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THIN-LAYER CHROMATOGRAPHY ANALYSIS AND SCAVENGING ACTIVITY OF MARIGOLD (*Calendula officinalis* L.) EXTRACTS

Gordana S. Ćetković, Sonja M. Đilas, Jasna M. Čanadanović-Brunet and Vesna T. Tumbas

The methanol, petroleum ether, chloroform, ethyl acetate, n-butanol and water extracts were obtained by extraction of marigold flower (Calendula officinalis L.). The content of total phenolic compounds, determined by UV spectrophotometric method using the Folin-Ciocalteu reagent, was 15.12 mg/g. The content of total flavonoids, determined by UV spectrophotometric method according to Markham, was 5.13 mg/g. Qualitative determination of phenolic compounds in the extracts was performed by one- and two-dimensional thin-layer chromatography (TLC) procedures.

The results of one- and two-dimensional TLC analyses showed that different flavonoids and phenolic acids were present in the investigated extracts. The greatest number of flavonoids (rutin, quercetin and some unidentified flavonoid glycosides) and phenolic acids (chlorogenic, caffeic, coumaric and vanillic acid) were deteminated in methanol extract.

The influence of marigold extracts, in concentration range 0.6-1.2 mg/mL, on 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radicals was investigated by electron spin resonance (ESR) spectroscopy. All extracts showed scavenging activity (SA) in the following order: ethyl acetate > n-butanol > methanol > water > chloroform > petroleum ether. The SA increased with increasing concentration of extracts. The ethyl acetate and n-butanol extracts exibited the most significant SA. These extracts in concentration of 1.2 mg/mL eliminated completely DPPH radicals. The lowest SA had chloroform and petroleum ether extracts (in concentration of 0.6 mg/mL SA=0%). The SA of marigold extracts is attributed to its hydrogen-donating ability and scavenging effect.

KEY WORDS: Marigold (*Calendula officinalis* L.), extraction, TLC, scavenging activity, DPPH, ESR

Dr. Gordana S. Ćetković, Assist. Prof., Dr. Sonja M. Đilas, Prof., Dr. Jasna M. Čanadanović-Brunet, Assoc. Prof., Vesna T. Tumbas, B. Sc., University of Novi Sad, Facutly of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia and Montenegro

INTRODUCTION

In the last decades natural alternatives to synthetic antioxidants have been intensively studied and the effects of antioxidative substances obtained from natural sources elucidated (1,2). Many natural products show antioxidant activity, for instance, tocopherols, flavonoids, coumarins, and, more generally, phenolic compounds widespread in the plant kingdom. Among them, flavonoids are a particularly attractive class of polyphenols, as they often occur in significant concentrations (0.5-1.5 %) (3).

Marigold (*Calendula officinalis* L., *Asteraceae*) grows as a common garden plant throughout Europe and North America. In our country the yellow or golden-orange flowers of marigold are traditionally used as spices, tea and medicine. They may be used as fresh or dried, and can be made into tea, tinctures, ointments and creams. The active constituents of this plant include carotenoids (hence the orange colour), essential oils, flavonoids, sterols, tannins, saponins, triterpene alcohols, polysaccharides, a bitter principle, mucilage, and resin (4).

This paper is concerned with the thin-layer chromatography (TLC) qualitative analysis of phenolic compounds in marigold extracts obtained by successive extraction as well as with the scavenging activity of these extracts on DPPH radicals by ESR spectroscopy.

EXPERIMENTAL

Methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol, *tert*-butanol, acetic acid, formic acid and sodium acetate trihydrate were products of "Zorka" Šabac, Serbia and Montenegro. The 2,2'-diphenyl-1-picrylhydrazyl (DPPH), aluminium chloride hexahydrate, 2-aminoethyl diphenylborinate, Folin-Ciocalteu reagent, standards of flavonoids (rutin and quercetin) and phenolic acid (chlorogenic, caffeic, coumaric and vanillic acid) were purchased from Sigma Chemicals Co., USA. These chemicals were of analytical reagent grade.

