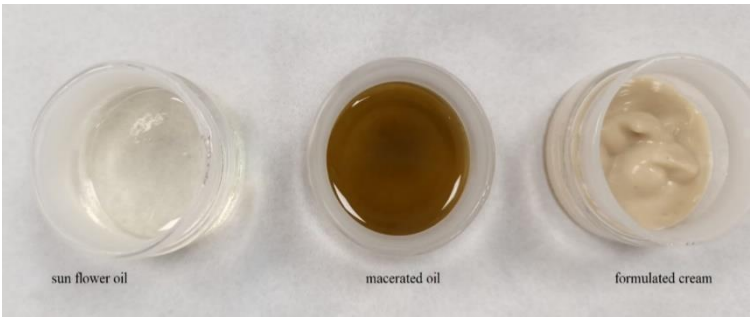

Figure 3. Physical properties: Appearance, color

The topical formulation was evaluated for some physicochemical properties: appearance, color, texture, phase separation, homogeneity, viscosity and Ph. The results are expressed as greenish-brown, semisolid, opaque cream (figure 3), with characteristic odour of plants, with smooth texture, and with a homogeneous distribution free from phase separation and foreign particulate matter. The pH is 6.03 and the viscosity is 1538.38cP.

Formulated cream TLC identification

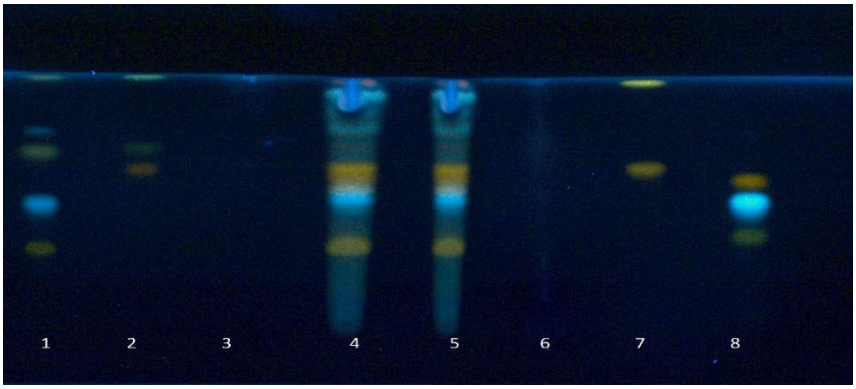

Figure 4. Standard 1 - Rutin, Chlorogenic acid, Apigenin-7-glucoside, Apigenin (1), Standard 2 - Isoquercetin, Kaempferol, Kaempferol-3-glucoside (2), Sunflower oil (3), UAE Mixture (4), Formulated cream (5), Cream base (6), Standard 3 - Luteolin, Luteolin-7-glucoside (7), Standard 4-Izoramnetin-3-rutinoside, Chlorogenic acid, Hyperoside (8)

The TLC fingerprint of the cream is similar to the fingerprint of the UAE mixture, demonstrating the presence of polyphenolic compounds and flavonoids in the cream composition.

The content of alpha-tocopherol was determined by a GC-MS method and the evaluation of the antioxidant activity was performed by applying the DPPH indirect spectrophotometric method. The test results for alpha-tocopherol and for the antioxidant activity can be found in table 4.

The results in Table 4 indicates the following aspects: (1) The pure sunflower oil contained 1.15(±0.011) mg/g of alpha-tocopherol, while the mixture of plants in oil 1.53(±0.012) mg/g, which was a significant increase of 33% and (2) Hypericum perforatum and Calendula officinalis UAE samples have the lowest values for EC50 meaning better antioxidant activity than the rest of the selected plants.

