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Effect of chemical modification of hydrodistillation on yield, composition and biological activity of *Rosmarinus officinalis* essential oil

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Studies on the chemical modifications of *Rosmarinus officinalis* essential oil hydrodistillation process (HD) by using 5% citric acid (CA-HD) and 5% trisodium citrate (TSC-HD) as a water phase were performed. Composition of essential oils obtained in conventional and modified conditions was analyzed by gas chromatography with mass selective detector method (GC-MS) and compared. Antioxidant activity of all essential oils was determined spectrophotometrically by using DPPH radical scavenging method. It was found that applied modifications of hydrodistillation process enhanced yields and antioxidant activity and the best results were obtained using 5% citric acid as a modifier. Effect of this modification on fungicidal activity of essential oils against 8 various fungi strains (*Alternaria alternata*, *Botrytis cinerea*, *Fusarium culmorum*, *Phythophtora cactorum*, *Rhizoctonia solani*, *Phythophtora infestans*, *Sclerotinia sclerotiorum* and *Ascosphaera apis*) was also determined and in most cases enhanced activity was observed.

Keywords: *Rosmarinus officinalis* essential oil, chemical modification of hydrodistillation, antioxidant activity, fungicidal activity.

INTRODUCTION

Rosmarinus officinalis (rosemary) is a flowering plant from the *Lamiaceae* family that grows widely in Mediterranean countries, southern Europe and some regions of Asia. Rosemary is popular aromatic spice, traditionally used not only in cooking but also in the treatment of various diseases including depression, insomnia or arthritic pain. Essential oils obtained from rosemary are precious aromatic mixtures of compounds characterized by broad biological activity, including antioxidant[1–4], antibacterial[4–6], antiviral[7–8], antifungal[9–11], anticancer[1, 12–14] and anti-inflammatory properties[15]. They are used as active ingredients in cosmetic products, food products, as well as in medicine and aromatherapy[16, 17]. Moreover, rosemary essential oils can be used as natural pesticides for protection of crops against a broad range of diseases and pests[10, 18].

The main components of rosemary essential oil are: 1,8-cineole (15–55%), camphor (5–21%), α-pinene (9–26%), camphene (2–12%), β-pinene (2–9%), borneol (1–5%)[12, 13, 15, 18]. Contents of particular compounds vary due to vegetative stage of plant, as well as different environmental and climatic conditions in specific geographical regions. Composition of essential oils can be also affected by all steps of plant material processing after harvesting, including drying, storage and isolation process[19]. Although such changes in composition are often subtle, they can influence their biological activity.

Last years, growing interest in applications of essential oils in different areas led to searching of some more effective processes of their isolation. Moreover, obtaining of high quality products characterized by specific composition and enhanced biological activity has become a target and a challenge. Studies have shown that in some cases it is possible to increase the effectiveness of conventional techniques like hydrodistillation (HD) and steam distillation (SD) by application of some innovative physical and chemical modifications[20]. In the literature there are described methods of supporting the hydrodistillation process using ultrasounds (US-HD)[20–21], microwaves (MSD, MS-HD)[22–24] or pulsed electric field treatment before hydrodistillation (PEF)[25]. As chemical modifications, the effect of salts[24, 26, 27], surfactants[28] and enzymes[20, 29] on the efficiency and composition of essential oils obtained by hydrodistillation from various plant materials were studied.

In the case of *Rosmarinus officinalis* essential oil not much data related to chemical modification of hydrodistillation process was found in the literature. It was reported that application of 5% NaCl during hydrodistillation did not influenced its yield and composition, but addition of ionic liquids (ILs) improved the yield by about 25% with no significant changes in the composition of the essential oils[30]. Enhancing of rosemary essential oil yields by 5, 50 and 20% was achieved after enzymatic pretreatment before hydrodistillation using respectively cellulase, hemicellulase and their combination, what also resulted in decreasing of 1,8-cineole content and significant enhancing of antimicrobial activities against the series of pathogens (*Escherichia coli*, *Salmonella typhimurium*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Enterococcus feacium* and *Candida albicans*)[29].

Because it was reported that neutral compounds like NaCl were not effective modifiers of hydrodistillation process[24, 27, 30], in presented research, chemical modification of *Rosmarinus officinalis* hydrodistillation was performed by using compounds which change pH for acidic (citric acid) and weakly basic (trisodium citrate) as additives to water phase, what was not found in the literature. The influence of these modifications on yields, composition and antioxidant activity of essential oils was examined. Moreover, fungicidal activity of rosemary essential oil obtained in the best selected conditions was compared to the reference essential oil obtained by conventional hydrodistillation.

