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Valorization of waste of *Calendula officinalis* - obtaining of ethanol extracts

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Valorization of wastes of food industry and agriculture became a priority in the last years. Novel promising waste materials are residues from the essential oils and natural pigment industry. Such underexplored waste is obtained from *Calendula officinalis* (marigold) which is widely used as herbal medicine and industrially for extraction of lutein. The aim of our work was to investigate the possibility for valorization of the waste of marigold. Two kinds of residues were investigated – one obtained after extraction of marigold flowers by freon (1,1,1,2-tetrafluoroethane) and second obtained after steam distillation. The total polyphenols and antioxidant activity of the ethanol extracts were investigated and was found that extraction of the raw materials by freons preserves to a large extent polyphenols – wastes after freon extracted marigold had 1543.8 mg/L total polyphenols compared to 336.5 mg/L for the after hydro-distilled marigold flowers. The same results were confirmed from the data for antioxidant activity – wastes from freon treated flowers showed 15856.2 μmol TE/l (by ORAC method) and 5011.3 μmol GAE/l (by HORAC method) while waste of hydro-distilled marigold flowers showed almost 5 times lower values. The individual phenolic acids and flavonoids were determined by HPLC and the main compounds found were chlorogenic and caffeic acids (from the phenolic acids), and naringin from the flavonoids. By GC-MS were investigated polar metabolites and aroma compounds in the ethanolic extracts. The results from the present investigation showed that the marigold waste could be a valuable source for obtaining of by-products with pronounced antioxidant activity.

Key words: *Calendula officinalis (marigold), waste valorization, antioxidant activity, polyphenols*

INTRODUCTION

Marigold (*Calendula officinalis*) is a very common flower grown across Europe as garden plant. It has been used also as herb, edible plant and it has well known medicinal properties [1]. The flower is found in the wild but also it has an important commercial application due to the facts that its seed oil contains large amounts of calendic acid which is used for coatings, in formulation of cosmetics etc. [1]. Marigold flowers are also a valuable source for commercial production of lutein [2]. Due to the fact that the concentration of active compounds in the plant materials are relatively low after extraction or distillation of the important biologically active substances large quantities of wastes remain. Throwing simply away or using as compost is among the very often used procedures to eliminate these wastes. But they could also serve as initial materials for extraction of valuable by-products. In a previous work of us we have investigated the possibility for extraction of water-soluble pectic polysaccharides from several plant wastes including marigold [3]. The aim of the present work was to explore further the possibilities for valorization of wastes of marigold obtained after extraction with freons and steam distillation.

MATERIALS AND METHODS

Materials

The waste *Calendula officinalis* mass (steam distilled) was obtained from the region of Chehlare (region of Plovdiv, crop 2015). The *Calendula officinalis* (crop 2015, region of Plovdiv) was extracted by freon (1,1,1,2-tetrafluoroethane, R134a) as described by Nenov [4]. After treatment the residues were cooled down, inspected for elimination of impurities and dried under vacuum at 50 °C. The mass was stored at -18 °C until further treatment. All the solvents used were of analytical grade and purchased from local distributors.

Methods

The 70 % ethanolic extracts from two wastes of *Calendula officinalis* (extracted by freon and steam distilled) were obtained according to Kratchanova *et al.* [5].

The total polyphenol content of ethanolic extracts was determined using the method described by Singleton and Rossi, [6]. The antioxidant activity by ORAC and HORAC assays was measured as described by Číž *et al*. [7].

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The content of individual phenolic and flavonoid components was analyzed on Agilent 1220 HPLC system (Agilent Technology, USA), equipped with binary pump and UV-Vis detector. Wavelength of λ= 280 nm was used. Separation was performed using Agilent TC-C18 column (5 μm, 4.6 mm x 250 mm) at 25ºC. Mobile phases constituted of 0.5 % acetic acid (A) and 100% acetonitrile (B) at flow rate 0.8 ml/min. The gradient conditions started with 14% B, between 6 min and 30 linearly increased to 25% B, then to 50% B at 40 min. The standard compounds (gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, rutin, naringin, myricetin, quercetin, naringenin and kaempherol) were purchased from Sigma-Aldrich (Steinheim, Germany).

