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Effect of enzyme-assisted extraction on yield, composition, and antimicrobial activity of essential oils from *Rosmarinus officinalis* L. grown in Lam Dong Province, Viet Nam

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ABSTRACT

In the present study, viscozyme L pre-treatment for aerial parts of Rosmarinus officinalis L. (rosemary), grown in Lam Dong province, was performed to evaluate the effective extraction of essential oils by hydro-distillation. Enzymatic pre-treatment with 1 g viscozyme L enzyme mixed with 5 mL sodium chloride 15% at 50°C for 1 hour, followed by hydro-distillation, was seen to slightly increase the yield of rosemary essential oils from 0.96% to 1.08%. GC/MS analysis showed the presence of 32 compounds in rosemary essential oils, of which the major components were α-pinene (29.71 - 32.17%) and cineol (17.55 - 18.74%) in both the control and the enzymatically treated samples. The results also revealed that rosemary essential oils obtained from both the control and the enzymatically treated samples exhibited moderate antimicrobial activity against Escherichia coli, Staphylococcus aureus, and Candida albicans in all tested concentrations.

1. INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.), an important commercial plant belonging to the family Lamiaceae, is mostly found in the southern parts of European countries and North Africa (Turrill, 1920). Additionally, it has been cultivated worldwide for medicinal, culinary, cosmetic, and ornamental purposes (Heywood, Skoula, 1999). The essential oils extracted from rosemary are widely used in food, flavoring and fragrances, cosmetics, and pharmaceutical industries (Bousbia et al., 2009; Abu-Al-Basal, 2012). There are various techniques that are used to extract the essential oils of rosemary, e.g., hydro-distillation, steam distillation, microwave hydro-diffusion and gravity, supercritical carbon dioxide, and organic solvent extraction, in which hydro-distillation is the most commonly used (Carvalho et al., 2005; Bousbia et al., 2009). Utilizing hydro-distillation, rosemary was found to contain 0.35–1.9% of volatile oil (Özcan, Chalchat, 2008; Bousbia et al., 2009; Ngan et al., 2019). The main reported constituents of rosemary oil are camphor (5.0–21%), 1,8-cineole (15–55%), α-pinene (9.0–26%), borneol (1.5–5.0%), camphene (2.5–12%), β-pinene (2.0–9.0%), and limonene (1.5–5.0%) (Begum et al., 2013; Hosni et al., 2013). Studies show that rosemary oil from Lam Dong province in Vietnam contains α-pinene (35.54 %), eucalyptol (20.902%), camphene (4.384%), bicyclo[3.1.1]hept-3-en-2-one (7.794%), caryophyllene (1.225%), endo-borneol (4.147%), and bornyl acetate (4.065%) (Ngan et al., 2019).

Rosemary essential oils exhibit various biological activities such as antibacterial, anti-inflammatory, antifungal, anticancer, antiviral properties, and insecticidal activities (Dörrie et al., 2001; Bousbia et al., 2009; Okoh et al., 2010; Hosni et al., 2013; Jordán et al., 2013). Consequently, the market demand for rosemary essential oils and extracts has increased remarkably. This has prompted subsequent research efforts investigating optimal extraction procedures to improve the yield of essential oils without alteration of the qualitative features of the end product. Among those, the enzyme-assisted extraction technique is widely used to improve the extraction efficiency of essential oils from plants. Enzyme-assisted extraction has been successfully used to enhance the recovery of essential oils from garlic, celery seeds, cumin (Sowbhagya et al., 2009; 2010; 2011), thyme and rosemary (Hosni et al., 2013), lavender (Calinescu et al., 2014), and ginger (dos Santos Reis et al., 2020). According to Hosni et al., (2013), pre-treatment of rosemary with cellulase, hemicellulase and the combination of both enzymes has been shown to improve yields of essential oils from Tunisia. The results demonstrated that enzymatic pre-treatment of rosemary dried leaves raised essential oils yields by 50% depending on the enzyme employed (Hosni et al., 2013). At the same time, the antibacterial activity of rosemary essential oils was also evaluated, and the result showed that enzymatic pre-treatment of rosemary dried leaves could be useful for the antimicrobial activity of this essential oil (Hosni et al., 2013). Although the enzyme-assisted extraction procedure has been proven to increase the yield of rosemary essential oils from Tunisia, there are no reports on its application in rosemary from Lam Dong province, Vietnam. Moreover, Saei-Dehkordi et al. (2010) showed that the composition of essential oils of plant species also varied with geography. Moreover, until now, there are also no reports on using viscozyme L to treat rosemary samples before the extraction of essential oils of this species. While, previous reports on the application of viscozyme L to some plants for the extraction of volatile oil such as garlic, basil, and celery seeds show an increase in the yield of oil in the range of 6.1% to 50% (Sowbhagya et al., 2009; Sowbhagya et al., 2010; Manasa et al., 2012; Morsy & Hammad, 2021). Therefore, our study aims to examine enzyme-assisted extraction to improve the extraction efficiency of essential oils from rosemary in Vietnam, and the antimicrobial activities of its essential oils.

