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Research Article

Simultaneous extraction of oil and protein from perilla seed by three-phase partitioning and their application in serum

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Abstract

Perilla seeds contain high amount of oil and protein. This study was aimed to simultaneously extract oil and protein from perilla seed by using three-phase partitioning method (TPP). The extraction conditions were optimized by varying the ratio of crude extract to *t*-butanol of 1:2, 1:1 and 2:1 (v/v) and ammonium sulfate concentration of 20, 30 and 40% (w/v). The crude extract to *t*-butanol at 1:2 and 30% (w/v) ammonium sulfate resulted in the highest recovery of perilla oil (35.13±2.67%) and protein (13.18±0.65%). This provided 14 folds of time saving when compared to conventional method of oil and protein extraction separately. The obtained perilla protein and oil extracts were included in the facial serum formulation. Stability and satisfaction of the serum formulation were tested compared to its base formula. The perilla facial serum showed its good stability after accelerated conditions of centrifugation and heating-cooling for 4 cycles. The phase separation and pH changing was not observed. The viscosity and the color were slightly affected by the accelerated condition. The satisfaction test including moisturizing sensory of the serum was evaluated by 20 volunteers. The product was satisfied with average score 5.47 of the maximum 6 level. This study emphasized the valuable perilla seed protein and oil could be extracted by single step of TPP and feasibly included into moisturizing facial serum product.

Keywords: perilla seed, protein, oil, serum, three-phase partitioning (TPP)

Introduction

Perilla frutescence (perilla) is an aromatic herb originated in some Asian countries including Thailand, Korea, India and China and now throughout world-wide. The perilla stalks, leaves and seeds have been repoted as a rich source of omega-3 polyunsaturated fatty acids (PUFAs), specifically alpha-linolenic acid (ALA). It also contains omega-6 and omega-9 fatty acids which are the essential for health (Mohammad & Atul, 2010). The seeds of perilla are a good source of oleic acid, stearic acid and palmitic acid (Chang et al., 2009; Peiretti et al., 2011). Earlier studies have shown that perilla seed is a potential source of fat (51.7%) and protein (17.0%) (Longvah & Deosthale, 1991). The lipids consisted of 91.2-93.9% neutral lipids, 3.9-5.8 % glycolipids and 2.0-3.0 % phospholipids (Shin & Kim, 1994). The contents of tocopherol were 734 mg/kg in perillain which γ-tocopherolis dominating isomer contributing 94.3% in perilla to the total amount of tocopherols (Scapin et al., 2017). The leaves and seeds

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of perilla have been found to have many other components such as beta-sitosterol and ampesterol (Honda et al., 1986), flavonoids such as apigenin, luteolin, chrysoeriol, quercetin, catechin, and phenolic acids such as caffeic acid and rosmarinic acid (Lee et al., 2013; Mohammad & Atul, 2010). Perilla possesses various bioactivities, such as antioxidant (Jung et al., 2011), anti- inflammatory (Ueda et al., 2002), anti-allergic (Makino et al., 2013; Takano et al., 2004) and anticancer (Narisawa et al., 1994). In the North of Thailand, the perilla seeds are widely consumed as flavoring and nutritional sources. Perilla oil is widely used as dressing or cooking (Shin & Kim, 1994). Volatile oils of the plant are also used in aromatherapy and perfume (Povilaitytee & Venskutonis, 2000). Moreover, the perilla extract from the leaves, seeds and oils has also been used in nutritional and cosmetic formulations (Mohammad & Atul, 2010; Terranova et al., 2006). However, the perilla protein which is mostly readily digestible (Oita et al., 2008) has not been documented for industrial application.

Several methods have been reported for the extraction and purification of protein, such as alcohol and salt precipitation, ion exchange chromatography, membrane separation (Azarkan et al., 2003), and aqueous two-phase extraction (Tan et al., 2015). Although these methods are effective, there are many disadvantages such as high cost and time demands, or difficult to scale-up (Tan et al., 2015). Various methods for extracting the oil from the seeds have been reported and conventional method of solvent extraction is the most widely used technique, due to its high efficiency in oil recovery. However, the major disadvantage in using solvent extraction technique is its high energy input and toxicity from large amount of solvent used (Shivani et al., 2011).