EXPERIMENTAL

Essential oils isolation techniques

Studied material were leaves of rosemary (*Rosmarinus officinalis*) cultivated in Poland. Plant material was collected in July 2019, dried naturally in darkness at room temperature for 2 weeks and grinded using electric mill before experiments. Essential oils were obtained using Deryng apparatus by classic hydrodistillation method (HD) and hydrodistillation modified by replacing water with 5% solutions of citric acid (CA-HD) or trisodium citrate (TSC-HD). In each experiment, plant material (50.00 g) and 500 mL of redistilled water or 5% aqueous solution of modifying agent were placed in a 1000 mL round bottom flask and connected to Deryng apparatus. The hydrodistillation was conducted under reflux for 3 hours. After completion of the process, the essential oil was separated, dried over anhydrous sodium sulfate and stored in sealed vials at 4 °C until analyses were performed. The yields of essential oils were expressed in % (v/w), by calculations using the volumes of isolated essential oils and the weights of dry plant material.

Analysis of essential oils composition by GC-MS

Composition of all obtained *Rosmarinus officinalis* essential oils was determined by gas chromatography with mass selective detector (GC-MS) method. Directly before chromatographic analyses, 20 μL of essential oil was dissolved in 1 mL of acetone. Three samples were prepared for each essential oil and analyzed. The analyses were carried out using a 6890N gas chromatograph (Agilent Technologies) with a 5973 Network Mass Selective Detector. For chromatographic separation of analytes HP-5MS capillary column (30 m x 0.25 mm x 0.25 μm) was used with the temperature program from 50 °C to 290 °C at a rate of 4 °C/min. Helium (flow: 1.2 mL/min) was applied as a carrier gas. Analytical samples (2 μL) were dosed to a column using a 7683 Series Injector Autosampler in a split mode (10:1). The temperatures of Mass Selective Detector were respectively: ion source 230 °C, quadrupole 150 °C. The mass spectra of separated compounds were obtained via electron impact ionization (70 eV) by scanning in the range of 20–600 m/z.

Identification of the particular compounds in the isolated essential oils was performed on the basis of the mass spectra compared with NIST02 MS library. Additionally, it was verified using the calculated values of the linear retention indices (LRI), compared with the values published in the literature[31] and by comparison of retention times with the standards available in the laboratory. To determine the linear retention indices, the C7-C30 n-alkanes standard mixture in hexane (Supelco) was analyzed in the same chromatographic conditions. The internal normalization method was used for quantitative analysis and the relative contents of particular compounds were determined as the peak area percentages in TIC (Total Ion Chromatogram).

Antioxidant activity

The antioxidant activity of the obtained *Rosmarinus officinalis* essential oils was determined using DPPH radical scavenging method. For this purpose, for each essential oil several solutions in methanol with concentrations in a range of 5–50 mg/mL were prepared and analyzed. Spectrophotometric measurements were performed using UV-1600 PC spectrophotometer (VWR) in 1-cm cuvettes with methanol as a reference. Stock solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was prepared directly before analysis by dissolving of 0.0197 g DPPH in 25mL of methanol (C = 0.002 mM/mL) and diluted to obtain working solution (C = 0.12 μM/mL). For determination of antioxidant activity, 3 mL of DPPH working solution was mixed with 0.5 mL of essential oil solution and incubated for 30 minutes, protecting from light. The absorbance at 517 nm was measured for 3 independent samples and the results were averaged. Analogously, a blank sample which contained 0.5 mL of solvent (methanol) was prepared and analyzed. The value of RSA (Radical Scavenging Activity) was calculated from the obtained values of sample absorbance after 30 minutes (A_{30}) and blank sample absorbance (A_0), using following formula[32]:

$$RSA = 100\ (A_0 - A_{30}) / A_0\ [\%]$$

To calculate IC_{50} parameter, referred as concentration which cause a 50% inhibition of free radical activity, for each essential oil the dependence of RSA value on the concentration was determined. For this purpose, the RSA values for several different concentrations of particular essential oils in methanol (5–50 mg/mL) were determined. Next, the plots of RSA (%) versus concentrations C (mg/mL) were prepared and the mathematical equations of this dependences were determined. On the basis of the obtained equations, for the RSA = 50%, the essential oils concentrations providing 50% inhibition of free radical activity (IC_{50}) were calculated. Because IC_{50} parameter is inversely correlated with the radical scavenging activity, the lower values represents the stronger antioxidant properties of the essential oils.