Plant material, marigold flowers, was collected in the region of Žabalj (Vojvodina, Serbia and Montenegro) and dried in air.

Extraction. Dried flowers of marigold (5 g) were extracted with 70% methanol (250 mL) at room temperature for 24 h. Aliquot of the obtained extract (20% v/v) was evaporated to dryness (methanol extract). The amount of 80% v/v of the extract was concetrated under the reduced pressure and the obtained product was fractioned using different organic solvents: petroleum ether, chloroform, ethyl acetate and *n*-butanol. The methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and the remaining water extracts were evaporated to dryness under reduced pressure. The yields of extracts were:

Methanol	m = 0.2392 g	Ethyl acetate	m = 0.0984 g
Petroleum ether	m = 0.0717 g	*n*-Butanol	m = 0.2391 g
Chloroform	m = 0.0847 g	Water	m = 0.6819 g

Total phenolic compounds. Total phenolic compounds were determinated spectrophotometrically using Folin-Ciocalteu reagent and the results are expressed as chlorogenic acid equivalents per g dry weight (5).

Total flavonoids. Total flavonoids (expressed as mg rutin per g dry weight) were estimated spectrophotometrically according to Markham (6).

Thin-layer chromatography. TLC was performed on the 20 × 20 cm plates precoated with microcrystalline cellulose (Camag, Muttanez, Switzereland). A volume of 1 μL of 1% methanolic solutions of standards and investigated extracts was spotted on the plates. One-dimensional TLC analysis was performed with ethyl acetate : formic acid : acetic acid : water in volume ratio 100 : 11 : 11 : 26 as mobile phase. The mobile phases for two-dimensional TLC analysis were:

1. *tert*-butanol : acetic acid : water in volume ratio 3:1:1
2. 15% acetic acid

Spots were observed under UV light at 366 nm before and after spraying with the 2-aminoethyl diphenylborinate.

DPPH radical assay. Blank probe was obtained by mixing 600 μL of 0.4 mM methanolic solution of DPPH and 200 μL methanol. A volume of x μL of 1% methanolic solution of investigated extracts was added to (200-x) μL methanol and 600 μL 0.4 mM methanolic solution of DPPH. The concentration range of the investigated extracts was 0.60-1.20 mg/mL. After that the mixture was stirred for 2 min, transferred to a quartz flat cell ER-160FT and analysed by ESR spectroscopy.

The scavenging activity (SA) of the extracts was defined as:

$$SA=100\cdot(h_o-h_x)/h_o\ [\ \%\ ]$$

h_o the height of the second peak in the ESR spectrum of DPPH free radicals of the blank

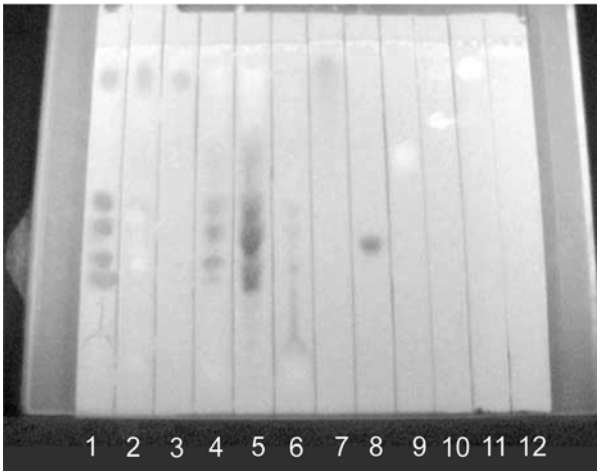
h_x the height of the second peak in the ESR spectrum of DPPH free radicals of the probe

The ESR spectra were recorded on an ESR spectrometer Bruker 300E (Rheinstetten, Germany) under the following conditions: field modulation 100 kHz, modulation amplitude 0.226 G, time constant 40.96 ms, conversion time 671.089 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C.

