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A Study of the Chemical Composition and Biological Activity of Extracts from Wild Carrot (*Daucus carota* L.) Seeds Waste.

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ABSTRACT

Waste (wild carrot seeds, *Daucus carota* L.) formed after the industrial extraction of 96% ethanol were re-extracted with 40% and 70% ethanol in order to recover of valuable substances for potential use as a safe food additives and functional food ingredients or nutraceuticals. In this study we compared chemical composition and activity of two obtained extracts (70% EtOH and 40% EtOH). The results indicated that the 70% EtOH extract has a higher total phenol and flavonoid content than 40% EtOH extract. The antioxidant activity of 70% EtOH extract was 86.88 ± 3.018 , whereas of 40% EtOH extract scavenging activity was 78.72 ± 3.276 . The extracts were also active against bacteria with MIC against 2 Gram-positive bacteria being in the range of 1.56-3.125 mg/mL and against 3 strains of Gram-negative bacteria of 3.125-12.50 mg/mL whereas against 1 strain of yeast in the range of 3.125-6.25 mg/mL. The results obtained from the studies on photo-protective activity clearly show that the incorporation of wild carrot extract into gel formulation can be used to prepare some types of sunscreens.

Keywords: wild carrot seeds, *Daucus carota*, phenolic compounds, flavonoids, antimicrobial activity, photoprotective activity, antioxidant activity

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INTRODUCTION

In recent years have seen back to the traditional folk medicine and use of extracts of natural origin as medicines or health-promoting agents, resulted in increased interest in substances with a specific biological activity.

One of the sources of particular interest regarding these compounds are by-products, that are rich sources of active substances, including phenolic compounds. The availability of phenolic compounds from agricultural and industrial residues, their extraction and antioxidant activity have been the subject of a many papers [1-3].

Phytochemical studies done on wild carrot have been concentrated on composition of volatile oil obtained from both the fresh and dried fruits (seeds). It is obvious that the composition of the oil varies between different cultivars. Anyway, preclinical studies have documented a variety of pharmacological activity of carrot seed oil including CNS-depressant, spasmodic and antispasmodic, hypotensive and cardiac-depressant activities. However, there is a lack of robust clinical research assessing the efficacy and safety of wild carrot. The major traditional use of wild carrot oil is as a diuretic agent. This activity has not been documented in animal studies, but the seed oil of wild carrot does contain significant level of terpinen-4-ol, the diuretic principle of juniper.

Constituents of wild carrot include flavonoids – flavones (e.g. apigenin, chrysin, luteolin), flavonols (e.g. kaempferol, quercetin) and various glycosides [4]. Furanocoumarins – 8-methoxypsoralen and 5-methoxypsoralen (0.01-0.02 µg/g fresh weight) are also present in fresh plant with their concentrations increasing in the diseased plants [5] and reach up to 0.66-1.65% of oils. Various components include λ-pinen, β-pinen, geraniol, geranyl acetate, limonene, λ-terpinen, λ-terpineol, terpinen-4-ol, β-bisabolene, β-elemene, caryophyllene, caryophyllene oxide, carotol, daucol, asarone [6, 7].

Plants have always been one of the main sources of medicinal agents. This has resulted in the increasing search for promising plant sources with a sufficient resource base for biologically active substances [8, 9]. The increased demand for medicinal compounds of natural origin leads to the search for new species of plants with a certain range of pharmacological benefits and also augments the use and in-depth study of the materials traditionally used in folk and official medicine [10, 11]. This leads to the systematic usage of raw plants and intensive repurposing of traditional materials in order to expand their scope. Considering this, we decided to undertake a more careful examination of the possible uses for the fruits of wild carrot.