The individual volatile and non-volatile compounds in the ethanolic extracts were determined according to the following procedures:

1). Non-volatile substances – 0.2 ml ethanolic extract was lyophilized and 50 μL pyridine and 50 μL N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were added. The sample was incubated at 70 °C for 40 min. For analysis 1.0 μL from the solution was injected on gas chromatograph Agilent GC 7890 with mas-selective detector Agilent MD 5975 and column HP-5ms (30 m with diameter 0.32 mm and 0.25 μm thicknesses). The following temperature regimen was used – initial temperature 100 °C (hold for 2 min) then increase to 180 °C with 15 °C/min (hold for 1 min) and increase of the temperature to 300 °C with 5 °C/min (hold for 10 min); injector and detector temperatures – 250 °C, helium was used as carrier gas at 1.0 ml/min. The scanning range of mass-selective detector was m/z = 50 – 550 in split-split mode (10:1).

2). Volatile substances – 1.0 ml ethanolic extract was extracted with 1.0 ml dichloromethane (triple). The combined organic layers were dried under vacuum at 30 °C. To the dry residue 100 μL dichloromethane was added. For analysis 1.0 μL from the solution was injected on gas chromatograph Agilent GC 7890 with mas-selective detector Agilent MD 5975 and column HP-5ms. The following temperature regimen was used – initial temperature was 40 °C and then increase to 300 °C with 5 °C/min (hold for 10 min); injector and detector temperatures – 250 °C, helium was used as carrier gas at 1.0 ml/min. The scanning range of mass-selective detector was m/z = 40 – 400 in splitless mode.

The individual compounds were identified comparing the retention times and the relative index (RI) with those of standard substances and mas-spectral data from libraries of The Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) and NIST'08 (National Institute of Standards and Technology, USA).

RESULTS AND DISCUSSION

Treatment of the wastes with aqueous-ethanolic solutions is usually applied before extraction of polysaccharides from the raw materials. It aims to remove some low-molecular substances (pigments, sugars, etc.) which will hamper the future extraction. In our case it also aimed at obtaining of extracts rich on polyphenolic substances [3, 8]. In previous experiments we have investigated the influence of the ethanol concentration on extractability of polyphenols and subsequent polysaccharide extractions (Slavov, unpublished results). Our findings showed that extraction with 70% ethanol solutions gave the optimum results for possibilities of combined valorization of the waste materials of *Rosa damascena* and for this reason we have decided the treatment of the marigold residues after extraction or steam distillation to be performed with 70 % ethanol. The extracts obtained were subjected to preliminary analysis for their total phenolic substances and antioxidant activity. The results from the analysis are shown in Table 1.

The results from the preliminary experiments suggest that steam distillation led to significant reduction of polyphenols due to their partial extraction. Extraction with freons preserves to a large extent substances which contribute to the antioxidant activity of the ethanolic solutions.

It seems also that this extraction led to better disintegration of the cell walls of the plant materials which is beneficial for the further extractions of biologically active substances. In this respect extraction of the plant materials by halocarbons resembles extraction with supercritical CO_2 although the state of the freons used is above their critical point.

Furthermore we had investigated the polyphenolic profile of the 70 % ethanolic extracts obtained – Table 2.

Table 1. Polyphenols and antioxidant activity of 70 % ethanol extracts of marigold wastes

№	Waste material	Total phenolics, mg/L	ORAC, μmol TE/l	HORAC, μmol GAE/l
1	Marigold – after freon	1543.8 ± 84.9	15856.2 ± 1427.0	5011.3 ± 267.6
2	Marigold – after steam distillation	336.5 ± 36.2	3654.2 ± 211.3	981.2 ± 79.8

Table 2. Phenolic acids and flavonoids in 70% ethanolic extracts.

