การประเมินผลของสเปรย์ดับกลิ่นเท้าที่มีสารสกัดของใบพลู

Evaluation of Betel Leaf Extract Deodorizing Foot Spray

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บทคัดย่อ

ปัญหากลิ่นเท้าเป็นปัญหาที่หลาย ๆ คนต้องเผชิญในชีวิตประจำวัน ในการศึกษานี้จึงได้ทำการศึกษาการสกัดสารสกัดจากใบพลูด้วยเอทานอล เพื่อนำสารสกัดที่ได้ไปเป็นส่วนผสมในผลิตภัณฑ์ป้องกันการเกิดกลิ่นเท้าและพัฒนาให้เป็นตำรับสเปรย์ ในการศึกษาการกลบกลิ่นของสารสกัดใช้เทคนิคเครื่องแก๊สโครมาโทรกราฟี-แมสสเปคโทรมิเตอร์และวิธีการดมกลิ่น การพัฒนาสูตรตำรับสเปรย์บำบัดกลิ่นเท้า พบว่าสูตรที่มีสารสกัดจากใบพลู ร้อยละ 0.1 มี Aluminium Chlorohydrate Solution ร้อยละ 30 มี Propylene Glycol ร้อยละ 5 มี Glycerin ร้อยละ 2 มี Butylhydroxyl Anisole ร้อยละ 0.1 และมี Paraben Concentrate ร้อยละ 1 ให้สูตรตำรับที่ดีคือ ละอองของสเปรย์แห้งเร็วและไม่มันเยิ้มเคลือบผิวหลังการใช้งาน การศึกษาการยับยั้งเชื้อแบคทีเรียที่ก่อให้เกิดกลิ่นเท้าด้วยสูตรสเปรย์ที่มีสารสกัดจากใบพลูพบว่า ผลิตภัณฑ์สเปรย์ที่มีสารสกัดจากใบพลูสามารถยับยั้งการเจริญของเชื้อ *Staphylococcus epidermidis* ATCC12228 และ *Bacillus subtilis* ATCC6633 โดยมีเส้นผ่านศูนย์กลางของโซนใสยับยั้งเชื้อเป็น 14.77 ± 0.21 mm และ 14.0 ± 0.35 mm ตามลำดับ

คำสำคัญ: กลิ่นเท้า สารสกัดจากใบพลู การสกัดด้วยเอทานอล

Abstract

Foot odor is a problem that many people have to deal with on a daily basis. The present study was designed to evaluate the ethanolic extract of betel leaves as an ingredient in deodorizing foot spray product, and the formulation was developed as a prototype spray. The deodorizing activity over foot malodor was studied by GC-MS technique and olfactory method. The formula of developed spray for foot malodor treatment consists of

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0.1 % betel leaf extract, 30 % aluminium chlorohydrate solution, 5 % propylene glycol, 2 % glycerin, 0.1 % butylhydroxyl anisole and 1 % concentrated paraben in purified water by weight. This formula had a good appearance, fast-drying, non-greasy and thin coating on the skin surface after application. The product exhibited anti-bacterial activity against *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 with diameters of inhibition zone of 14.77 ± 0.21 mm and 14.0 ± 0.35 mm, respectively.