Fungicidal activity

The fungicidal activity of *Rosmarinus officinalis* essential oils was determined in Łukasiewicz Research Network – Institute of Industrial Organic Chemistry (Warsaw, Poland). In the experiments 8 various species of fungi were applied, including 7 pathogens of cultivated plants*: Alternaria alternata, Botrytis cinerea, Fusarium culmorum, Phythophtora cactorum, Rhizoctonia solani*, *Phythophtora infestans, Sclerotinia sclerotiorum* and pathogen *Ascosphaera apis* causing infectious disease of honeybee brood - calcareous mycosis. All fungi strains was from the Institute collection. The agar growth medium poison technique was applied for *in vitro* assays of the essential oils fungicidal activity. The samples of essential oils were dissolved in acetone and the stock solutions (C = 3%) were prepared. Next, the proper amounts of these solutions were mixed with molten PDA (Potato Dextrose Agar, DIFCO) growth medium after autoclaving in order to obtain concentrations of 5.0 and 2.0 mg/mL. The obtained homogeneous mixtures were introduced to the sterilized Petri dishes. After solidification of PDA, at the center of each plate the mycelial circle disk of fungi taken from the edge of a 8-day-old culture was inoculated. Next, after a few days incubation at 25 °C, depending on the mycelium growth in a control sample (PDA with acetone), the inhibitory effects of the

essential oils on linear growth were determined by the fungal colony diameter measurement. The fungicidal activity was expressed as a percentage inhibition of mycelium compared to the control sample. Percentage of the mycelia inhibition was calculated as averaged value from three replications of each measurement.

Statistical analysis

The statistical analysis was performed using Microsoft Excel 2016 (Microsoft) and TIBCO Statistica 13.3 (TIBCO Software Inc.). The data are presented as the means ± standard deviations (s) from the three replications. In each case 3 independent experimental samples were analyzed and it was assumed that results have a normal probability distribution. To analyze the obtained yields and antioxidant parameters of essential oils, the analysis of variance (ANOVA) was performed. The differences among mean values of experimental data were evaluated by using of Tukey's HSD post-hoc test at a 5% significance level.

RESULTS AND DISCUSSION

The studies showed that replacing of water by 5% citric acid solution (pH = 2.0) resulted in 21% enhancing of essential oil yield, from 2.73±0.06 to 3.30±0.10% (v/w). Addition of the same amount of trisodium citrate to water phase resulted in slight alkalization (pH = 8.0) and increased essential oil yield to 3.07±0.06% (v/w), so the observed enhancing effect was smaller (12%). Statistical analysis showed that the obtained results differ at $P < 0.05$. The influence of these modifications of hydrodistillation process on yields of *Rosmarinus officinalis* essential oils is presented in Figure 1.

GC-MS analysis enabled identification and quantification of 19 compounds present in *Rosmarinus officinalis* essential oils. The main components were: 1,8-cineole (about 31%), α-pinene (17–20%) and camphor (17–19%). Composition of essential oils obtained in conventional and modified conditions varied only in changing of relative contents of particular compounds. It can be noticed

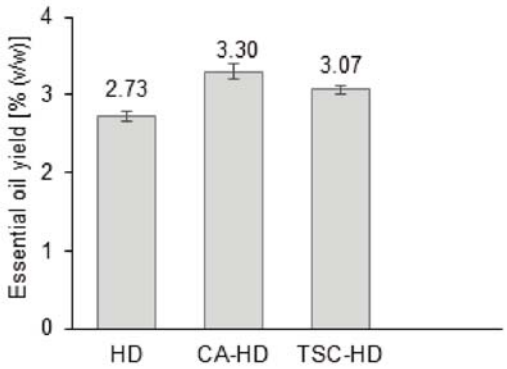

Figure 1. Yields of *Rosmarinus officinalis* essential oils obtained by conventional hydrodistillation (HD) and hydrodistillation modified with 5% citric acid (CA-HD) and 5% trisodium citrate (TSC-HD)

that replacing of water with 5% citric acid reduced content of α-pinene from 20.06 to 18.86% and increased the content of camphor from 17.30 to 19.07%. Similar tendencies were obtained for hydrodistillation with 5% trisodium citrate: lowering of α-pinene content from 20.06 to 17.03% and increasing the content of camphor from 17.30 to 18.80%. Moreover, isolation process with 5% trisodium citrate resulted in almost doubling the content of β-caryophyllene (4.72%) in comparison to conventional process (2.49%). For other compounds, including the main component 1,8-cineole, the differences in relative contents of particular compounds did not exceed 1%. The retention times, the linear retention indices (determined experimentally and compared with the literature values) and the relative contents of particular identified compounds are summarized in Table 1.