An effective technique for the separation and purification of enzymes, proteins and edible oils called three–phase partitioning (TPP) has been introduced (Chaiwut et al., 2010; Rawdkuen et al., 2010; Gagaoua et al., 2014). This method uses ammonium sulfate to achieve a saturation and precipitation of protein and *t*-butanol was added to make three distinct phases and the purified biomolecules was found in the interphase (Chaiwut et al., 2010). It is simple, less time consuming, and maintains the nature of the protein (Rachana & Lyju, 2014). Purification of aloe polysaccharide (APS) and protein resulting in highly effective APS extract (Tan et al., 2015) was an example of using the TPP for single step extraction of bioactive compounds. Extraction of protein and oil from the plant source by using one step method has not been reported. Therefore, the aims of the study were to simultaneously extract the protein and oil from perilla seed by using the TPP method and develop facial serum containing perilla protein and oil.

Materials and methods

Plant sample and chemical reagent

Dry perilla seeds were collected from Hangdong district, Chiang Mai province, Thailand during January to Febuary 2016. The seeds were air dried for 3 days. Tert-butanol (2-methyl-2-propanol) and ammonium sulfate ($(NH_4)_2SO_4$) were obtained from Merck (Darmstadt, Germany). Coomassie Brilliant Blue G-250 was purchased from Sigma Chemical (St. Louis, MO, USA) and 95% ethanol was supplied from Quality Reagent Chemical. Bovine serum albumin (BSA) was obtained from Fluka (Buchs, Switzerland). Alpha tocopheryl acetate (Vitamin E acetate) was supplied from Eisal Co. Cosmetic raw materials were purchased from suppliers: Godrej Industries, Chemico Inter Corparation, Qingdao Fuso Refining & Processing, Innospec, Nam Siang Trading, and Lubrizol.

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Preparation of crude extract from perilla seeds

The seeds of perilla were dried in a tray-dryer at 55°C until the weight was constant. The seeds were ground in a blender and then homogenized with water at a ratio of 1:2 w/v. Then the homogenate was filtered through a Whatman filter paper No.1. The filtrate was centrifuged at 5000 *g* at 4°C for 20 min. The obtained supernatant was referred to the "crude extract" and stored at -20°C until used.

Extraction of perilla protein and oil by three-phase partitioning (TPP)

The TPP composed of crude extract, *t*-butanol and $(NH_4)_2SO_4$. The mixture was partitioned by orbital shaking at 150 rpm for 10 min and centrifuged at 5000×*g* at 4°C for 20 min. The top phase (*t*-butanol) were collected and evaporated by rotary evaporater for oil determination and the interphase was collected for protein quantification. The partitioning compositions of the sample per *t*-butanol ratio and the concentration of $(NH_4)_2SO_4$ were varied for studying their effect on simultaneous extraction of protein and oil from perilla seed. The ratio of crude extract to *t*-butanol was varied as 1:2, 1:1 and 2:1 (v/v) with a constant 30% w/v $(NH_4)_2SO_4$ and the extraction was carried out as mention above. The ratio of crude extract to *t*-butanol which provided the highest recovery was chosen for study of the effect of $(NH_4)_2SO_4$ concentration on protein and oil partitioning. The $(NH_4)_2SO_4$ concentration was varied at 20, 30 and 40% (w/v).

Determination of protein and oil

The concentration of protein in the interphase was measured by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as protein standard. Protein recovery (%) was calculated from protein amount of interphase compared to initial protein amount in crude extract. Top phase from the TPP was subjected to remove *t*-butanol by using rotary evaporator. Oil recovery (%) was calculated from oil weight of the top phase compared to initial weight of perilla seeds.

Analysis of fatty acid compositions

Fatty acid compositions of perilla oil were analyzed by using GC-MS. The fatty acid methyl esters (FAMEs) of perrila seed oil was prepared by saponification of 3 grams oil with 0.9 M H_2SO_4 in methanol (3 mL) and 1 mL of toluene (Siriamornpun et al., 2006). The mixture was refluxed for 2 hours at 75-80°C and the solvent was removed by rotary evaporator at 40°C. Recovered FAMEs sample was adjusted to pH 6. The GC-MS analysis was carried out as the system composed of 0.25mm×30mm×0.25μm capillary column (Agilent 190915-43 HP-5MS) at 250°C using helium as a carrier gas with 1.0 mL/min. The injector and detector temperature were set at 220°C. The FAMEs was detected at 20-250 Da and identified by NIST08 database.