Table 4. Alpha-tocopherol and antioxidant activity results

Name	Alpha-tocopherol content (GC-MS), mg/g	DPPH (EC_{50}mL)
Calendula officinalis UAE sample	1.35±0.012 RSD = 0.8%	1,512± 0.016 RSD = 0.3%
Hypericum perforatum UAE sample	1.28±0.015 RSD = 1.2%	1,415± 0.015 RSD = 1.6%
Galium verum UAE sample	1.17±0.006 RSD = 0.5%	5,141±0.021 RSD = 0,9%
Taraxacum officinale UAE sample	1.18±0.006 RSD = 0.5%	-
Achillea millefolium UAE sample	1.22±0.010 RSD = 0.8%	6.425±0.017 RSD = 1.2%
Equisetum arvense UAE sample	1.21±0.017 RSD = 1.4%	-
Rubus idaeus UAE sample	1.28±0.015 RSD = 1.2%	-
Sunflower oil	1.15±0.011 RSD = 0.9%	8.490±0.045 RSD = 1.8%
UAE Mixture	1.53±0.012 RSD = 0.7%	0.089±0.011 RSD = 1.1%
Cream base	-	-
Formulated cream	0.49±0.006 RSD = 1.2% (Recovery = 87%)	0.5g formulated cream reduces DPPH by 15%

Also, it can be seen that only a volume of 0.089mL of UAE of the plants mixture reduces DPPH by 50%, which means almost 17 times lower compared to the volume of the pure sunflower oil. Therefore, if the compounds responsible for the antioxidant activity are liposoluble, an increase in the antioxidant activity of our UAE mixture was expected.

Regarding the results for UAE samples, to the best of our knowledge, there is no literature data on the bioactive compounds ultrasound-assisted extraction to sunflower oil from any medicinal plants. In a survey of past literature reports we found studies of extraction from an edible wild plant - *Crithmum maritimum* L. [18] or from a brown macroalga - Pelvetia canaliculate L. [19] to sunflower oil during ultrasound-assisted treatment, with the purpose of increasing the nutritional value and oxidative stability of the oil. In both studies, the extraction yields of flavonoids to the oil were rather low, ranging from 1.9% to 3.4% and from 0.85% to around 9.40%, respectively for different concentration of each lyophilized plant added to sunflower oil (5-20% m/v). These results were explained by the presence of low soluble flavonoid compounds of C. maritimum and of *P. canaliculata* in the oil which acts as a non-polar solvent. Also, the authors reported higher antioxidant activity in supplemented sunflower oil samples

from Crithmum maritimum and Pelvetia canaliculate, respectively than in the non-supplemented oil, indicating that the compounds with antioxidant activity that migrated from the plants studied to the oil are mainly non-polar.

CONCLUSIONS

Our study presents, for the first time, the extraction of bioactive compounds from the selected medicinal plants to sunflower oil by non-pollutant ultrasound-assisted technique and the evaluation of total flavonoid content, alpha-topherol content of these extracts, as well as their antioxidant activity.

TLC fingerprints showed the chemical composition similarity of the plants extracts obtained by ultrasound-assisted extraction in sunflower oil with the plants studied in this work, concerning the presence of characteristic polyphenolic acids and flavonoids. UV-VIS spectrophotometry and GC-MS analysis were used to quantify total flavonoids and alpha-tocopherol content.

The antioxidant potential was investigated by DPPH assay. The UAE sample of the all plants mixture showed a higher antioxidant activity compared to the pure sunflower oil, probably due to a synergistic effect of phenolic and flavonoid compounds with the antioxidant molecules of α-tocopherol.

The active principles from the selected plants incorporated by ultrasounds-assisted extraction into sunflower oil, are found in the cream formulation presented in this work. This fact was demonstrated by the presence of the same bioactive compounds as in the UAE sample of the plants mixture revealed by TLC analysis and by a level of 87% of alpha-tocopherol which makes the cream preparation a good candidate for repairing the epidermis. Also, due to the oily plant extract, formulated cream has a certain antioxidant activity (0.5g reduces DPPH by 15%).

Thus, this green approach of ultrasound-assisted extraction is of great interest providing a good alternative to conventional extractions; it can be used for the extraction of valuable compounds from a large number of plants. The use of sunflower oil as natural solvent facilitates the application of the ultra-assisted extracts from medicinal plants for dermatocosmetic preparations.