According to the handbook *Martindale: The Complete Drug Reference*, nine mixtures of compounds present in seeds of wild carrots and are registered worldwide as drugs: *Aroma-detox* (Omega Pharma, France), *Evamilk* (Laboratori Gambar S.r.l., Italy), *Hepatalgina* (Altana Pharma SA, Argentina), *Metiogen* (Química MedicalArg. SACI, Argentina), *Natursel-C*, *Natur-Zin* (Laboratorio Ximena Polanco, Chile), *Palatrobil* (Monserrat y Eclair SA, Argentina), *Sciargo* and *Watershed* (Potter's, HerbalSupplies Ltd, UK) [12]. They are produced in the form of mixtures, oral drops, pills and capsules; prescribed as dietary supplements for detoxification, increased lactation, in the case of digestive disorders, lumbago, radiculitis, hepatobiliary disorders and as antioxidants.

The major extractant that is used by pharmaceutical manufacturers to obtain carrot oils is 96% ethanol. It ensures maximum extraction of essential oils from native materials. On the other hand, it is a well-known fact that 70% and 40% solution of ethyl alcohol is an excellent extractant for complex polyphenolic compounds including pharmaceutically important flavonoids.

Therefore, the aims of the present study were (i) to evaluate the efficiency of the extraction with 40% and 70% aqueous ethanol to receipt polyphenolic compounds and (ii) to determine the biological activity of 70% and 40% ethanol mixtures obtained.

Chemical analysis of the extracts by high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) was carried out to identify the polyphenols responsible for the biological activities.

MATERIALS AND METHODS

Plant and extraction

The dried waste obtained after extraction of wild carrot seeds with 96% EtOH (1:1) were extracted with 70% EtOH (1:5.33) and 40% EtOH (1:2.2) for 24 h at room temperature. The extracts were filtered and concentrated under reduced pressure, removing all solvents.

Dry residue of extracts

In a flat-bottomed dish of about 50 mm in diameter and about 30 mm in height, 2.00 mL of extract is introduced. Solvents were evaporated to dryness on a water-bath and by drying in an oven at 100-105°C for 3 hours. After cooling in a desiccator over *anhydrous silica gel R* the extracts had been weighted [13].

Total phenolic content (TPC)

The total phenolic was measured using spectrophotometry by a modified Folin-Ciocalteu method [14, 15]. Each extract (0.32 mg and 0.28 mg respectively) was dissolved in (70% methanol) (1 mL). 20 µL of the extract solution was then diluted with distilled water (1.58 mL) and was mixed with 100 µL of Folin-Ciocalteu reagent. After 5 min, 300 µL of Na₂CO₃ (7 g/100 mL distilled water) solution was added. After 2 hours of incubation at room temperature, the absorbance was read against a blank (70% methanol) at 760 nm in a 10 mm quartz cuvette. The same procedure was applied to the standard solutions of gallic acid. Total phenol content, expressed as milligrams of gallic acid equivalent (GAE) per gram (mg GAE/g) of extract, was calculated using a calibration curve prepared with fresh Gallic acid standard solutions. All measurements were performed in triplicate.

Total flavonoid content (TFC)

Quantitative detection of the total content of flavonoids was carried out using Cary-50 Varian spectrometer in relation to luteolin. In a 25 mL volumetric flask of 2 ml of the extract was placed and 2 mL of 3% aluminium chloride, 0.1 mL acetic acid 1M and 96% EtOH were added tend the total volume 25 mL was fulfilled [16]. After 40 minutes of incubation at room temperature, the optical density of the solution was measured at a wavelength in the range of 360-415 nm in a 10 mm quartz cuvette. Standard sample solution of luteolin was used as control. Approximately 10.60 mg (accurate weight) of luteolin, previously dried at a temperature of 130 - 135 °C for 3 hours was dissolved in 85 mL of 96% alcohol in 100 mL volumetric flask by heating in a water bath, cooled, adjusted by the same alcohol to the mark and mix. Total flavonoid content, expressed as milligrams of luteolin equivalent per gram (mg luteolin/g) of extract, was calculated as follows:

$$\text{mg Luteolin/g extract} = \frac{\text{Abs sample} * \text{m control} * 2 * 25 * 1000}{\text{Abs control} * 100 * 25 * 2 * 15.97}$$