Development of serum containing perilla protein and oil

Oil/water facial moisturizing serum was developed by using ingredient listed on Table 1. The ingredients of the oil phase (phase A) were mixed at room temperature. In the same time, the aqueous part was mixed and then the oil phase was added to the water phase under mechanical stirring. The emulsion was continuously stirred and the pH was adjusted by using citric acid solution of part C. The proper texture of base formula was obtained after alteration amounts the ingredients. Stability of the base serum was checked by using centrifugation and heating-cooling accerelation test for 4 cycles. The formula providing the highest stability (pH, color and viscosity) and organoleptical characteristics (aspect, color and odor) was selected for

the base serum. In case of the serum containing oil and perilla protein, the perilla oil was added to replace jojoba oil and perilla protein solution was part D as described in the Table 1.

Stability test

The base serum and the serum containing perilla oil and protein (1.00 g) were centrifuged at 6000 rpm for 20 min. None of phase separation was observed. Then the base serum and the products were tested under accelerated condition of heating and cooling of 50°C and 4°C for 4 cycles. Before and after accerelation test, pH, viscosity and color of the products were measured. The pH of the serums was measured by using pH meter (NeoMet/pH-200L) at room temperature. The viscosity was measured by using the viscometer (Bookfield/RVD-II+P), 10 rpm and spindle no.4 under room temperature. Color measurement of base serum and sample were performed by using Chroma meter (Konica Minolta) through L*, a*, b* color scale of CIE. The L* value represents the brightness from black (0) to white (+100), a* value represents from green (-60) to red (+60) while b* value represents from blue (-60) to yellow (+60). The measurements were performed in three replicates.

Table 1. Formulas of base serum and serum containing perilla protein and oil

Part	Ingredients	Base Serum	Serum sample
A	Tocopheryl Acetate	0.10	0.10
	Caprylic/Capric Triglyceride	0.50	0.50
	Jojoba Oil	2.00	-
	Perilla Oil	-	2.00
	Isopropyl Palmitate	1.00	1.00
	Isopropyl Isostearate	1.00	1.00
	Dimethicone	1.00	1.00
	C12-15 Alkyl Benzoate	1.00	1.00
	PEG-40 Hydrogenated Castor Oil	0.20	0.20
B	DI Water	qs to 100	qs to 100
	Methyl Gluceth-20 and Glycerin	1.50	1.50
	Sodium Acrylates/Beheneth-25 Methacrylate Crosspolymer (and) Hydrogenated Polydecene (and) Lauryl Glucoside	1.50	1.50
	Hydroxyethyl Acrylate/Sodium Acryloyldimethyl Taurate Copolymer (and) Squalane (and) Polysorbate 60	2.50	2.50
C	DI Water	2.00	2.00
	Citric acid	0.15	0.15
D	Perilla Protein	-	1.00
	DMDM Hydantoin	1.00	1.00

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Sensory evaluation

Sensory satisfaction of the finished products was evaluated by 20 volunteers (10 males and 10 females). The different skin feel attributes were selected to properly discriminate the serum on distinct stages of evaluation (Lee et al., 2005). These attributes were spreadability, moisturizing, absorbability, smell, feeling after application and texture and appearance. A 0-6 scale was used where 0 was the less and 6 was the most sensory satisfaction.

Statistical analysis

All experiments were done in triplicate and all results are presented as mean±SD. The statistical analyses of collected datafor protein and oil recovery were performed using SPSS version 19.0 (IBM). One-way analysis of variance (ANOVA) and multiple comparisons by Tukey's were performed to analyze the difference among data. Statistical differences were considered to be significant at $P<0.05$.

Results and discussion

Effect of crude extract to *t*-butanol ratio on protein partitioning

Effect of the crude extract to *t*-butanol ratio on protein partitioning and oil extraction is revealed in Figure 1. The highest protein recovery (13.09%) and oil recovery (36.63%) were obtained from the interphase and top phase of the TPP system at 1:2 crude extract:*t*-butanol. The high amount of *t*-butanol makes a flocculate and increase buoyancy and lead protein favor to be extracted in the interphase (Tan et al., 2015). The increasing of *t*-butanol providing a higher oil yield was agreed with the previous study of TPP system (Duttaet et al., 2015). From this result, the ratio of crude extract to *t*-butanol at 1:2 was selected to study the effect of ammonium sulfate concentration on protein partitioning.