2. MATERIALS AND METHODS

2.1. Materials

The aerial parts of rosemary (*Rosmarinus officinalis* L.) were collected in March 2021 from a farm in Lam Ha district, Lam Dong province, Vietnam (Fig. 1). Viscozyme L enzyme was procured from Novozymes, Bagsvaerd, Denmark. Viscozyme L includes arabanase, cellulase, hemicellulase, beta-glucanase, and xylanase. The rosemary oils obtained from enzyme pre-treatment and control experiments were analyzed by GC–MS. Gram-positive bacteria strains namely *Staphylococcus aureus* (ATCC 6538); Gram-negative bacteria strains namely *Escherichia coli* (ATCC 8739); Fungal strains namely *Candida albicans* (pathogenic yeast), were selected to test the antimicrobial capacity of rosemary essential oils. Two bacteria strains were obtained from the Institute of Drug Quality Control in Ho Chi Minh City. The fungal strain was obtained from the General Hospital of Lam Dong province, Vietnam.

Figure 1. The aerial parts of rosemary (*Rosmarinus officinalis* L.)

2.2. Enzyme pre-treatment and extraction of essential oils

One kg of rosemary fresh aerial parts was thoroughly mixed with 5 mL sodium chloride 15% solution containing 1 g viscozyme L enzyme.

Thereafter, the samples were incubated at 50°C for 1 hour, and enzyme-treated samples were subjected to hydro-distillation. In a control experiment, rosemary fresh aerial parts (1 kg) without enzyme pre-treatment were also extracted using the hydro-distillation method. Essential oils from the control and enzymatically treated samples were extracted by hydro-distillation for 3 hours. The essential oils were recovered, dried over Na_2SO_4, weighed, stored in a brown bottle, and kept refrigerated at 4°C until being analyzed by GC-MS. The extractions were performed in triplicate. The yield of oil was expressed as volume/ fresh material percentage (v/w %).

2.3. Chemical composition of essential oils

The qualitative analysis of the rosemary essential oils obtained from enzyme pre-treatment and control rosemary samples were performed by gas chromatography-mass spectrometry (GC-MS) method on an Agilent Technologies HP 6890N Plus Chromatograph connected to a mass spectrometer HP 5973 MSD. The GC-MS system was operated using the following conditions: Column: Agilent DB-5MS: length: 30 m, film thickness: 0.25 µm, diameter: 0.25 mm; MS transfer line temperature: 220°C; Ion source temperature: 200°C; Injector temperature: 220°C; Temperature program: from 70°C (15 min) up to 250°C with increments of 10°C/min; Flow: 1.2 mL/min; Mass range (m/z): 50−450. The essential oils compounds were identified based on the retention times (RT) and the MS with those values found in the literature of Adams (2007) (Adams, 2007), and by the computerized matching of the acquired mass spectra with those stored in the NIST08 and WILEY 275 mass spectral libraries of the GC-MS data system.