RESULTS AND DISCUSSION

The contents of total phenolic compounds and flavonoids in marigold flowers are 15.12 mg/g and 5.13 mg/g, respectively.

TLC chromatogram of the methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol extracts and water extract, as well as of standard compounds (quercetin, rutin, chlorogenic, caffeic, coumaric and vanillic acid), is presented in Fig. 1. Spots were characterized by *Rf*-values and color under UV light before (UV) and after spraying with 2-aminoethyl diphenylborinate (UV-NA, Table 1).



SPOTS:			
1.	methanol extract	7.	quercetin
2.	petroleum ether extract	8.	rutin
3.	chloroform extract	9.	chlorogenic acid
4.	ethyl acetate extract	10.	caffeic acid
5.	n-butanol extract	11.	vanillic acid
6.	water extract	12.	coumaric acid

Fig. 1. TLC chromatogram of the investigated extracts

Table 1. *Rf*-values and color of spots of the investigated extracts``

Spot	*Rf*	UV/UV-NA*	Compound
1	0.06	yellow/yellow	flavonoid-glycoside
	0.21	violet/yellow	flavonoid-glycoside
	0.30	dark violet/yellow	rutin
	0.42	violet/yellow	flavonoid-glycoside
	0.64	blue/light blue	chlorogenic acid
	0.79	blue/light blue	caffeic acid
	0.92	blue/light blue	coumaric acid
	0.96	yellow/yellow	quercetin
	0.98	blue/light blue	vanillic acid
2	0.93	blue/light blue	coumaric acid
	0.96	yellow/yellow	quercetin
	0.99	blue/light blue	vanillic acid
3	0.93	blue/light blue	coumaric acid
	0.96	yellow/yellow	quercetin
4	0.34	dark violet/yellow	rutin
	0.43	violet/yellow	quercetin-glycoside
	0.62	blue/light blue	chlorogenic acid
	0.80	blue/light blue	caffeic acid
	0.92	blue/light blue	coumaric acid

Table 1. continuation

5	0.30 0.33 0.42 0.62 0.79 0.93	dark violet/yellow dark violet/yellow violet/yellow blue/light blue blue/light blue blue/light blue	flavonoid-glycoside rutin flavonoid-glycoside chlorogenic acid caffeic acid coumaric acid
6	0.18 0.26 0.33 0.62	yellow/yellow violet/yellow dark violet/yellow blue/blue	flavonoid-glycoside flavonoid-glycoside rutin chlorogenic acid
7	0.96	yellow/yellow	quercetin
8	0.30	dark violet/yellow	rutin
9	0.62	blue/light blue	chlorogenic acid
10	0.79	blue/light blue	caffeic acid
11	0.92	blue/light blue	coumaric acid
12	0.98	blue/light blue	vanillic acid

* Spots were observed under UV light after spraying with 2-aminoethyl diphenylborinate

Rf-values and color of spots of all extracts are presented in Tables 2-7.

Table 2. *Rf*-values and color of spots of the ethyl acetate extract

Spot	*Rf*		UV/UV-NA*	Compound
	TBA	15% CH_3COOH		
1	0.78	0.39	yellow/yellow	quercetin
2	0.72	0.41	violet/yellow	flavonoid-glycoside
3	0.74	0.49	violet/yellow	flavonoid-glycoside
4	0.65	0.58	dark violet/yellow	rutin
5	0.71	0.63	blue/light blue	phenolic acid
6	0.70	0.71	blue/light blue	phenolic acid
7	0.68	0.74	blue/light blue	phenolic acid
8	0.75	0.76	violet/yellow	flavonoid-glycoside
9	0.63	0.84	blue/light blue	phenolic acid
10	0.96	0.75	yellow/yellow	flavonoid
11	0.90	0.84	violet/yellow	flavonoid-glycoside