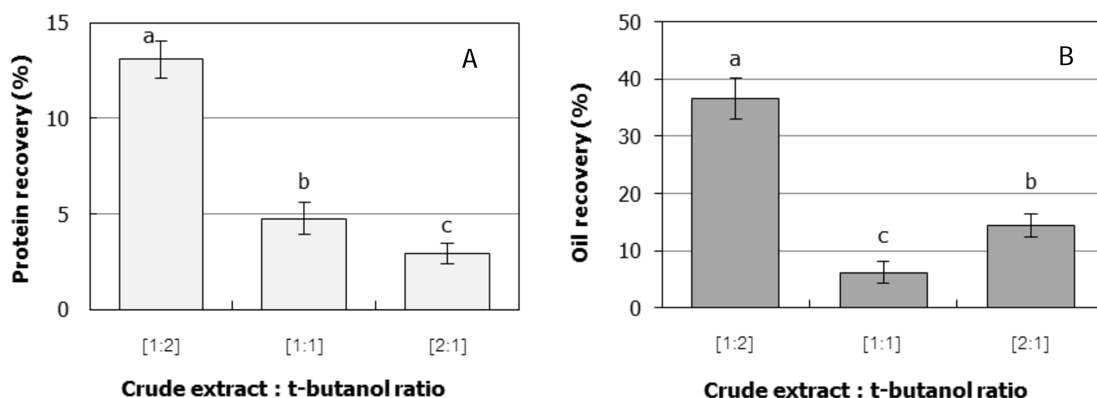

Figure 1. Effect of crude extract to *t*-butanol ratio on protein recovery (A) in the interphase and oil recovery in the top phase (B) of TPP

Effect of ammonium sulfate concentration on protein partitioning

The study on effect of ammonium sulfate concentration was explored by using the ratio of crude extract to *t*-butanol at 1:2 with varying the concentration of $(NH_4)_2SO_4$ at 20, 30 and 40% (w/v). As shown in Figure 2A, the salt concentration of 30% provided the highest recovery of protein (13.18±0.48 %). However, it was not significantly different from that of the 40%

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$(NH_4)_2SO_4$ which exhibited 13.06±0.65 % recovery. Increasing the concentration of $(NH_4)_2SO_4$ increased protein amount in the interphase of TPP (Chaiwut et al., 2010). The 40% $(NH_4)_2SO_4$ giving the maximum protein in the interphase was revealed (Gagaoua et al., 2014). However, it has also been reported that the 30% (w/v) $(NH_4)_2SO_4$ was the best concentration for applying to TPP effectively (Rawdkuen et al., 2010). From the Figure 2B, the highest oil recovery (36.50±3.48 %) was obtained from top phase at 40% $(NH_4)_2SO_4$. This value was not significantly different from 35.13±2.67 recovery of the 30% salt. This might be attributed to the highest polarity in the aqueous bottom phase due to high amount of salt rendering the oil to be moved to the top phase.

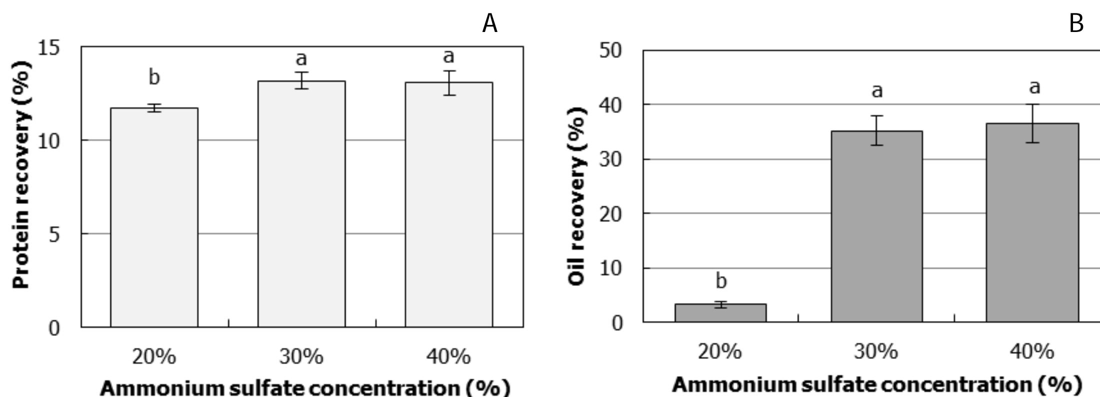

Figure 2. Effect of ammonium sulfate concentration on protein recovery in the interphase (A) and oil recovery in the top phase (B) of TPP