Phenolic acids, mg/100 ml	70 % ethanol extracts	
	Marigold – after freon	Marigold – after steam distillation
Chlorogenic acid	3.44±0.42	0.24±0.01
Neochlorogenic acid	1.55±0.10	-
Vanillic acid	1.02±0.03	0.42±0.08
Caffeic acid	3.08±0.89	0.13±0.04
p-Coumaric acid	1.64±0.09	0.09±0.01
Ferulic acid	0.38±0.05	0.01± -
Ellagic acid	0.61±0.03	0.09± -
Cinnamic acid	0.16±0.01	0.07± -
Gallic acid	1.19±0.20	0.31± 0.04
TOTAL, mg/100 ml	**13.07**±0.90	**1.36**±0.8
Flavonoids, mg/100 ml		
Quercetin	1.39±0.08	0.46±0.04
Quercetin-3-β-glucoside	1.32±0.40	0.30±0.06
Myricetin	1.67±0.06	-
Kaempferol	0.96±0.08	0.16±0.01
Naringin	40.99±1.20	3.35±0.58
Naringenin	6.69±0.90	2.86±0.09
Catechin	4.43±0.40	2.68±0.10
Epicatechin	-	-
TOTAL, mg/100 ml	**57.45**±1.20	**9.81**±0.80

The highest contents of the phenolic acids were observed for chlorogenic and caffeic acids – two compounds which are known for their pronounced antioxidant activities [9]. Other phenolic acids contributing to the higher antioxidant activity of the ethanolic extract from marigold treated with freon were p-coumaric, neochlorgenic, gallic and vanillic acids. Comparing the total amount of phenolic acids could be concluded that ethanolic extract from marigold treated with freon has 10 times more than steam distilled residues. The 70% ethanolic extract of freon treated marigold is also rich of flavonoids (5 times higher) from which almost 85% is due to presence of naringin and naringenin.

In the next analysis were determined by GC-MS the volatile and non-volatile polar compounds present in the 70% ethanolic extracts. The results are presented in Tables 3 and 4.

The results from the analysis showed that even after extraction / distillation a high amount of linoleic and linolenic acids (which also includes amounts of calendic acid – a conjugated linoleic acid) [10] remain in the wastes. The extract from marigold waste (freon) also is rich on malic, quinic, caffeic and syringic acid. The results from the analysis confirm the observation that extraction with freons preserves to a larger extent in the plant material the non-volatile biologically active substances.

Table 3. Polar non-volatile substances in ethanolic extracts. RI - relative index (Kovats retention index); % of TIC - total ion current.

Compounds	**RI**	% of TIC	
		Marigold – after freon	Marigold – after steam distillation
Succinic acid	1310	0.763±0.012	0.342±0.065
Fumaric acid	1355	0.407±0.015	0.367±0.008
L-Threonine	1390	0.319±0.020	0.192±0.015
L-Homoserine	1446	0.112±0.009	0.088±0.005
Malic acid	1488	2.505±0.090	0.776±0.079
Salycilic acid	1516	0.226±0.089	0.313±0.068
L-Threonic acid	1528	0.677±0.078	0.228±0.072
Vanillic acid	1758	0.241±0.085	0.144±0.079
Protocatechuic acid	1813	0.299±0.056	0.233±0.046
Quinic acid	1843	1.372±0.120	2.615±0.111
Syringic acid	1888	1.310±0.110	1.399±0.099
Gluconic acid	1991	1.762±0.090	1.197±0.068
Glucaric acid	2013	0.722±0.085	0.302±0.078
Caffeic acid	2140	0.693±0.054	0.211±0.045
Linoleic acid	2209	3.113±0.154	3.684±0.132
α-Linolenic acid	2217	2.675±0.123	3.403±0.097
Stigmasterol	3315	0.547±0.045	0.852±0.056
β-Sitosterol	3355	0.693±0.047	1.595±0.095

Table 4. Polar volatile substances in ethanolic extracts. RI - relative index (Kovats retention index); % of TIC - total ion current.