EXPERIMENTAL SECTION

Materials: We used cold-pressed sunflower oil from a local producer from Horezu, Valcea area and 7 dried and crushed plants (aerial parts and leaves) bought from Dacia Plant: *Calendula officinalis, Hypericum perforatum, Galium verum, Taraxacum officinale, Achillea millefolium, Equisetum arvense* and *Rubus idaeus.*

Reagents: polyphenolic acid standards - Chlorogenic acid (Sigma), flavones: Apigenin (Toronto Research Chemicals), Isoquercetin (Chromadex), Kaempferol (Santa Cruz), Luteolin (Santa Cruz) and glycosides of flavones (flavonoids) - Rutin (Sigma), Kaempferol-3-glucoside (Santa Cruz), Apigenin-7-glucoside (EP), Luteolin-7-glucoside (Sigma), Izoramnetin-3-rutinoside (PhytoLab), Hyperoside (TRC); Methanol (Carlo Erba, HPLC grade); Aluminum chloride (Sigma,); Ethyl acetate (Carlo Erba); Formic acid (Merck, 99%); Sodium acetate (Sigma, 99%); Diphenylboric acid aminoethyl ester (Merck); Macrogol 400 R (Merck), Alpha-tocopherol (Sigma), Hexane (Merck), Dimethylsulfoxide (DMSO, Sigma), 2,2-Diphenyl-1-Picrylhydrazyl (DPPH, Sigma).

Ultrasound-assisted extraction: Each crushed plant sample is covered with sunflower oil in a plant-oil ratio of 1:10(w/w) and is ultrasonicated at room temperature (23–25°C) for 3hours, in a ultrasonic bath Bandelin Sonorex (ultrasounds were applied with a frequency of 35 kHz). Each extract is filtered and stored at 4 °C in airtight brown containers until further use.

Samples for thin layer chromatography analysis

Every extract was homogenized in methanol (HPLC grade) by ultrasonication, then centrifuged. An aliquot of the methanol layer was applied on the chromatographic plate.

For comparison, each crushed plant sample was mixed with methanol (HPLC grade), ultrasonicated, filtrated, and the filtrate was used for TLC analysis.

For identification, four mixtures of standard substances were prepared: Standard 1 - Rutin, Chlorogenic acid, Apigenin-7-glucoside, Apigenin; Standard 2 - Isoquercetin, Kaempferol, Kaempferol-3-glucoside; Standard 3 - Luteolin, Luteolin-7-glucoside; Standard 4-Izoramnetin-3-rutinoside, Chlorogenic acid, Hyperoside.

Samples for UV-VIS spectrophotometry analysis: Every UAE sample was mixed with purified water, ultrasonicated for 15 minutes for flavonoids extraction, then transferred into a separation funnel for layers separation. The aqueous layer was filtrated through 0.45µm Nylon syringe filter and used for quantitative UV-VIS spectrophotometry analysis.

In parallel, an amount of each crushed plant was mixed with purified water, ultrasonicated for 15 minutes, filtrated through 0.45µm Nylon syringe filter and used for analysis.

Qualitative analysis by thin layer chromatography (TLC)

The analysis method was adapted, starting from the European Pharmacopoeia method (11th edition, monograph 1297 "Calendula flower (Calendula flos)"[20-22].

TLC separation was performed on HPTLC Silicagel 60 F254 chromatographic plates, 2-10µm, 20x20cm (Merck). 4µL of each sample prepared for TLC analysis were applied. Then, the plates were developed in the mobile phase: anhydrous formic acid - water - ethyl acetate (10:10:80 v/v/v) on a migration distance of 70mm from the lower edge of the plate. The plates were dried for 5 minutes in air flow, at room temperature and then heated at 100-105°C for 5 minutes. The detection was carried out immediately by spraying with a solution of aminoethyl diphenylboric acid ester 10g/L in methanol R, and then with a solution of macrogol 400 R 50g/L in methanol, after which it was left to dry in the air for 1 minute. Finally, the plates were visualized by ultraviolet examination at a wavelength of 366nm.

Quantitative analysis of TFC by UV-VIS spectrophotometry

TFC was determined using aluminium chloride in a colorimetric method described in the Romanian Pharmacopoeia X [23] modified for this work .

The determination was based on a complexation reaction of flavonoids with aluminum chloride and measuring the complex absorbance at a wavelength of 430nm [24, 25]. UV-VIS measurements were carried out on Specord 250 Plus instrument using 10mm optical path cells.