HPLC analysis

The HPLC analyses of 124 plant extracts were performed using an Ultimate 3000 Dionex HPLC system with photodiode array detector (PDA) and using Chromeleon 6.8 software (Dionex, Sunnyvale, CA, USA). Separations were carried out with reversed phase column Gemini 5 u C-18 (Merck, Darmstadt, Germany; 250 x 4.60 mm, particle sized 5 µm), with mobile phase consisting of 0.01 mol/L aqueous phosphate buffer pH 2.5 (solvent A) and methanol (solvent B). Constant solvent flow rate (1 mL/min) was applied. The following gradient elution scheme was applied (A/B ratio): 10:90% t = 0 min; 40:60% t = 13.5 min; 90:10% t = 39 min; 100:0% t = 42 min; 10:90% t = 55 min. The temperature of the column oven was set to 30 °C. The chromatograms were monitored at 214 and 280 nm, since most of the carrot phenolic compounds show their UV absorption maxima around these two wavelengths. The comparison of UV spectra and retention times with standard compounds enabled the identification of phenolic acids and flavonoids presented in analysed extracts. They were quantified against their external standards.

Antioxidant activity

The antioxidant activity of the plant extracts was determined by applying the 2,2 diphenyl 1-picrylhydrazyl (DPPH) radical scavenging method [17]. For the measurement of the samples scavenging activity, in test tubes, 1.5 mL DPPH stock solution (2.2 mg was dissolved in 100 mL of methanol) was added to aliquots of 0.2 mL of methanolic solutions (0.32 mg/mL and 0.28 mg/mL respectively) of extracts. After mixing, the samples were left at room temperature for 30 minutes in the dark. The control was prepared by using 0.2 mL of methanol instead of the antioxidant extract solution, while a blank containing only methanol was used. The ascorbic acid was used as a reference compound. Absorbance at 517 nm was measured using UV/Vis spectrophotometer. Triplicate measurements were carried out. The radical effect was calculated as follow:

$$\text{Scavenging activity (\%)} = 100(\text{Abs control} - \text{Abs sample}) / \text{Abs control}.$$

Photo protection properties

The model system, based on the UV-induced discoloration of paprika gel (*Capsicum annum*), was used to check the efficiency of phenolic antioxidants in protecting paprika carotenoids against UV induced discoloration. Agar (20 mg/mL) was added to water and heated at 80 °C for 15 minutes and then at 50 °C for 15 minutes to prepare a jelly-like substance. Paprika was then solubilized in acetone and added to jelly (0.5 mg/mL) [18-20]. A reference gel was also prepared by adding acetone to jelly in place of paprika. The jelly mixture was poured into Petri dishes with a reference jelly or the same amount of methanol as controls. The dishes were exposed to UV radiation 360 nm in a bio-safety cabinet for a period of up to 120 minutes. Photographs were taken after regular intervals of time, namely every 15 minutes [21].

Antimicrobial activity

Test-microorganisms

To evaluate the antimicrobial activity following bacterial species were used: Gram-positive *Staphylococcus aureus* ATCC 6538-P, *Staphylococcus hyicus* – isolated from the soil, *Micrococcus luteus* – isolated from soil, criptogamic culture of *Bacillus subtilis* ATCC 6633, Gram-negative *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739 and *Salmonella Abony* CIP- 8039, and *Acinetobacter johnsonii* – isolated from the environment, *Moellerella wisconsensis* – isolated from the environment and the fungi: *Candida albicans* ATCC 10231, *Candida utilis* Lia-01, *Saccharomyces cerevisia* ATCC 9763, *Aspergillus brasiliensis* ATCC 16404. Test - strains of bacteria were grown in a soybean casein digestive broth for 24 hours at 33 °C, test – strains of fungi were grown on a dense nutrient medium of Sabouraud-Dextrose agar, yeast for 48 hours and *Aspergillus brasiliensis* ATCC 16404 for seven days at 23 °C. The suspension test – organisms for inoculation were prepared by serial dilutions in buffer solution of sodium chloride and peptone pH 7.0.