Keywords: Foot Malodor, Betel Leaf Extract, The Ethanolic Extract

Introduction

Foot odor is one of the body odors. People with foot odor might face the problems of skin disease such as hyperhidrosis, irritation and painful skin problems. In addition, they might loss self-confidence as well as social relationship [1]. There are 3 ways to control foot malodor [2]. The first method is elimination of the bacteria causing smelly foot, such as inhibition the growth of odor causing bacteria by using antimicrobial products. The second method is antiperspirants to eliminate the source of bacterial feeding and growth. The third method is covering over the bad smell with fragrances. Nowadays, people use natural product in their daily life, especially personal care products. Betel is one of the fragrance plants that have been popular in Asian countries such as India, Myanmar, Laos, Cambodia, Malaysia, Indonesia, Philippines and Thailand. It contains many biologically active components [3] such as antimicrobial agent [4], and antifungal agent [5-7]. Especially essential oil of betel leaves possess a strong pungent and aromatic flavor [4]. Betel has been used to make perfume [8]. So it is interesting to be applied in anti-malodor product, especially the foot odor. Moreover, previously published data showed that betel has the effectiveness against *Staphylococcus epidermidis* and *Bacillus subtilis,* microorganisms which was referred as the main cause of foot odor [7, 9]. However, the evaluation of deodorizing foot spray products has not ever been discussed before. So the present study was designed to evaluate the ethanolic extract of betel leaves as an ingredient in deodorizing foot spray product, and the formulation was developed as a prototype spray. The preparation was evaluated antibacterial activity against the foot odor causing bacteria, concealing activities against the foot malodor, and the stability test under room temperature for 3 months and acceleratory 6 heating, as well as cooling cycles. Therefore, we hope that this study will initiate the development of deodorizing foot spray products in the future with a proper evaluation.

Materials and Methodology

1. Preparation of Betel Leaf Extract

The ethanolic extract of betel leaves was prepared by using the modified method of Subashkumar [10]. Fresh betel leaves were collected from a local market of Hat Yai, Songkla. The leaves were washed and

cut into smaller pieces and then were dried in the oven at 45 °C for 24 hours. Ten grams of dry leaves were macerated by soaking in 500 mL of 95 % ethanol for 24 hours, then sealed the bottle with aluminum foil. After filtration, the filtrate were evaporated in rotary vacuum evaporator at 50 °C or below, until the crude plant extract with thick mass in dark green color were obtained.

2. Study of Compound in Betel Leaf Extract

The crude betel leaf extract was then analyzed by GC-MS of Thermo Scientific™ Ultra/ISQ system, fitted with capillary column of 30 m×0.25 mm×0.25 µm. Helium was carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was programmed from 80 °C to 250 °C at a rate of 2.5 °C/min. The full-scan mass spectra within the scan range 35-500 amu. The experiment was carried out in triplicate.

The contents of eugenol, the main fragrance in betel leaf extract, were quantified according to the study of Inam [11], by high performance liquid chromatography (HPLC) on a Hypersil GOLD column (C18 with 250×4.6 mm). The mobile phase was 70:30 v/v (methanol: water); isocratic conditions at a flow rate of 0.7 mL/min. The UV absorbance was detected at 282 nm.

3. Study of the Solubility of Betel Leaf Extract

Solubility of betel leaf extract was studied by adding 0.01 mg of crude betel leaf extract in a 25 mL test tube. Then 0.5 mL of solvent per time was gradually added into the test tube with continuously shaken. The solvent was added until the extract dissolve in the solvent. The solvent used for solubility studies were water, 70 % ethanol, 95 % ethanol, glycerin ,and propylene glycol.

4. Study of the Activity of Betel Leaf Extract Against the Bacteria Causing Foot Malodor

The microorganisms used in this study were collected from the Department of Microbiology, Faculty of Sciences, Prince of Songkla University. *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 were chosen as the representative of foot odor microbes. The selected strains were then cultured in Mueller Hinton broth and adjusted to the turbidity of 1.5x108 CFU/mL (0.5 McFarland standard). The broth cultures were incubated at 37 °C for 24 hours. The activity against bacteria causing foot malodor was studied by broth micro-dilution method according to the study of Ara [9]. Betel leaf extract in 2.5 % dimethyl sulfoxide, free of bacteria causing foot odor, was a negative control. The standard tetracycline was a positive control. The minimum inhibitory concentration was observed and noted.

5. Study of the Deodorizing Activity Over Foot Malodor of Betel Leaf Extract

Isovaleric acid was represented as foot odor, according to the study of Ara [9]. The 1:1 mixture of 0.1 g/mL isovaleric acid solution was first mixed with 0.1 g/mL of betel leaf extract solution. The deodorizing activity over foot odor was then studied by 2 methods. The first one was instrumental method, according to the method of Caroprese [12]. The mixture was analyzed by GS-MS at various times of 0, 2, 4, 6 and 10 minutes. The other

method was olfactory method, modified from the study of Kim [13]. The 7 members at least of olfactory panel were set up. They were trained how to evaluate and to score the sample, into 6 hedonic scales of deodorizing activity over foot malodor. The criteria for characterize odor into 6 hedonic scales, were as follows:

The score would be 0, if the isovaleric acid odor was obvious, no betel leaf extract odor.