Compounds	**RI**	% of TIC	
		Marigold – after freon	Marigold – after steam distillation
Hyacinthin	1075	0.205±0.025	0.101±0.021
Benzeneacetic acid	1274	0.188±0.054	0.125±0.065
Syringol	1336	0.333±0.065	0.099±0.066
Eugenol	1358	0.265±0.075	0.142±0.078
2-Methylbenzoate	1372	0.154±0.021	0.076±0.019
α-Copaene	1379	0.317±0.032	0.085±0.026
β-Cubebene	1391	0.296±0.065	0.102±0.059
Methyleugenol	1405	0.144±0.012	1.358±0.025
β-Caryophyllene	1419	0.208±0.016	0.126±0.014
β-Copaene	1431	0.129±0.024	1.283±0.048
γ-Cadinene	1513	1.985±0.089	1.566±0.087
Viridiflorene	1518	2.133±0.087	0.908±0.068
δ-Cadinene	1529	1.922±0.095	0.322±0.069
Veridiflorol	1592	1.511±0.086	0.077±0.008
α-Cadinol	1641	3.180±0.113	0.713±0.063
α-Bisabolol oxide B	1648	3.514±0.124	3.085±0.114
7-Methoxy-coumarin	1737	1.595±0.102	8.747±0.254
α-Bisabolol oxide A	1792	2.221±0.098	5.949±0.168
7-hydroxy-coumarin	1813	0.755±0.085	0.578±0.077

Results from analysis for volatile substances in ethanolic extracts showed that even after the extraction / distillation of the plant materials remain significant amounts of aroma compounds in the residues. The highest quantities of floral aroma substances were found for α-Bisabolol oxide B, α-Cadinol, α-Bisabolol oxide A and viridiflorene. The above-mentioned compounds were found in ethanolic extracts from the two investigated wastes (marigold extracted with freons and steam distilled) in relatively high concentrations and even more some compounds (7-Methoxycoumarin, methyleugenol, α-Bisabolol oxide A, etc.) were preserved to a larger extent due to their insolubility in water in steam-distilled marigold residues.

CONCLUSIONS

The present study focused on valorization of marigold wastes showed that the 70% ethanolic extracts have pronounced antioxidant effects and also were rich on phenolic acids and flavonoids. To a larger extent the extraction with non-polar solvents (halocarbons) led to significant preservation in the wastes of valuable biologically active substances. Steam distillation due to the formation of water phase led to extraction and loss of some of the substances. The investigated approach of treatment of the wastes with ethanol and obtaining of valuable by-products also have the advantage that it could be combined with further extraction of the plant residues in order to obtain polysaccharides and thus will lead to a better valorization of the wastes.

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ОПОЛЗОТВОРЯВАНЕ НА ОТПАДЪЦИ ОТ *Calendula officinalis* – ПОЛУЧАВАНЕ НА ЕТАНОЛНИ ЕКСТРАКТИ

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(РЕЗЮМЕ)

Оползотворяването на отпадъците от хранителната индустрия и селското стопанство е един от основните приоритети в последните години. Едни нови и с голям потенциал като източник на биологично активни вещества отпадъци са тези, получени при преработката на етерично-маслени и медицински растения. Недостатъчно изследвани са отпадъците получени при преработка на цветове *Calendula officinalis* (невен) които са широко използвани в козметиката, както и за получаване на лутеин. Целта на настоящата работа е да се изследва възможността за оползотворяване на отпадъците, получени при преработка на невен. На изследвания са подложени два вида материали – получени след екстракция на цветове невен с фреон (1,1,1,2-тетрафлуороетан) и получени след водно-парна дестилация. Общите полифеноли и антиоксидантната активност на екстрактите са изследвани и е намерено, че екстракцията с фреон запазва до голяма степен полифенолните съединения – отпадъците, получени след екстракция с фреон имат 1543.8 mg/L общи полифеноли в сравнение с 336.5 mg/L за отпадъците от невен, получени след водно-парна дестилация. Подобни резултати са получени и при изследване на антиоксидантната активност – отпадъците след екстракция с фреон показват 15856.2 µmol TE/l (чрез ORAC метода) и 5011.3 µmol GAE/l (по HORAC метода), докато екстрактите, получени от отпадъци на невен, дестилирани чрез водно-парна дестилация имат 5 пъти по-малки стойности. Чрез HPLC са определени индивидуалните фенолни киселини и флавоноиди и е показано, че основните компоненти са хлорогенова и кафеена киселини, и наринген (от флавоноидите). Чрез GC-MS са изследвани полярните нелетливи метаболити и ароматичните вещества в етанолните екстракти. Резултатите показват, че отпадъците от преработката на цветове невен могат да бъдат един добър източник за получаване на вторични продукти с изразена антиоксидантна активност.

Ключови думи: *Calendula officinalis (невен), отпадъци, оползотворяване, антиоксидантна активност, полифеноли.*