2.4. Antimicrobial activity

The antimicrobial effect of the obtained rosemary essential oils was evaluated by the agar well diffusion method (Devillers et al., 1989; Valgas et al., 2007). The growth media was nutrient agar (NA) for tested microorganisms. The microorganisms were inoculated by the spread plate method on base plates containing 7 mL nutrient agar in sterile 90 mm Petri dishes (containing approximately 10^6–10^8 CFU/mL of the microorganisms). In the center of each dish, wells of approximately 6 mm diameter were created by using a cork-borer, and 40 µL of essential oils solution, dimethyl sulfoxide (DMSO), and chloramphenicol were pipetted in the middle of each disk, in which DMSO and chloramphenicol (chloramphenicol 250 mg of Vidipha Central Pharmaceutical Joint Stock Company, Vietnam) were used as a negative control and positive control, respectively. Then, plates were pre-incubated at 4°C for 30 minutes for the complete diffusion of the oil and incubated at 30°C for 24 h for microorganism growth. In the present study, three dilutions of essential oils (comprising 50%, 75%, and 100%) were used to evaluate antimicrobial activity. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zones (mm) around the wells.

2.5. Data analysis

The experiments to evaluate the yield and antimicrobial activity of essential oils were carried out in triplicates, and the results were presented as mean values ± SD. One-way analysis of variance (ANOVA) and the Ducan test were conducted to reveal the mean difference (being significant when $p < 0.05$). All statistical analyses were performed by using SPSS 22.0 software package (Chicago, Illinois, USA).

3. RESULTS AND DISCUSSION

3.1. Effect of enzymes on yield and chemical composition of rosemary leaf essential oils

Results of the yield and components analysis of rosemary oil obtained by enzyme pre-treatment and control experiments are detailed in Table 1 and Table 2.

Table 1. Effect of enzyme pre-treatment on the yield of rosemary essential oils

Treatment	Essential oils (v/w) (%)
Control	0.96% ± 0.05
Viscozyme L	1.08% ± 0.02

The percentage yield of essential oils obtained by only hydro-distillation (without enzyme pre-treatment) was 0.96%, and this result is in accordance with that reported by Özcan, Chalchat, 2008, Bousbia et al., 2009 and Ngan et al., 2019. In contrast, the yield of essential oils obtained from the enzyme-treated samples was 1.08%. The yield of essential oils from plants that can be exploited varies widely, and the broad range is 0.05–18.0%. (Sankarikutty & Narayanan, 2003). Therefore, enzymatic pre-treatment of rosemary resulted in an increase of up to 0,12% in the yield of volatile rosemary oil, which is extremely economically significant. This increase could be attributed to viscozyme L (including arabanase, cellulase, hemicellulase, beta-glucanase, and xylanase) that

attacks and depolymerizes the plant cell wall polysaccharides, thus facilitating the release of essential oils (Gil-Chávez et al., 2013). Using the enzyme pre-treatment technique partially affects the oil yield after 1 hour of enzyme incubation, as indicated in this study. This result was different from that reported by Hosni et al., 2013, which showed that enzyme pre-treatments are not sufficiently efficient for better recovery of essential oils from *R. officinalis.*

The volatile constituents of the *R. officinalis* essential oils are shown in Table 2 (Supplement 1 2). Thirty-two compounds were identified in the essential oils of rosemary, present in both control and enzymatically treated samples. Results in Table 2 indicated that α-pinene (29.71 - 32.17%), cineol (17.55 - 18.74%), camphene (4.15 - 4.43%), D-limonene (3.81 - 4.13%), linalool (2.99 - 3.19%), endo-Borneol (3.67 - 3.85%), levoverbenone (3.37 - 3.77%), geraniol (4.06 - 5.22%), and borneol acetate (3.19 - 3.53%) were major compounds of the essential oils of *R. officinalis* in Lam Ha district, Lam Dong Province, Vietnam. According to Anh et al., 2019 the main compounds of the untreated essential oils of rosemary grown in the same area include α-pinene (22.25%), levoverbenole (12.12%), cineole (14.49%), borneol acetate (4.93%), linalool (3.045%), and geraniol (6.36%), which are in partial agreement with our results. In contrast, the dominant compounds of the rosemary essential oils in Lam Dong province are different from those reported from previous studies in India and Tunisia, where the main component of rosemary essential oils is camphor (21%) and 1,8-cineol (55%), respectively (Begum et al., 2013; Hosni et al., 2013). However, the constituents of essential oils within the same plant species are affected by local geographic conditions where that species distributes (Saei- Dehkordi et al., 2010). Therefore, the difference in dominant compounds of the rosemary essential oils between the present study and previous studies is not contradictory.