Table 3. *Rf*-values and color of spots of the methanol extract

Spot	*Rf*		UV/UV-NA*	Compound
	TBA	15% CH_3COOH		
1	0.75	0.71	blue/light blue	phenolic acid
2	0.71	0.74	blue/light blue	phenolic acid
3	0.68	0.84	blue/light blue	phenolic acid
4	0.79	0.17	yellow/yellow	quercetin

Table 4. *Rf*-values and color of spots of the petroleum ether extract

Spot	*Rf*		UV/UV-NA*	Compound
	TBA	15% CH_3COOH		
1	0.66	0.28	blue/light blue	phenolic acid
2	0.83	0.27	yellow/yellow	quercetin
3	0.89	0.83	blue/light blue	phenolic acid

Table 5. *Rf*-values and color of spots of the chloroform extract

Spot	*Rf*		UV/UV-NA*	Compound
	TBA	15% CH_3COOH		
1	0.71	0.18	yellow/yellow	quercetin
2	0.80	0.16	violet/yellow	flavonoid-glycoside
3	0.70	0.34	violet/yellow	flavonoid-glycoside
4	0.62	0.57	violet/yellow	flavonoid-glycoside
5	0.63	0.65	dark violet/yellow	rutin
6	0.53	0.75	blue/light blue	phenolic acid
7	0.30	0.72	blue/light blue	phenolic acid
8	0.30	0.76	blue/light blue	phenolic acid

Table 6. *Rf*-values and color of spots of the *n*-butanol extract

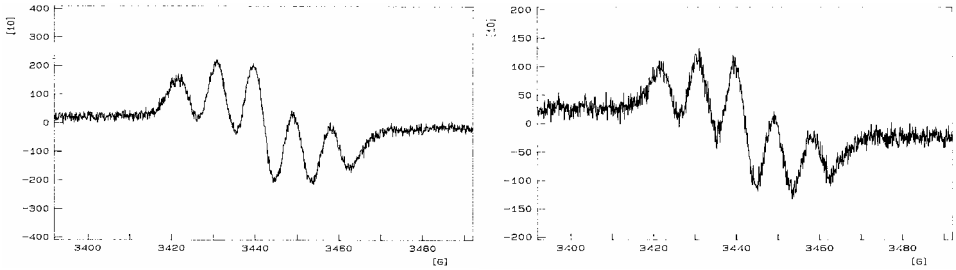
Spot	*Rf*		UV/UV-NA*	Compound
	TBA	15% CH_3COOH		
1	0.79	0.30	yellow/yellow	flavonoid
2	0.93	0.36	yellow/yellow	quercetin
3	0.66	0.53	violet/yellow	flavonoid-glycoside
4	0.57	0.54	dark violet/yellow	flavonoid-glycoside
5	0.60	0.61	dark violet/yellow	rutin
6	0.52	0.69	blue/light blue	phenolic acid
7	0.82	0.62	blue/light blue	phenolic acid
8	0.89	0.51	violet/yellow	flavonoid-glycoside

Table 7. *Rf*-values and color of spots of the water extract

Spot	*Rf*		UV/UV-NA*	Compound
	TBA	15% CH_3COOH		
1	0.63	0.57	violet/yellow	flavonoid-glycoside
2	0.57	0.59	violet/yellow	flavonoid-glycoside
3	0.62	0.64	dark violet/yellow	rutin
4	0.84	0.62	blue/light blue	phenolic acid

The results of one- and two-dimensional TLC analyses show that different phenolic compounds, flavonoids and phenolic acids, are present in the investigated extracts. A largest number of flavonoids (rutin, quercetin and some unidetified flavonoid-glycosides) and phenolic acids (chlorogenic, caffeic, coumaric and vanillic acid) was found in methanol extract. Rutin and some unidentified flavonoid-glycosides are present in the ethyl acetate, *n*-butanol and water extracts. The ethyl acetate and *n*-butanol extracts also contain coumaric, caffeic and chlorogenic acid, while water extract contains only chlorogenic acid. The petroleum ether and chloroform extracts are poor in phenolic compounds. Bilia et al. (7) established that the marigold flower contains some other quercetin glycosides such as isoquercitrin and quercetin-3-*O*-rutinosylrhamnoside. It can be supposed that these flavonoids are also present in the methanol, ethyl acetate, *n*-butanol and water extracts.

