Scientia Pharmaceutica (Sci. Pharm.) 75, 35-46 (2007)

Antioxidant and Antimicrobial Activities of *Hyptis suaveolens* Essential Oil

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Abstract

The essential oil of *Hyptis suaveolens* obtained by steam distillation was examined for its antioxidant and antimicrobial activities. The antioxidant activity was determined by means of the DPPH radical scavenging test and ABTS free radical decolorization assay. Results from both methods indicate that the antioxidant activity of *H. suaveolens* oil is time and concentration dependent. The antioxidant potential of *H. suaveolens* oil determined by the DPPH method expressed as IC_{50} was 3.72 mg/ml whereas the TEAC value determined by the ABTS assay was 65.02 µM/mg. The antimicrobial activity of the essential oil was assessed by the dilution and well diffusion methods. The results show that the antifungal potential of *H. suaveolens* oil is more pronounced than its antibacterial properties. Its fungal growth inhibitory activity was dose dependent with a MID value of 1:640. The 20% ethanolic solution of *H. suaveolens* oil had antifungal power similar to 6% boric acid, 2% benzoic acid, or 5% salicylic acid but higher than 4% phenol. The activity decreased when the oil was stored at high temperature (> 40°C). The results of GC-MS analysis indicated the changes in oil composition which led to the decrease in antifungal activity.

Keywords

Essential oil, Antioxidant, ABTS, DPPH, Antimicrobial, *Hyptis suaveolens*

Introduction

It is well-known that free radicals and other reactive oxygen species formed in living cells play an important role in the origin of life and biological evolution [1, 2, 3]. However, it has been found that those reactive species also play a cardinal role in oxidative damage to cellular constituents which leads to cell injury and death. This has been associated with pathogenesis of various chronic diseases, e.g. carcinomas, coronary heart disease, and many other health problems related to advancing age [4, 5, 6]. Thus, to increase the antioxidant intake in the human diet is one important way to minimize such oxidative damage. A great number of plant species in different regions of the world have been screened for their antioxidant activity [7, 8, 9]. However, most of them are not edible plants. *Hyptis suaveolens* (Labiatae) is a medium aromatic annual shrub distributed in the tropical and subtropical regions. It is usually applied in Asian food recipes as an appetizer because of its flavored essential oil. It was reported to be used for traditional medicine as an anticancer agent [10]. The ethanolic extract from its leaves exhibited healing properties with a supportive role of antioxidant enzymes [11].

Studies are on going throughout the world in search for compounds that are biologically active with a low profile of side effects. Essential oils from plants usually show antimicrobial activity against a wide range of microorganisms including antibiotic resistant bacteria and fungi [12]. They can affect both gram-positive and gram-negative bacteria in addition to yeasts and filamentous fungi [13, 14, 15]. Previous authors reported on the antibacterial activities of the essential oil from the leaves of *H. suaveolens* [16, 17]. However, there is no report on comparison of antimicrobial power of this oil against bacteria and fungi.

As mentioned above, this plant is used as an edible aromatic flavoring for food. The knowledge of the biological potential of its essential oil will provide valuable data concerning the plant, not only for its use as an appetizer but also as an agent with strong biological activity. Therefore, the aim of this study is to determine the antioxidant, antibacterial, and antifungal activities of

the essential oil of *H. suaveolens* grown in the northern part of Thailand. For assessment of antioxidant properties, two complementary methods for assaying free radical scavenging activity; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging test and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical decolorization assay were used. For the antimicrobial study, certain bacterial and fungal pathogens from human and animal sources were used. The stability of the oil with respect to antimicrobial action and chemical composition was also examined in order to suggest suitable conditions of storage for prolonged use of the oil.

Experimental

Plant material

The aerial part of *H. suaveolens* was harvested from the northern region of Thailand. The voucher specimen was deposited in the Herbarium of Chiang Mai University, Thailand. The fresh plant sample was submitted to steam distillation (3 h) using a Clevenger apparatus to obtain the essential oil.

Chemicals

Potassium persulfate, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethanol from Fluka Chemicals (Buchs, Switzerland). Mueller Hinton agar from Oxoid Ltd (Hampshire, England) and Sabouraud dextrose agar from Bacto-Difco Lab Co., Ltd (Detroit, MI, USA). All other chemicals were of the finest grade available.