GC-MS analysis of fatty acid compositions in perilla oil

As shown in Table 2, two polyunsaturated fatty acids (PUFA) were identified in the perilla oil. The main PUFA was linolenic acid (18:3) of 70.63% followed by linoleic acid (18:2). Saturated fatty acids accounted for 16.51% was belonged to palmitic acid, stearic acid and arachidic acid. High amount of linoleic and linolenic acid were the significant fatty acids in term of quantity and quality of perilla oil (Siriamornpun et al., 2006). The linolenic acid (ALA) content is predominantly about 55-60% (Siriamornpun et al., 2006). It is noticed that the ALA in this study was higher than all previous reports. Ultrasonic-assisted aqueous enzymatic extraction using response surface provided 31.34% oil yield containing ALA as major component of 64.05% (Li et al., 2017). Similarly, soxhlet extraction using hexane as solvent and cold pressing extraction exhibiting 35.20% and 28.60%, respectively of perilla oil yields gave lower content of ALA at 62.26% and 60.11%, respectively (Li et al., 2017). Supercritical fluid extraction of perilla oil using carbondioxide and LPG could extract the linolenic acid as a majority fatty acid of 58.00% and 57.24%, respectively (Scapin et al., 2017).

Stability and satisfaction test of serum containing perilla protein and oil

The facial moisturizing serum was prepared by cold process and mixed using homogenizer. After centrifugation at 6000 rpm for 20 min, phase separations were not observed in both the base and serum containing perilla protein and oil. Then the serums were subjected to acceleration test by heating-cooling cycle. As shown in Table 3, the pH values of

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the base before and after heating and cooling test were 5.50 and 5.61, respectively, while the perilla serum exhibited 5.54 and 5.60, respectively. The results of pH values slightly changed indicating their high stability against acceleration test. Determination of the pH of a formulation intended for cutaneous application is extremely important, since it must be compatible with the pH of the application site. The serum in this study was designed to have pH between 5.50-5.60. The natural pH of the skin comes from the secretions of sweat and sebaceous glands, and lactic acid production, which leads to the formation of a protective film over the entire skin surface, designated hydrolipidic film. The skin normally has an average pH of 5.5, although this may vary slightly depending on the area of the body (Tichota et al., 2014).

Table 2. Fatty acid composition of perilla oil extracted by the TPP

Fatty acids	% Content (w/w)
Saturated fatty acids	
Palmitic acid (16:0)	10.23 ± 0.56
Stearic acid (18:0)	6.07 ± 0.12
Arachidic acid (20:0)	0.21 ± 0.003
Unsaturated fatty acids	
Linoleic acid (18:2)	11.17 ± 0.14
Linolenic acid (18:3)	70.63 ± 2.49
Other volatile components	1.69 ± 0.06

Table 3. Values of pH, viscosity, and color of serums before and after heating-cooling cycles

Formula	Unit	Base serum		Perilla serum	
		Before	After	Before	After
pH		5.50±0.16	5.61±0.07	5.54±0.21	5.60±0.11
Viscosity	**cP**	13,553±233	13,493±141	12,713±95	12,540±103
Torque	**%**	67.77±2.16	62.47±1.14	63.57±3.86	57.70±1.95
Color	**L***	64.70±0.13	64.42±0.06	67.77±0.20	66.87±0.43
	a *	-0.52±0.01	-0.68±0.02	-0.59±0.04	-0.70±0.04
	b*	-2.22±0.06	-2.14±0.04	-1.60±0.30	-1.59±0.24
	ΔE	0.33±0.07		0.91±0.25	

The viscosities of the base serum before and after heating and cooling test were quite similar (Table 3). This implied the products contained enough stabilizing ingredients and the addition of perilla protein and oil was compatible with other ingredients. Amount of consistency agent was important to keep the viscosity of the formulation constant during the stress conditions applied (Miner, 1993). Presence of nonionic surfactant PEG–40 hydrogenated castor oil in the formula may cause a small reduction in viscosity (Miller & Loffler, 2006).