Stable DPPH radicals have been used to investigate scavenging activity of marigold extracts. The ESR spectra of DPPH radicals in the blank are characterized by their five lines of relative intensities 1:2:3:2:1 and hyperfine splitting constant a_N= 9.03 G (Fig. 2A). No change in the hyperfine structure in the ESR spectra was detected, but the intensities of lines corresponding to the concentration of DPPH radicals, decreased in the presence of the investigated extracts (Fig. 2B).



(A) (B)

Fig. 2. The ESR spectra of DPPH radicals in the absence of extracts (A, blank), and in the presence of 0.6 mg/mL ethyl acetate extract of marigold (B)

The SA of the different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extracts against DPPH free radicals is evident from the data given in Fig. 3.

As can been seen, all the investigated extracts showed SA on DPPH radicals. SA depends on the type and concentration of extracts.

The DPPH radicals SA of ethyl acetate, *n*-butanol, methanol and water extracts increases dose-dependently at all investigated mass concentration (0.6-1.2 mg/mL). Ethyl acetate extract has the strongest SA, ranging from 40.90% to 100%. *n*-Butanol extract has a similar activity but lower than ethyl acetate extract, ranging from 34.64% to 100%. The same concentration of methanol and water extracts exhibited SA from 25.32% to 80.08% and 12.38% to 68.24%, respectively.

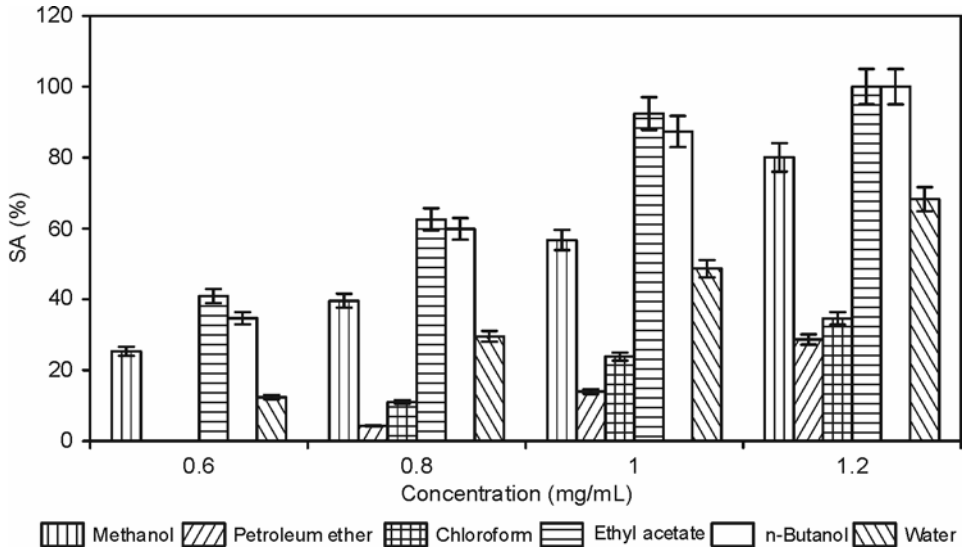


Fig. 3. SA of different concentrations of methanol, petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extracts of marigold on DPPH radicals.
Data represent the mean ±SD of three measurements

SA of the chloroform and petroleum ether extracts was not evident at the concentration 0.6 mg/mL, but at other investigated concentrations these extracts exhibited low SA, from 11.04% to 34.62%, and from 4.32% to 28.68%, respectively.