Antioxidant activity studies

In these experiments, two complementary methods of free radical scavenging activity; DPPH radical-scavenging test and ABTS free radical decolorization assay were used.

The scavenging effect on DPPH radical was determined by modifying the previous methods [18, 19]. The essential oil of *H. suaveolens* was mixed with ethanol to prepare an ethanolic test solution of 1 mg/ml. DPPH was dissolved in ethanol and mixed with the ethanolic test solution. The solution was adjusted to a final DPPH concentration of 100 µM. The mixture was shaken vigorously and left to stand for 5-60 min in the dark at room temperature. The amount of DPPH remaining after each time period was determined spectrophotometrically at 540 nm using a microtiter plate reader. To study the effect of concentration, ethanolic test solutions of different oil concentrations were used. After vigorous shaking, the mixtures were left to stand at 30 min. All measurements were performed in triplicate. The radical scavenging activity was calculated as % inhibition from the following equation; % inhibition = $\{(OD_{blank} – OD_{sample}) / OD_{blank}\} \times 100$.

In the ABTS free radical decolorization assay, the pre-formed radical monocation of ABTS is generated [20] by oxidation of ABTS solution (7mM) with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 12 h in the dark at room temperature. The ABTS solution was diluted with ethanol to the absorbance of 0.7±0.2 at 750 nm. Series of essential oil solutions of different concentrations were prepared by diluting with ethanol. An aliquot of 20 µl of the ethanolic dilution of each solution was added to 180 µl of ABTS radical cation solution. The absorbance, monitored for 60 min, was measured spectrophotometrically at 750 nm using a microtiter plate reader. All measurements were performed in triplicate. The antioxidant activity of each sample was expressed as Trolox equivalent antioxidant capacity (TEAC), as obtained by comparing the absorbance change at 750 nm in a reaction mixture containing an oil sample with that containing Trolox. This index is defined as the micromolar concentration per mg of the oil.

Microorganisms

The microorganisms used in this study were clinical strains from human and animal sources. *Trichophyton mentagrophytes,* representative of fungal

dermatophytes, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, representative of gram-positive and gram-negative bacteria respectively, were clinical isolates from patients at Chiang Mai University Hospital, Thailand. The other gram-negative bacteria, *Escherichia coli*, *Pasteurella multocida* and the gram-positive strains, *Erysipelothrix rhusiopathiac*, *Actinomyces pyogenes* and *Streptococcus suis* were isolated from the gastrointestinal tract of swine. The isolated bacteria and dermatophytes were cultured overnight in Muller Hinton agar and Sabouraud Dextrose agar respectively to reach the stationary phase of growth.

Antimicrobial activity study

The antimicrobial activity of the essential oil was determined by the dilution and well diffusion methods. In the dilution technique, two-fold dilutions of the oil in DMSO aqueous solution were serially prepared. For antibacterial testing, an aliquot of 2 ml of each dilution was mixed with prewarmed Muller Hinton agar. The mixture was then poured into a sterile petri dish. After the agar was congealed, a loop of each test bacterial suspension, concentration controlled by Mc Farland No 0.5, was inoculated. The plates were incubated in a reversed position at 30ºC. For antifungal study, the oil dilution was mixed with prewarmed Sabouraud dextrose agar and then an agar slant was prepared from this mixture. A loop of *T. mentagrophytes* at Mc Farland No 1.0 suspension was inoculated and incubated at 25ºC. The antimicrobial activity was expressed as the maximum inhibitory dilution (MID), defined as the maximum dilution of the oil that was still able to inhibit the growth of the test microorganism. The effect of concentration on antifungal activity of *H. suaveolens* oil in comparison with other antifungal agents was studied by the well dilution agar. The antifungal potential assessed by the latter method was expressed as the diameter of the clear inhibition zone. All experiments were performed in triplicate.