To a volume of 5mL of each sample for UV-VIS spectrophotometry analysis 5mL of sodium acetate solution 100g/L was added, then filtered on a 0.45µm nylon filter membrane. In each test tube 5mL of this filtrate were mixed with 2mL of $AlCl_3$ 25g/L solution and 3mL of purified water. The complex was stirred for 5 seconds, left to rest for 45 minutes and then the absorbance was measured at 430nm against a control sample in which aluminium chloride is replaced by purified water. The total flavonoid content was calculated using a calibration curve for rutin as standard. The result is expressed as mg rutin equivalents per gram and reported as the mean ± standard deviation (Mean±SD, n=3).

Quantitative analysis of alpha-tocopherol

The determination of the content in alpha-tocopherol is based on its separation by gas chromatography with mass spectrometry detection. Quantification was performed by the method of the external standard. Identification is based on the retention time of standard substance and confirmed with the help of the NIST mass spectra library. The equipment used was a 7890A gas chromatograph coupled with MSD 5975C (“Mass Selective Detector”; electron impact ionization source and quadrupole mass analyzer).

Chromatographic separation was achieved on a capillary column ZB-5MS (60mx0.25mmx0.25µm) [26], using helium with a flow rate of 1.2mL/min and sample injection in split mode (split ratio of 20:1). The mass spectra were

acquired in full scan mode with mass range of 50-600 a.m.u. The oven program increases from 100°C to 300°C with a rate of 10°C /min, where it stays for 30 min. The inlet temperature was 250°C, the interface temperature 260°C, ionization source temperature: 230°C and quadrupole temperature 150°C.

All samples were solubilized in hexane by ultrasonication for 10 min and centrifugation for 15min at 20°C. 1µL of the hexane layer was injected into the GC-MS. The concentration of alpha-tocopherol was calculated on from a calibration curve plotted in the concentration range 0.102-0.340mg/mL (R^2=0.9908). The limit of quantification LOQ was 0.102mg/mL. The result is expressed as mg alpha-tocopherol per gram and reported as the mean ± standard deviation (Mean±SD, n=3).

Antioxidant/antiradical activity

The evaluation of the antioxidant/antiradical activity of the plant extracts in sunflower oil was performed by applying the indirect DPPH spectrophotometric method, according to M. Rahmani et all [27], which involves recording the decrease in absorbance at wavelength λ= 520nm (maximum DPPH absorption). This decrease is proportional to the concentration of free radicals reduced in solution. The ability of extracts to capture the DPPH* radical is determined by the magnitude of the oxidation-reducing potential of molecules with antioxidant properties present in the composition of the extract. Antiradical activity (AAR) was defined as the amount of antioxidant needed to reduce the initial concentration of DPPH* by 50% and represents the effective concentration, EC50. The absorbance was recorded using a PerkinElmer Lambda 25 UV-Vis spectrophotometer equipped with a sample thermostat system.

All samples were solubilized in DMSO, then different volumes of sample solution were incubated for 30min with 0.004% DPPH solution. After 30min, the absorbance was measured at wavelength λ = 520nm. A curve is plotted for different sample volumes and the absorbance measured values. The volume required for EC50 inhibition was estimated based on this curve. The antioxidant activity is expressed as EC_{50} in mL. The results are reported as the mean ± standard deviation (Mean±SD, n=3).

Physicochemical tests for the formulated cream

Physical appearance and color were evaluated by visual observation. Homogeneity and texture were tested by pressing a small quantity of the formulated cream between the thumb and index finger. The consistency of the formulation and presence of coarse particles (*particule grosiere*) were used to evaluate the texture and homogeneity of the formulation.

Viscosity Measurement

A Brookfield viscometer DV-I (Brookfield Engineering Laboratories, Middleboro, MA) was used with a concentric cylinder spindle to determine the viscosity of the formulated cream. The tests were carried out at 21°C. The spindle was rotated at 0, 0.5, 1, 2, 2.5, 4, 5, 10, 20, 50, and 100 rpm values. All measurements were made in triplicate.

pH Values

The pH was determined using a pH-meter (Mettler-Toledo Seven470). Measurements were made in triplicate. The InLaB ISM sensor was calibrated with standard buffer solutions (pH 2, 4, 7 and 9.21) before each use.

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