Determination of antimicrobial activity using the diffusion into agar

Nutrient medium – soybean casein digest broth was melted and inoculated at a temperature of 48-50 °C, using the suspension of test organisms to obtain concentration of 10⁷ CFU/mL, to provide suitable test microorganisms growth inhibition zones. Immediately after the administration of the test microorganism, 20 mL of medium was poured into 90 mm Petri dishes [22]. Dishes were placed under a laminar air flow till their surfaces became completely dry. The study was conducted using the hole method. 7 mm diameter holes were made in the medium using a stainless steel cylinder. Extractants: 40% and 70% aqueous-alcoholic solutions were used as positive controls, there were used, as well as alcoholic solution (10 mg/mL) of well-known antimicrobial agents from the leaves of eucalyptus - *Chlorophyllipt*.

Into the holes 0.1 mL of every solution was placed, with the number of repetitions for each concentration being 6. Samples were kept at room temperature of 23 °C for a 4 hours for a complete diffusion of solutions into culture medium. The dishes with soybean casing digestive broth were incubated at 33 °C for 18-24 hours, the dishes with Sabouraud Dextrose Agar were incubated at a temperature of 23 °C for 48 hours.

In the experiment there were 9 standard (museum) strains and 3 strains isolated from the environment were used. Each test was repeated six times.

Determination of the minimum inhibitory concentration by serial dilutions in liquid nutrient medium

The method of serial dilutions enables to determine the antimicrobial activity of the test samples. Bean casein digestive broth nutrient medium was poured into test tubes; 2 mL into each one. In the first tube 2 mL of the prepared solution 1:5 with 1:5 purified water was added and mixed, followed by removal of 2 mL in order to transfer to the next test tube until the dilution became 1:80. At the same time a positive control was prepared, namely bean casein digest broth nutrient medium. Into each test tube, including the control one, 0.2 mL of the prepared culture was injected of 10^7 - 10^8 CFU/mL. The standard of inoculum was established according to the Mac Farland optical standard at Densimat Biomerieux. Inoculants were placed into the thermostat for 18-24 h at 33 °C. The results were manifested by the presence or absence of medium clouding. The concentration of the extract in the test tube with a transparent medium (no growth of test microbe) of lowest concentration corresponds to the minimum inhibiting concentration of the drug (MIC). Each test was done in six repetitions using 7 standard (museum) strains.

Determination of antibacterial activity

Standard procedure was used during all the studies for comparison purposes. The growth inhibition zones were formed due to the diffusion of the test solution into the culture medium. The diameters of growth inhibition zones were measured using electronic calliper with measurement accuracy of 0.1 mm. The mean arithmetic value for each extract was calculated

To determine the bactericidal concentration, the 0.1 mL of the test tubes content with transparent medium (lack of visible growth) were inoculated with dense nutrient medium – bean casein digest broth. The incubation was performed for 18-24 h at 33 °C. The minimum concentration of the extract (no growth on agar or in broth was visible), was determined. This amount corresponds to minimal bactericidal concentration. If the growth of individual colonies of microorganisms, compared to controls was 1.10 %, such test tubes was treated as those with bacteriostatic concentration.

RESULTS AND DISCUSSION

Dry residue of extracts

Dry residue of 70% EtOH extract was equal 15.97 mg/mL whereas of 40% EtOH extract was 14.27 mg/mL. Differences in extraction efficiency were observed when the extraction medium contained various proportions of ethanol.

Total phenolic and total flavonoid content

The phenolic contents of the studied extracts were determined spectrophotometrically according to the Folin-Ciocalteu method and was expressed as gallic acid equivalent (mg gallic acid per g of extract) whereas level of flavonoids as luteolin equivalent (mg luteolin per g of extract). The amounts of total phenolic and flavonoid contents are shown in Table 1.

Table 1: Total Phenolic and total flavonoids content of dried extracts from the waste of wild carrot seeds

Extracts	The total phenolic content (mg GA g^{-1} of dried extract)	The total flavonoids content (mg luteolin g^{-1} of dried extract)
70% EtOH	92.381 ± 4.8%	15.6 ± 2.2 %
40% EtOH	173.777 ± 8.6%	6.65 ± 1.2 %

The results show that the 70% EtOH extract has lower total phenolic content with flavonoids being more effectively extracted with 40%. The difference of results obtained might possibly be due to the different solvents polarities.