The score would be 1, if the isovaleric acid odor was much more than betel leaf extract odor.

The score would be 2, if the isovaleric acid odor was more than betel leaf extract odor.

The score would be 3, if the isovaleric acid odor was equal to betel leaf extract odor.

The score would be 4, if the betel leaf extract odor was more than isovaleric acid odor.

The score would be 5, if the betel leaf extract odor was obviously more than isovaleric acid odor.

6. Preparation of Product Containing Betel Leaf Extract for Deodorizing Foot Spray

The formula of spray was modified from the formula of Natthaya [8], based on the commercial formulation of spray products. The concentration of betel leaf extract was referred to the studied minimum inhibitory concentration (MIC) and the deodorizing activity over isovaleric acid. The developed foot spray consists of 30-40 % aluminium chlorohydrate solution (50 %), propylene glycol (1-5 %), glycerin (2-4 %), butyl hydroxyanisole 0.1 %, concentrated paraben 1 % and purified water added to give a 100 %.

7. Evaluation of Spray Product Containing Betel Leaf Extract for Deodorizing Foot Spray

Table 1 The spray products were evaluated for the various criteria summarized on

Evaluation Criteria	Details
Physical properties	clarity, color, flavor, applicable texture
Chemical properties	pH-meter, eugenol content (HPLC)
Anti-bacterial activity	*Staphylococcus epidermidis* ATCC 12228 and *Bacillus subtilis* ATCC6663.
(Disc diffusion method)	(tetracycline as positive control and spray base as negative control)
Deodorizing activity	Olfactory method (mixture of 1 mL of 0.1 g/mL isovaleric acid solution 1 ml mixed with 1 mL spray solution)
Stability test	Normal condition: Store at 30 ºC for 3 months
	Accelerated condition: heating at 45 °C storage for 24 hours and cooling at 4 °C (6 repeated cycles)

8. Statistical Analysis

All experiment data were represented as mean ± SD and were done as triplicate. One way analysis of variance (ANOVA, single factor) was used to analyze with the level of significance at $p < 0.05$. The content of eugenol was tested by using linear regression and presented in term of correlation coefficient (R^2).

Results and Discussion

1. Preparation of Betel Leaf Extract

The ethanolic extract of *Piper betle* leaves was thick, sticky mass and dark-green color with a strong pungent odor. The percentage yield of extract was found to be 11.0 ± 0.31 % of wet weight.

2. Study of Compound in Betel Leaf Extract

The compounds found in ethanolic extract of betel leaf extract were methyl D-glucopyranoside, 2-methoxy-4-vinylphenol and eugenol. Eugenol was reported the odor of eugenol was a spicy strong aromatic odor. Therefore; this study followed eugenol as a chemical marker for foot odor management. Eugenol content in the extract was 15.60 ± 0.24 % w/w, as shown in Table 2. It was confirmed by compared with standard data.

Table 2 Gas chromatographic and mass spectral data for the ethanolic extract of *Piper betle* leaves

Retention Time (min.)	Name of Compound	Molecular Formula	Molecular Weight	Similarity Index	% Peak Area
13.03	2-methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	927	2.33
14.46	2-methoxy-4-(2-propenyl)- phenol (Eugenol)	$C_{10}H_{12}O_2$	164	901	1.24
22.93	Methyl D-glucopyranoside	$C_7H_{14}O_6$	194	772	82.15

3. Study of the Solubility of Betel Leaf Extract

The betel leaf extract was sparingly soluble in 70 % ethanol, water, propylene glycol and glycerin. It was insoluble in 95 % ethanol because the predominant compound found in the extract was methyl D-glucopyranoside. It was showed that betel leaf extract is semi-polar compound which are more soluble in semi-polar solvents like 70 % ethanol.