The antioxidant activity of the *Rosmarinus officinalis* essential oils expressed by the IC_{50} parameter varied in a range of 14.65–18.78 mg/mL. The results indicate that applied modifications of hydrodistillation process positively influenced antioxidant activity. The essential oil gained in hydrodistillation modified by 5% citric acid showed the highest activity, representing by the lowest IC_{50} concentration (14.65±0.15 mg/mL).

Table 1. Composition of *Rosmarinus officinalis* essential oils obtained by conventional hydrodistillation (HD) and hydrodistillation modified with 5% citric acid (CA-HD) and 5% trisodium citrate (TSC-HD)

No.	Compound	RT [min]	Retention index		Relative content ± s [%]		
			LRI_{exp}	LRI_{lit}	HD	CA-HD	TSC-HD
1	Tricyclene	4.86	920	923	0.21±0.01	0.20±0.01	0.14±0.04
2	α-Pinene	5.17	933	936	20.06±0.34	18.68±0.30	17.03±0.13
3	Camphene	5.49	946	950	7.03±0.14	7.05±0.09	6.09±0.15
4	Thuja-2,4(10)-diene	5.64	952	956	0.22±0.08	0.20±0.07	0.12±0.01
5	β-Pinene	6.18	974	978	0.88±0.04	0.87±0.01	1.22±0.02
6	β-Myrcene	6.62	992	989	0.83±0.03	0.77±0.05	0.77±0.04
7	α-Phellandrene	6.95	1005	1004	2.58±0.11	2.35±0.14	2.23±0.16
8	α-Terpinene	7.30	1017	1017	1.43±0.02	1.37±0.05	1.18±0.03
9	1,8-Cineole	7.76	1033	1032	30.89±0.34	31.03±27	31.61±0.37
10	γ-Terpinene	8.59	1058	1060	1.01±0.02	1.04±0.04	1.14±0.06
11	Terpinolene	9.48	1089	1087	0.47±0.10	0.50±0.01	0.57±0.03
12	Camphor	11.33	1148	1143	17.30±0.33	19.07±0.16	18.80±0.24
13	Borneol	12.04	1169	1166	4.68±0.17	5.20±0.15	5.17±0.15
14	Terpinen-4-ol	12.44	1180	1177	0.96±0.05	1.04±0.06	1.03±0.02
15	α-Terpineol	12.93	1195	1190	1.70±0.09	1.90±0.09	2.01±0.14
16	Verbenone	13.52	1214	1206	5.23±0.11	4.92±0.14	4.79±0.20
17	Isobornyl acetate	15.89	1285	1286	0.64±0.02	0.75±0.02	0.70±0.02
18	β-Caryophyllene	20.07	1418	1420	2.49±0.07	2.11±0.07	4.72±0.13
19	α-Humulene	21.13	1451	1453	0.41±0.01	0.32±0.01	0.70±0.02

RT – retention time; LRI_{exp} – linear retention index determined on the HP-5MS capillary column; LRI_{lit} – linear retention index from literature[31]; Relative content – percentage of the peak area in Total Ion Chromatogram; s – standard deviation (n=3).

Addition of trisodium citrate also slightly increased activity of essential oil (IC_{50} = 15.43±0.12 mg/mL) in comparison to conventional hydrodistillation process (IC_{50} = 18.78±0.14 mg/mL). The differences in the values are significant at $P < 0.05$. The improvement of antioxidant activity in the case of essential oils obtained in processes modified by citric acid and trisodium citrate can be correlated with the observed slightly increased percentage of some oxygenated components like camphor, 1,8-cineole or borneol, which are known for their antioxidant potential. In Table 2, the antioxidant activities expressed as IC_{50} parameters determined by DPPH radical scavenging method are collected.