The comparison of the rosemary essential oils compositions between the control and the enzymatically treated samples showed that although there were no qualitative differences, quantitative differences were noted. In this present study, regarding the individual compounds, a remarkable decrease in the amount of the major compounds α-pinene and borneol acetate was observed in the enzyme-treated samples.

Table 2. Composition of the rosemary essential oils obtained without enzymatic pre-treatment (control) and with enzymatic pre-treatment

No	Compound	RT	Percentage (%)	
			Control	Viscozyme L treated
1	2-Thujene	3.08	0.43	0.40
2	*α-pinene*	3.19	32.17	29.71
3	Camphene	3.38	4.43	4.15
4	*β-pinene*	3.68	2.76	2.78
5	*β*-myrcene	3.74	1.98	1.84
6	*β*-terpinene	4.1	0.89	0.78
7	*o*-cymene	4.19	0.70	1.28
8	D-limonene	4.26	4.13	3.82
9	Cineol	4.31	17.55	18.74
10	*δ*-terpinene	4.59	1.85	1.45
11	Terpinolen	4.93	1.52	1.15
12	Linalool	5.05	2.99	3.19
13	Filifolone	5.12	0.33	0.38
14	Cis-verbenol	5.63	0.33	0.34
15	Trans-verbenol	5.68	0.50	0.57
16	Alcanfor	5.73	2.50	2.65
17	Pinocarvone	5.9	0.36	0.37
18	*α*-phellandren-8-ol	5.98	0.41	0.41
19	Endo-borneol	6.02	3.67	3.85
20	3-pinanone, cis	6.08	0.07	0.78
21	Terpinen-4-ol	6.11	1.24	1.17
22	*α*-terpincol	6.28	2.24	2.09
23	Exo-borneol	6.38	0.78	0.71
24	Levoverbenone	6.45	3.77	3.37
25	Citronellol	6.59	0.39	0.80
26	Geraniol	6.88	5.22	4.06
27	Borneol acetate	7.35	3.53	3.19
28	Genanyl acetate	8.35	0.92	0.83
29	Methyl eugenol	8.59	0.49	0.37
30	Caryophyllene	8.96	1.20	1.02
31	Humulene	9.34	0.21	0.19
32	Caryophyllene oxide	10.67	0.44	0.56

Note: RT (Retention times)

Moreover, the four other major compounds including camphene, D-limonene, levoverbenone, and geraniol did not show a remarkably decreased in the enzyme-treated samples. In contrast, the amounts of the remaining major compounds including cineol, linalool, and endo-borneol increased after the enzymatic pre-treatment step. These results agree with those reported in previous studies, where some main components of rosemary (*R. officinalis*) essential oils including 1,8-cineol, α-pinene were decreased in enzymatic treatment samples (Hosni et al., 2013). Various reports showed that enzyme types, enzyme concentration,

and extraction time are the most important independent parameters that can influence the yield of essential oils (Gai et al., 2013), whereas a few data reported their effect on the number of individual components of essential oils (Puri et al., 2012).

3.2. Antimicrobial activity of rosemary aerial parts essential oils

The antimicrobial activity of the rosemary essential oils from the control and enzymatically treated samples were evaluated by determining the inhibition zone diameter on gram-positive and gram-negative bacteria, as well as fungal strains after 24 hours of culture. The anti-microorganism effectiveness of rosemary essential oils on the test microorganisms is shown in Table 3. The essential oils from both control and enzymatically treated samples of *R. officinalis* showed growth inhibition against both two bacteria strains and fungal strains in a dose-dependent manner. The comparison of the inhibition zone diameters in Table 3 between rosemary essential oils of control and enzymatically treated samples show that there was no significant difference. This can be explained based on the quality of rosemary essential oils derived from both untreated and enzymatically treated samples, and the result in Table 2 highlighted that there are no qualitative differences between them.