4. Activity of Betel Leaf Extract Against the Bacteria Causing Foot Malodor

The minimum inhibitory concentration of ethanolic extract was determined against two selected pathogens; tetracycline inhibits *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 at the concentration of 30 μg/mL and 1 μg/mL, respectively [14]. The extracted from betel leaves had the minimum inhibitory concentration against *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 was 500 μg/mL and 1000 μg/mL, respectively. The minimum inhibitory concentration of Tetracycline against *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 was 32μg/mL and 2μg/mL, respectively.

5. The Deodorizing Activity Over Foot Malodor of Betel Leaf Extract

The GC-MS results showed that whilst the time increased, the peaks of isovaleric acid at retention time of 2.17 to 2.55 minutes were decreased. Whereas the peaks of the betel leaf extract at retention time of 9.53 and 10.94 minutes were increased (Figure 1). Therefore, it can be concluded that the odor of betel leaf extract can cover up the odor of isovaleric acid (represented as foot odor). However, the deodorizing activity over foot malodor was not immediately appeared. According to the result, evaporation of isovaleric acid showed the tendency to relate with time which should be studied in the details in the future. Moreover, the deodorizing activity over the odor might be further calculated as covering index for identifying the strength of the activity. The mean scores of deodorizing activity over foot malodor by olfactory method were 4; which indicated that betel leaf extract could cover the odor of isovaleric acid.

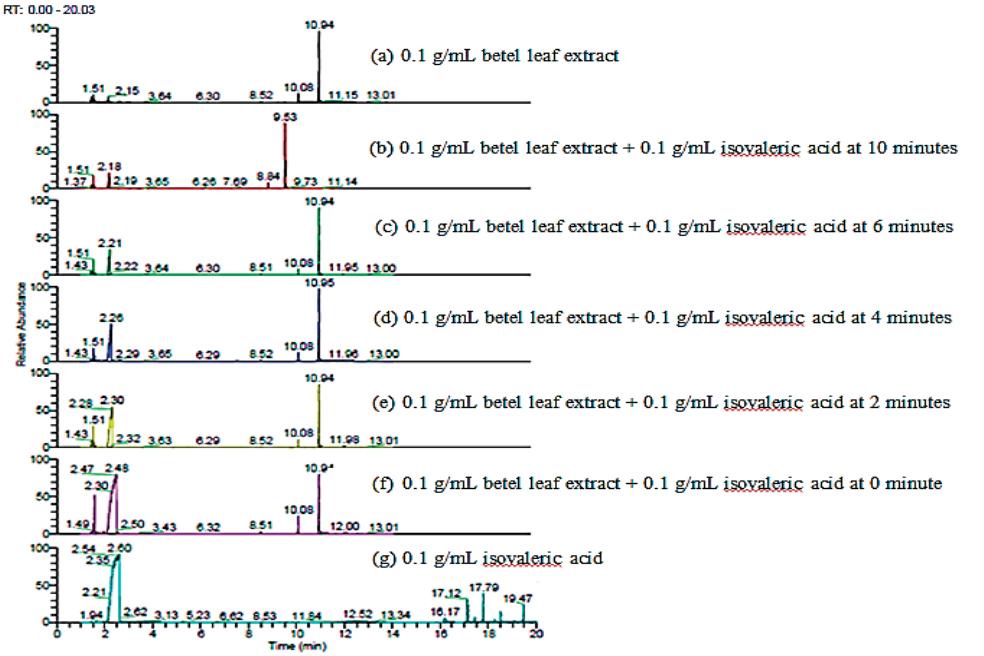

Figure 1 GC-MS chromatogram of (a) 0.1 g/mL of betel leaf extract, (b) 0.1 g/mL isovaleric acid and the mixture solutions at 10 minutes, (c) 6 minutes, (d) 4 minutes, (e) 2 minutes, (f) 0 minute and (g) 0.1 g/mL of isovaleric acid

6. Preparation and Evaluation of Spray Product Containing Betel Leaf Extract for Deodorizing Foot Spray

All the results were shown in Table 2. The concentration of betel leaf extract used in the formulation was 0.1 g. The best spray base formula consisted of 30 % aluminium chlorohydrate solution (50 %), 5 % propylene glycol, 2 % glycerin, 0.1 % butylhydroxyl anisole and 1 % paraben concentrate. The product was clear with a mild pungent scent. It made the skin feel comfortable when applied on the foot, because of its pH, was skin pH range

(5.5). And it composed of aluminium chlorohydrate solution, which served as antiperspirant, and two humectants (glycerin and propylene glycol).