According to the literature (8-11) phenolic compounds such as flavonoids and phenolic acids, detected by TLC analysis, are principal contributors to the SA of the investigated extracts. The SA of quercetin, rutin, flavonoid-glycosides and phenolic acids (chlorogenic, caffeic, coumaric and vanillic acid), present in the extracts, is attributed to their hydrogen-donating ability and scavenging effect (one $DPPH^{\bullet}$ molecule forms complex with one aryl radical formed from phenolic compounds).

CONCLUSION

- Flavonoids (rutin, quercetin and some unidetified flavonoid-glycosides) and phenolic acids (chlorogenic, caffeic, coumaric and vanillic acid) are deteminated in the investigated extracts by TLC analysis;
- A largest number of flavonoids and phenolic acids are present in methanol extract;
- Employing the ESR spectroscopy, the SA of marigold extracts, in a concentration range of 0.6-1.2 mg/mL, on DPPH radicals was established;
- SA depends on the concentration and type of the extract;
- SA increases with increasing concentration of extracts;
- The following order of SA has been established: ethyl acetate > *n*-butanol > methanol > water extacts > chloroform > petroleum ether extract;

- The most significant SA exhibited the ethyl acetate and *n*-butanol extracts. The concentration of 1.2 mg/mL of these extracts completely eliminated DPPH radicals;
- The much lower SA had the chloroform and petroleum ether extracts. SA of these extracts was not evident at the concentration of 0.6 mg/mL;
- The SA of the investigated marigold extracts is probably due to the hydrogen donor ability and scavenging effect of the phenolic compounds (flavonoids and phenolic acids) that were identified by TLC.

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ТАНКОСЛОЈНА ХРОМАТОГРАФСКА АНАЛИЗА И "СКЕВИНЏЕР" АКТИВНОСТ ЕКСТРАКАТА НЕВЕНА (*Calendula officinalis* L.)

Гордана С. Ћетковић, Соња М. Ђилас, Јасна М. Чанадановић-Брунет и Весна Т. Тумбас

Екстракцијом цветова невена (*Calendula officinalis* L.) добијени су метанолни, петролетарски, хлороформски, етилацетатни, н-бутанолни и водени екстракти. Садржај укупних фенолних једињења у невену, одређен УВ спектрофотометријском методом користећи Folin-Ciocalteu реагенс, био је 15,12 mg/mL, док је садржај укупних флавоноида, одређен УВ спектрофотометријском методом по Markham-у, 5,13 mg/mL.

На основу једно- и дводимензионалне танкослојне хроматографске (TLC) анализе утврђено је присуство различитих флавоноида и фенолних киселина у испитиваним екстрактима. Метанолни екстракт је најбогатији флавоноидима (рутин, кверцетин и неки неидентификовани флавоноид-гликозиди) и фенолним киселинама (хлорогенска, кафена, кумаринска и ванилинска киселина).

Утицај екстраката невена, концентрација 0,6-1,2 mg/mL, на стабилне 2,2′-дифенил-1-пикрилхидразил (DPPH) радикале испитан је електрон спин резонантном (ESR) спектроскопијом. Сви екстракти су показали скевинџер активност (СА).

Утврђен је следећи редослед активности екстраката: етилацетатни > н-бутанолни > метанолни > водени > хлороформски > петролетарски. СА екстраката је у корелацији са концентрацијом. Етилацетатни и н-бутанолни екстракти при концентрацији од 1,2 mg/mL потпуно уклањају DPPH радикале. Хлороформски и петролетарски екстракти, при концентрацији од 0,6 mg/mL, не показују СА. СА испитиваних екстраката највероватније је условљена способношћу фенолних једињења да отпуштају водоников атом као и арил радикала, насталих отпуштањем водониковог атома, да "хватају" DPPH радикале.

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