Antioxidant activity

The antioxidant activity of the plant extracts were determined by applying the DPPH radical scavenging method. For both extracts similar values have been obtained with 86.88 %± 3.02 for 70% extract and 78.72% ± 3.28 for the 40% EtOH extract The total phenolic and total flavonoid contents show the correlation with the results of scavenging activity.

HPLC-DAD-UV analysis of the ethanolic extracts

The results of the identification and quantification of major polyphenolic compounds present in each obtained extract by HPLC-DAD-UV are shown in Table 2. Totally 12 compounds have been found in both extracts. 70% extract was lacking in catechin and 3-hydroxybenzoic acid. Rutin and *p*-coumaric acid were dominating amongst the identified compounds, with luteolin, chlorogenic acid, myricetin found in significant amounts. The identified compound constitute of 83.29% of all compounds seen in chromatograms.

Table 2: Results of HPLC of extracts from the waste of wild carrot seeds

№	Standards		70% EtOH		40% EtOH	
	Name of compounds	Ret. time [min]	Rel. area %	ppm	Rel. area %	ppm
1	Myricetin	14.115	0.97	5.49	0.71	1.39
2	Chlorogenic acid	7.668	1.64	5.64	1.52	1.80
3	Luteolin	16.803	2.37	7.66	2.06	1.00
4	Apigenin	19.135	0.94	1.75	0.82	1.53
5	Rutin	10.668	24.71	148.05	14.6	3.36
6	Catechin	8.043	0.0	0.00	1.95	3.16
7	Ferulic acid	12.117	0.70	3.83	5.38	0.65
8	<i>p</i> -Coumaric acid	11.682	14.67	10.44	25.42	1.70
9	3-Hydroxybenzoic acid	6.753	0.0	0.00	7.59	3.21
10	Hydroxybenzaldehyde	10.617	1.77	0.69	0.75	0.24
11	Caffeic acid	9.355	0.0	1.13	5.28	0.18
12	Cinnamic acid	17.28	2.37	0.58	3.21	0.79
	Summary:		83.29		69.29	

Photo protection properties

The results clearly show that the incorporation of wild carrot extract into gel formulation can be used to prepare sunscreens, since they possess photoprotective properties. Results shown in Fig. 1 indicate that the extract combined with quercetin appears to be the most active.

Antimicrobial activity

The results of antimicrobial activity studies, which were performed using diffusion into agar are shown Fig. 2. The obtained data regarding the antimicrobial action of 70 %, 40% aqueous-alcoholic extracts were helpful in determination of minimum inhibitory concentrations. 70% aqueous-alcoholic extract appear to be more effective than 40% extract, namely: the growth inhibition zone diameters reached 10.0-13.0 mm towards both Gram-positive and Gram-negative bacteria. In this means that both extracts have moderate antibacterial activity . Regarding yeasts, the diameter of the growth inhibition zone reached 10.0-13.0 mm, which also indicates moderate fungicidal activity of the extracts. On the contrary no fungicidal activity was found.

The results of the studies determining minimum inhibitory concentration, by means of extract dilution in a liquid nutrient medium are shown in Table 3. The minimum inhibitory concentration towards Gram-positive bacteria was 1.56-3.125 mg/mL, while towards Gram-negative bacteria was 3.125-6.25 mg/mL. For the fungi this value was equal was 3.125-6.250 mg/mL. This indicates moderate activity once more.