The anti-bacterial activity of the product, against *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 (the two selected species of microorganism caused foot malodor), analyzed by disc-diffusion method (presented as inhibition zone), were 14.77 ± 0.21 mm diameters and 14.0 ± 0.35 mm diameters respectively. The deodorizing activity over foot odor, using olfactory method and showed as olfactory score, was 4. The product was stable in both conditions.

7. Stability of Spray Product Containing Betel Leaf Extract for Deodorizing Foot Spray

Before the storage on stability test, the content of eugenol in formulation was 10.34 ± 0.11 % w/v. After the completion of stability tests; 6 freeze-thaw cycles and the storage at room temperature for 3 months, the content of eugenol was reduced to 8.89 ± 0.13 % w/v and 8.94 ± 0.12 % w/v, respectively. These results demonstrated that the both of products kept in room circumstance for 3 months and in the accelerated condition of 6 heating and cooling cycles were significantly decreased ($p < 0.05$) the eugenol content compared with the preferable formulated spray. Besides olfactory score against isovaleric acid of the product after both storages, were statistical confidently less from 4 to 3. All of spray product formula 4 still gave high deodorizing activity and were not significantly different ($p > 0.05$) from the preferable formulated spray. That meaned the product had lost some volatile substances, which could cover the odor of isovaleric acid or foot odor. However, the anti-bacterial activity against both *Staphylococcus epidermidis* ATCC12228 and *Bacillus subtilis* ATCC6633 of the product did still not change after both storages and were not significantly different ($p > 0.05$). The rational explanation for these coincident were volatile eugenol could always evaporate, so the quantity was less after storage. Nevertheless, its content still did the deodorizing activity but less than previous storage. But other compositions in the extract, which did not investigate in the study, still remained in the product and had anti-bacterial activity. We also observed the physical appearances of the product, did not change after any storages. So the product was still physical stable.

Table 3 Evaluation results of spray product containing betel leaf extract for deodorizing foot spray NB.

Topic	Before	After 6 Heating-cooling Cycle Storage	After 3 Months Normal Condition Storage [a]
Physical appearance	Clear solution and homogeneous	Clear solution and homogeneous	Clear solution and homogeneous
pH	5.52 ± 0.09	5.47 ± 0.11	5.44 ± 0.06
Eugenol content	10.34 ± 0.11 % w/v	8.89 ± 0.13 % w/v*	8.94 ± 0.12 % w/v*
Antimicrobial activity against *Staphylococcus epidermidis* ATCC12228	14.77 ± 0.21 mm [b]	14.17 ± 0.15 mm [b]	14.23 ± 0.25 mm [b]
Antimicrobial activity against *Bacillus subtilis* ATCC6633	14.00 ± 0.35 mm [b]	13.50 ± 0.50 mm [b]	13.27 ± 0.81 mm [b]
Deodorizing activity by Olfactory method (olfactory score)	4.00 ± 0.58	3.29 ± 0.49	3.14 ± 0.38

All value were shown as mean ± SD (n=3).

* The differences were considered to be significant when $p < 0.05$ compared with initial storage.

[a] After 3 months at room temperature.

[b] Inhibition zone, the value of diameter of the area with no micro-organism' growth.

Conclusions

Betel leaf extract showed the potential to be used as an active ingredient in deodorizing foot spray products. The designed betel leaf spray product was evaluated for the physicochemical properties as well as the anti-bacterial activity against microorganisms causing foot odor, and deodorizing activity over the foot odor. The olfactory method was also applied for the simple evaluation of the product but the evaluation with the distinct digital scale is recommended for the future study. The method of GC-MS is a decent technique to evaluate the deodorizing activity over the foot odor of the product. However, further study should design the method to calculate the deodorizing activity over foot odor as the scale to quantify the activity of the product.

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