GC-MS analysis of essential oils

The GC-MS analysis of the oil samples was performed on a Hewlett-Packard model 6890 Series GC System equipped with a HP 5973 MS detector (EI mode, 70 eV). A column type, HP-5 (5% phenyl dimethylsiloxane) with a length of 30 m, an inside diameter of 0.25 mm and a film thickness of 0.25 μm was used. The temperature of the column was programmed to increase after 5 min from 70 to 150ºC at the rate of 2ºC/min and then after 5 min from 150 to 250ºC at the rate of 1ºC/min. Helium was used as a carrier gas at a flow rate of 1 ml/min. The injector and detector temperature were 250 and 280ºC, respectively. The components of the essential oil were identified by comparison with gas chromatographic retention indices and mass spectra from the literature [21, 22].

Results and Discussion

Antioxidant activity

The results from DPPH and ABTS experiments both indicated that *H. suaveolens* oil possesses antioxidant activity. Its activity expressed as the percentage quenching of free radicals is time dependent as shown in Fig.1. The quenching of DPPH radicals increased rapidly during the first 5 min. Subsequently, it increased slowly and reached maximum quenching of about 12% in 30 min. The time dependent activity was more evident in the quenching of ABTS, as seen by the TEAC values in Fig. 2. The TEAC value increased rapidly during the first 30 min. After that, the rate of antioxidant activity was slow and reached the maximum at 60 min. It was found that the IC_{50} of *H. suaveolens* oil was 3.72 mg/ml. The TEAC value of *H. suaveolens* oil was found to be 65.02 μM/mg.

Antimicrobial activity

In the dilution method, the absence of any microorganisms in the test plates or slants after incubation indicated the inhibition of such strain by the

dilution of the oil. The values of maximum inhibitory dilution (MID) of *H. suaveolens* oil are shown in Tab. 1. MID values indicate the maximum dilution of the oil at which the inhibitory activity against the test microorganism still remained. The results show that the oil inhibits the growth of all test microorganisms, albeit at different concentrations. By comparison, it was found to have more potent activity in antifungal than antibacterial action. The oil is less active against gram-negative bacteria, particularly *P. aeruginosa* and *E. coli*, than gram-positive bacteria. This might be due to the protection by a hydrophilic outer membrane of the gram-negative bacteria which could suppress the passage of the lipophilic essential oil [23].

The oil was compared with other agents regarding antifungal activity against *T. mentagrophytes* using the well diffusion method. It was found that the antifungal activity of *H. suaveolens* oil is concentration dependent as shown in Tab. 2.

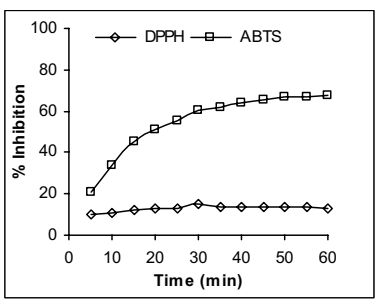

Fig. 1. Free radical quenching activity of *H. suaveolens* oil

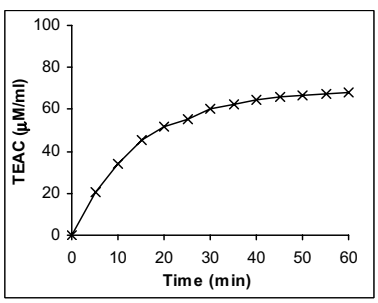

Fig. 2. TEAC of *H. suaveolens* oil

Microorganism	MID values
Trychophuton mentagrophytes	1:640
Staphylococcus aureus	1:160
Streptococcus suis	1:160
Erysipelothrix rhusiopathiac	1:80
Actinomyces pyogenes	1:80
Pasteurella multocida	1:80
Pseudomonas aeruginosa	1:20
Escherichia coli	1:20

Tab. 1. MID values of *H. suaveolens* oil against test microorganisms

Sustances	Concentration (%)	Inhibition zone (mm)
Hyptis suaveolens oil	0	NZ
	10	17.2
	20	34.1
	30	NG
	40	NG
	50	NG
Boric acid	0	NZ
	1	7.3
	2	17.2
	4	28.7
	5	31.4
	6	31.6
Benzoic acid	0	NZ
	1	24.6
	2	33.6
	4	39.7
	6	47.0
	8	51.3
Salicylic acid	0	NZ
	1	12.7
	2	25.8
	3	30.3
	4	32.0
	5	37.1
Phenol	0	NZ
	0.5	NZ
	1	9.1
	2	12.3
	3	17.6
	4	26.3