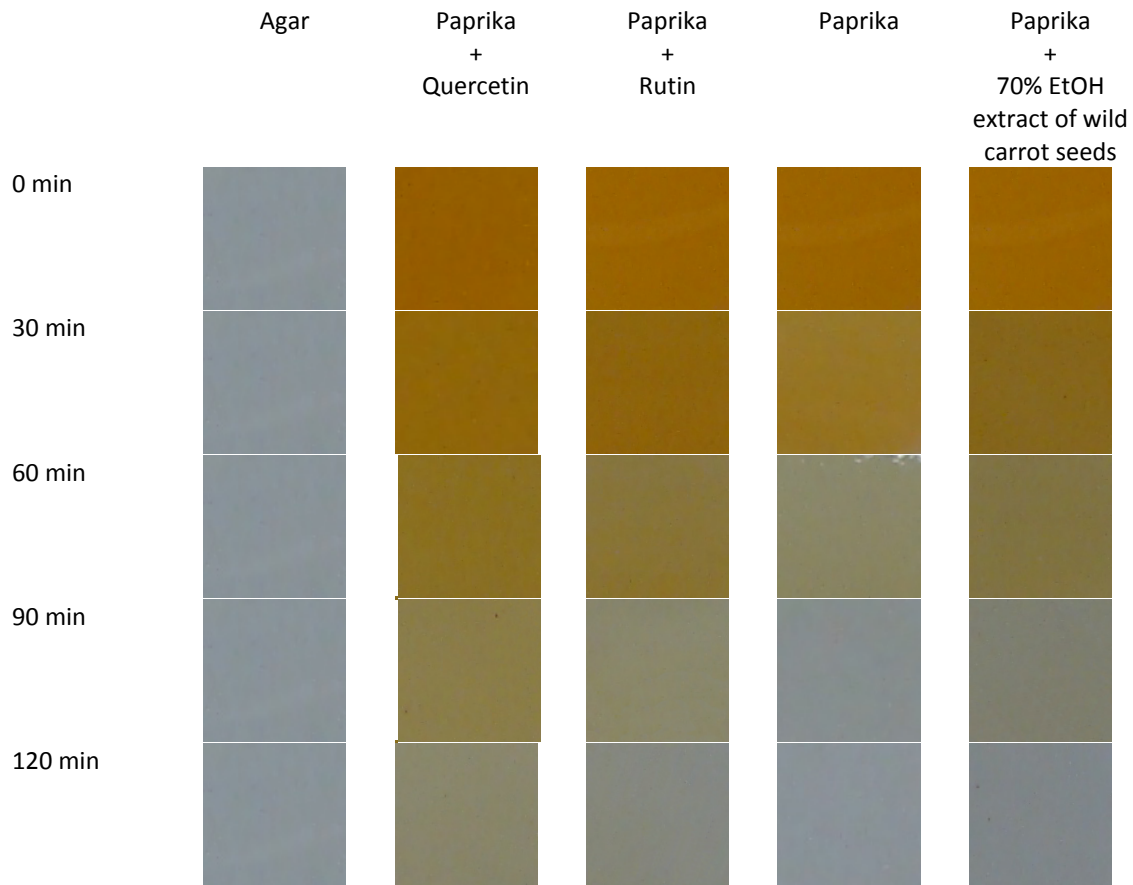


Figure 1: Photoprotection properties of extracts from waste of wild carrot seeds.