Table 2. Comparison of antioxidant activity of *Rosmarinus officinalis* essential oils obtained by conventional and chemically modified hydrodistillation processes

Isolation process	IC_{50} ± s [mg/mL]
Hydrodistillation (HD)	18.78±0.14 [a]
Hydrodistillation modified with 5% citric acid (CA-HD)	14.65±0.15 [b]
Hydrodistillation modified with 5% trisodium citrate (TSC-HD)	15.43±0.12 [c]

s – standard deviation (n=3); different letters in column indicate significant differences among the results ($P < 0.05$).

Comparing of results allowed to indicate, that hydrodistillation modified by citric acid resulted in the highest yield and the highest antioxidant activity of obtained essential oil. The sample of this essential oil was selected for fungicidal activity tests, as well as sample obtained in conventional hydrodistillation to evaluate the effect of this modification. Fungicidal activity against different pathogenic fungi strains was tested for two concentrations of rosemary essential oils: 5 mg/mL and 2 mg/mL (for *A. apis* only 5 mg/mL). Both tested essential oils showed a very good fungistatic effect (100%) against the strains of *R. solani*, *S. sclerotiorum* and *A. apis* at a concentration of 5 mg/mL. Additionally, essential oil obtained in hydrodistillation modified by citric acid fought *P. cactorum* in 100% and inhibited the growth of *A. alternata* with 80% effectiveness. For the remaining strains they showed a moderate activity (50–75%) and against *F. culmorum* their activity was weak (below 50%). Comparison of results shown in Table 3 indicate that except *F. culmorum,* essential oil obtained by hydrodistillation modified with citric acid (CA-HD) is significantly more active against tested fungi strains than obtained in conventional process (HD). It inhibited mycelia growth of most of tested fungi strains more effectively and was more active in lower concentration. Detailed explanation of this fact is not easy, because a lot of terpenes and terpenoids which are present in tested essential oil possess some antifungal properties, including the main component 1,8-cineole[33]. But it should be pointed out that fungicidal activity can be attributed to both major and minor components and some synergistic or antagonistic effects may also play a role.

Based on the results it can be concluded that applied modifications of hydrodistillation process by addition of citric acid (CA) and trisodium citrate (TSC) had an effect on yield, composition and biological activity of essential oils from *Rosmarinus officinalis*.

Table 3. Effect of *Rosmarinus officinalis* essential oils obtained by hydrodistillation (HD) and hydrodistillation modified with 5% citric acid (CA-HD) on the mycelia growth inhibition of various fungi strains

Fungi strains	Mycelia growth inhibition* [%]			
	HD		CA-HD	
	C=2 [mg/mL]	C=5 [mg/mL]	C=2 [mg/mL]	C=5 [mg/mL]
Alternaria alternata	38.0	50.0	42.0	80.0
Botrytis cinerea	31.3	54.3	50.0	71.7
Fusarium culmorum	34.8	41.9	30.4	39.5
Phythophtora cactorum	40.0	54.8	42.9	100
Rhizoctonia solani	71.7	100	100	100
Phythophtora infestans	27.2	72.7	57.4	75.0
Sclerotinia sclerotiorum	24.0	100	100	100
Ascosphaera apis	–	100	–	100

*evaluation of activity: 0–20% – no effect; 20.1–50% – weak activity; 50.1–90% – moderate activity; 90.1–100% – good activity. C – concentration of essential oil in PDA growth medium.

CONCLUSIONS

The results indicate that modification of hydrodistillation process by addition of compounds which modify pH of water phase can increase the efficiency of the isolation process of *Rosmarinus officinalis* essential oils. Replacing of water by 5% trisodium citrate or 5% citric acid improved the yield by 12 and 21%, respectively. Applied chemical modifications of hydrodistillation process did not drastically changed the composition of obtained essential oils, observed differences in relative contents of particular compounds not exceeded 3% and in most cases were below 1%. Nevertheless, these variations in composition, included slightly reduced content of α-pinene and increased content of some oxygenated compounds like camphor, 1,8-cineole or borneol, positively influenced the antioxidant activity of essential oils obtained in modified conditions. The best results were observed for essential oil obtained by hydrodistillation modified with 5% citric acid (CA-HD). Furthermore, its fungicidal activity tested against 8 different fungi strains was also in most cases significantly higher in comparison to activity of essential oil obtained in traditional process (HD). Summarizing, applied modification of hydrodistillation process can be useful for enhancing yield, antioxidant and antifungal activity of the rosemary essential oils and can bring some benefits for potential use in food industry and agriculture for protection against fungal diseases.

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