Table 3. Antimicrobial activity of rosemary essential oils

	Antimicrobial activity (mm)							
			Concentration (% of essential oils and DMSO)					
	Chloramphenicol	DMSO	EC			EE		
			100%	75%	50%	100%	75%	50%
E. coli	30.3 ± 0.05^{a}	-	7.0 ± 0.17^{ab}	9.0 ± 0.20^{b}	7.7 ± 0.06^{ab}	6.7 ± 0.06^{b}	7.3 ± 0.06^{bc}	8.3 ± 0.06^{c}
S. aureus	28.6 ± 0.15^{a}	-	4.7 ± 0.06^{ab}	5.8 ± 0.06^{b}	6.0 ± 0.10^{b}	5.7 ± 0.15^{b}	5.0 ± 0.10^{b}	4.7 ± 0.06^{b}
C. albicans	32.6 ± 0.05^{b}	-	5.3 ± 0.06^{a}	5.0 ± 0.17^{a}	5.0 ± 0.10^{a}	3.3 ± 0.06^{a}	4.3 ± 0.15^{ab}	2.7 ± 0.06^{a}

Notes: "-": No antimicrobial activity; Data within the same row followed by different superscripts are significantly different at $p < 0.05$. "EC": Essential oils from control samples; "EE": Essential oils from enzymatically treated samples.

Generally, the rosemary essential oils exerted a stronger antimicrobial effect against gram-negative bacteria (*E. coli*) than gram-positive bacteria (*S. aureus*) and fungus (*C. albicans*). However, the comparison between the inhibition zone diameters of the three microbial strains in Table 3 and the positive control (Chloramphenicol) showed that the antimicrobial activity of rosemary essential oils was much weaker than the positive control. According to de Billerbecrk (2007), the susceptibility of bacteria against antibiotics based on the diameters of inhibitory zones was ranked as follows: resistant: D < 6 mm; intermediate: 13 mm > D > 6 mm; and sensitive: D > 13 mm. For this, our results revealed that *S. aureus* and *C. albicans* were resistant to the essential oils from samples of *R. officinalis* in Lam Dong Province (with inhibition zone diameter < 6 mm in all dilutions of EC and EE). In contrast, *E. coli* was intermediate sensitive to these essential oils (with inhibition zone diameter 13mm> D > 6 mm in all dilutions of EC and EE). Previous reports showed that individual components and the combination of components within essential oils affected its antimicrobial activity. Diao et al., 2014 reported that major components of essential oils including monoterpene or sesquiterpene hydrocarbons and their oxygenated derivatives have potential antimicrobial activities. The activity against bacteria and fungus of the rosemary essential oils is related to α-pinene (29.71 - 32.17%) and cineol (17.55 -18.74%), which are the two major components of essential oils. According to de Sousa Eduardo et al., 2018, α-pinene is known to have efficient antimicrobial properties. However, previous reports showed that a combination of 1,8-cineole and α-pinene and cineol-rich oil of rosemary had reduced antimicrobial activity against *S. aureus*, *E. coli*, and *C. albicans* (Hosni et al., 2013; Jordán et al., 2013). These reports are in good agreement with our results.

4. CONCLUSIONS

This research confirmed that enzyme-assisted extraction using viscozyme L enzyme increased the yields of the essential oils extracted from rosemary aerial parts, grown in Lam Ha district, Lam Dong province, Vietnam. The yields of the extracted essential oils increased from 0.96% for the control to 1.08% for the enzymatically treated samples. However, enzyme pre-treatments did not significantly improve the recovery of essential oils from *R. officinalis* in Lam Dong province.

Furthermore, although the enzymatic treatment caused an in- crease in the amount of the extracted essential oils, the quality did not deteriorate. The main compounds' relative quantity comprises α-pinene (29.71 - 32.17%), and cineol (17.55 - 18.74%). In addition, this study showed that the rosemary essential oils obtained by both controls and enzymatically treated samples exhibited antimicrobial activity with tested bacteria and fungus strains.

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