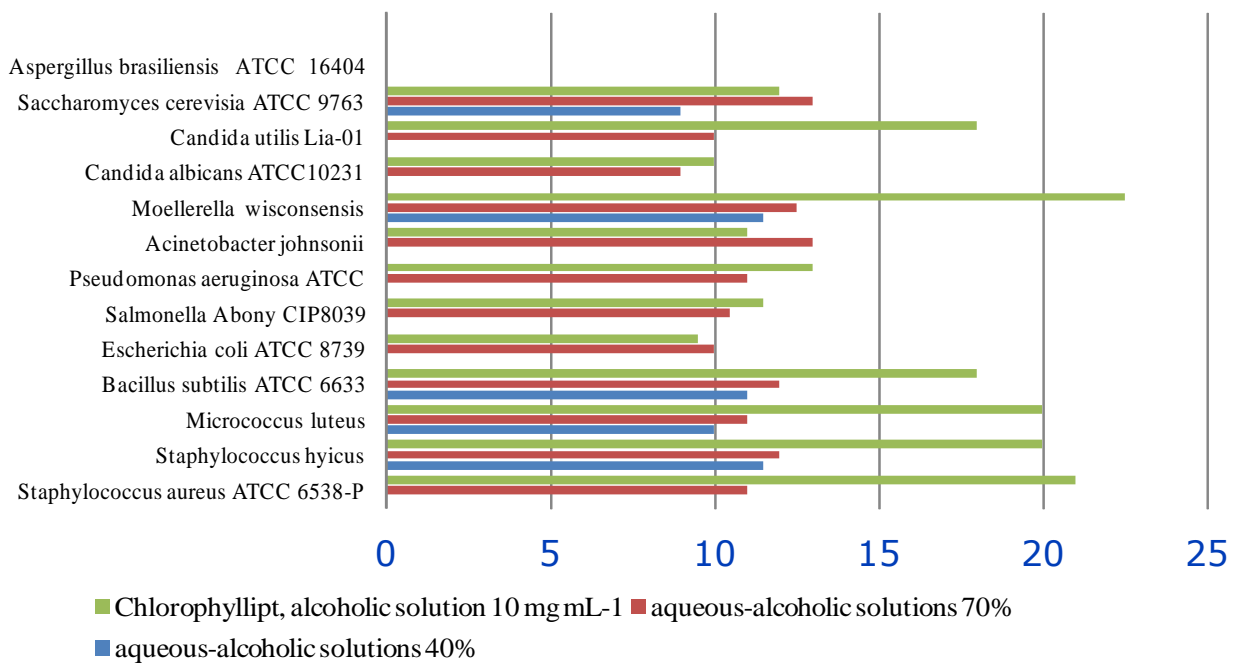


Figure 2: Antimicrobial activity of 70% and 40% EtOH extracts from wild carrot seeds waste compared to Chlorophyllipt, alcoholic solution 10 mg/mL.

Table 3: Minimum inhibition concentration of 70% EtOH dry extract from wild carrot seeds waste.

Test-microorganism	Concentration of wild carrot waste dry extract, mg mL ⁻¹	
	MBcC (mg mL ⁻¹)	MBsC (mg mL ⁻¹)
<i>Staphylococcus aureus</i> ATCC 6538-P	3.125	1.56
<i>Bacillus subtilis</i> ATCC 6633	3.125	1.56
<i>Escherichia coli</i> ATCC 8739	6.25	3.125
<i>Salmonella Abony</i> CIP 8039	12.5	6.25
<i>Pseudomonas aeruginosa</i> ATCC 9027	12.5	6.25
<i>Candida albicans</i> ATCC 10231	6.25	3.125

MBcC, minimum bactericidal concentration; MBsC, minimum bacteriostatic concentration; ND, inhibition not detected.

CONCLUSION

The utilization of by-products of plants processing as a source of biologically active compounds and their application in various branches of industries (cosmetics, pharmaceuticals) is a promising field which requires interdisciplinary research of chemists, microbiologist, biotechnologist and toxicologists. It is necessary to conduct researches focused on finding a suitably efficient and abundant potential sources, optimisation of extraction processes and knowledge of the biological activity of the obtained substance.

Our studies indicate that waste obtained after extraction of wild carrot seeds with 96% ethanol contain quite high amounts of interesting biologically active compound, including flavonoids and flavonoids and polyphenols. These compounds could be extracted by additional extraction with 70% and 40% ethanolic solutions.

These extracts appeared to have quite significant antioxidant activities as determined by DPPH test. The total phenolic and total flavonoid contents show the correlation with the results of the scavenging activity. The results clearly show that the incorporation of the wild carrot extract into gel formulation can be used to prepare sunscreens, which can serve a variety of purposes. Also antimicrobial activity of the obtained extracts have been studied and indicated that they possess moderate activity toward both Gram-positive and Gram-negative bacteria and fungi, being inactive against *Candida* yeast.

More detailed study is required in order to link the activity of the individual compounds with the observed activities.

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