NZ = no zone, NG = no gowth

Tab. 2. Inhibition zones of *H. suaveolens* oil against *T. mentagrophytes* in comparison with other agents

The oil concentration that showed the widest inhibition clear zone was 20%. When the oil concentration was 30% or more, there was no growth observed on the plates. This is considered to be due to the strong fumigant action from the oil volatile that suppresses the growth of the microorganism. It was noted that the inhibition zone due to 20% *H. suaveolens* oil was similar to that due to 6% boric acid, or 2% benzoic acid, or 5% salicylic acid. However, this clear zone was wider than that obtained with 4% phenol. After the oil had been kept at the high temperature of 45°C for 6 months, the MID against *T. mentagrophytes* was raised to 1:320. This result indicates the decrease in oil activity upon exposure to the high temperature. The antimicrobial activity is

considered to be due to the composition of certain active components in the oil. Therefore, a study of the chemical composition of the oil was undertaken.

Chemical composition study of H. suaveolens oil

The results of an oil composition analysis by means of GC-MS are shown in Tab. 3. It was found that the major compounds (ones present at concentrations higher than 10%) of *H. suaveolens* oil were sabinene, α-terpinolene, 1, 8-cineole, and β-caryophyllene.

	Compounds [a]	Percentage	
		Fresh oil	Oil stored at 45°C
1.	Cyclohexane	0.47	0.32
2.	α-Pinene	2.04	1.32
3.	Sabinene	25.40	9.97
4.	2-β-Pinene	6.72	4.80
5.	1-Octen-3-ol	2.42	0.66
6.	α-Terpinene	0.97	0.67
7.	para-Cymene	0.87	1.07
8.	Limonene	5.89	5.04
9.	1,8-Cineole	9.94	7.12
10.	γ-Terpinene	1.48	1.08
11.	α-Terpinolene	13.48	8.64
12.	Fenchol	0.78	ND
13.	Terpinen-4-ol	3.86	3.62
14.	β-Elemene	ND	0.60
15.	β-Caryophyllene	11.69	24.03
16.	α-Bergamotene	2.03	2.63
17.	α-Humulene	0.73	1.53
18.	β-Selinene	0.72	1.16
19.	Bicyclogermacrene	4.20	6.02
20.	Spathulenol	0.72	3.44
21.	Caryophyllene oxide	ND	2.99
22.	γ-Gurjunene	ND	0.62
23.	Bergamotol	0.64	2.76
24.	Napthalene	4.21	5.30
25.	Phenanthrene	0.72	1.89
	Total	99.98	97.28

[a] Compounds listed in order of elution from HP-5 column.
ND = Not detectable

Tab. 3 Composition of *H. suaveolens* oil

Regarding to the previously reported content of *H. suaveolens* essential oil [24], it is interesting to point out that there are significant differences. For example, α-bergamotene [25] was found to be one of the major constituents in *H. suaveolens* oil from Nigeria but was present at very low concentrations in our samples. Caryophyllene oxide which could not be detected in our sample

was detected as the main component in *H. suaveolens* oil from Cuba [26]. These results suggest that geographical environmental factors strongly influence the composition of the essential oil. The GC-MS data of the oil after storage at 45°C revealed the decrease in three major constituents, sabinene, α-terpinolene, and 1, 8-cineole. Hence, the decrease in antimicrobial activity of the oil after storage at high temperature is possibly due to the decrease in these major components of the oil.

Conclusions

The essential oil of *H. suaveolens* grown in the northern part of Thailand was found to possess antioxidant and antimicrobial activities. The in vitro antioxidant study shows that the oil has the ability to scavenge DPPH and ABTS free radicals. The antimicrobial activity study revealed that the oil possesses more potent antifungal than antibacterial activity. The study on composition of the oil indicates that the three main active components of the oil are sabinene, α-terpinolene, and 1,8-cineole. The decrease in antimicrobial activity of the oil at 45°C may be due to the decrease in concentration of these three main constituents.

Acknowledgements

The authors are grateful for the financial support under the Research and Development of Medicinal Plant Project awarded by the National Research Council of Thailand.

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