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The results of color measurement of the base serum and sample before and after heating-cooling test were aligned in Table 3. The L* means the amount of reflected light, and can range from 0 (black) to 100% (white); the a* and b* represent, respectively, the colors from green to red or blue to yellow, and the values range from –60 (close to green or blue) to +60 (close to red or yellow). Through the values of L*, a* and b*, the overall color change (ΔE) can be calculated. Minor changes of color in both base and serum were detected after acceleration test to give less ΔE of 0.33 and 0.91, respectively. This color changes were not visible to the naked eye. Thus, it was presumable that there were no changes in the color of the serum containing perilla protein and oil after heating-cooling cylcle. Color stability of the serum might suggest the absence of lipid degradation from oxidation reactions, and revealed lipid stability during thermal process and storage. Oxidative instability is a concern mainly associated with lipids consisting of unsaturated fatty acids (Jannin et al., 2008). Lipid oxidation reactions generate colored compounds and thus color change in the formulation can give information about lipid stability (Tichota et al., 2014; Gray, 1978).

The consumer satisfaction to a finish product received the mean value of very good score. Oils and proteins are generally considered as skin moisturizing agents. The oils provide occlusive barrier which prevents water loss from skin layer, on the other hand, the protein absorb water from atmosphere delivering skin moisturization. As shown in Figure 3, the perilla serum exhibited higher sensory satisfaction score than those of the base serum in all attribute tests. Moisturizing and sense of smell attributes of the perila serum possessed the highest satisfaction among the 20 volunteers. From the maximum scale of 6, they obtained similar mean score of 5.70±0.46, while the base serum showed lower satisfying scores of 4.80±0.46 and 4.30±0.85 for moisturizing and sense of smell, respectively. Spreadability property of perilla serum also showed higher gratifying score of 5.65±0.48 when compared to 5.20±0.61 of the base formula. The texture and feeling after application were also good satisfied which scored 5.45±0.50 and 5.35±0.74, respectively. The absorbability was less scored at 4.95±0.68 which might be from watery feeling of the product. The serum in this study was designed to have water breaking property during application which might need time for a while to absorb on to skin surface. However, feeling after application was satisfied by the volunteers and obtained high score as described above.

Conclusion

Protein and oil from perilla seed could be simultaneously extracted by using TPP. The TPP system consisted of the ratio of crude extract to *t*-butanol 1: 2 and 30% (w/v) $(NH_4)_2SO_4$ giving the highest protein recovery (13.18±3.48 %) and oil recovery (35.13±2.67 %). The oil and protein from perilla seed could be incorporated in moisturizing serum formulation providing high stability in pH, viscosity and color. The product also showed good satisfaction from 20 volunteers, especially spreadability, smell, moisturizing, feeling after application and texture properties.

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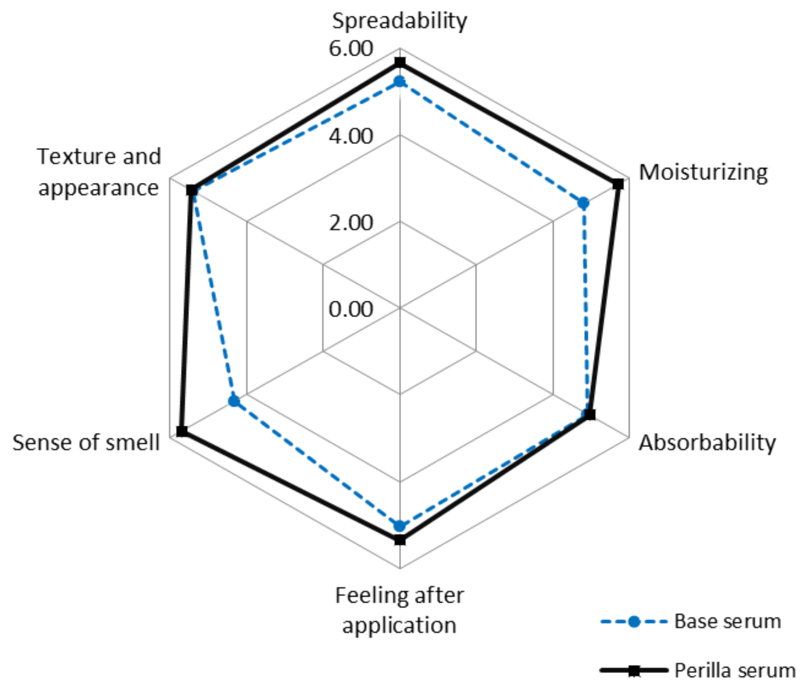

Figure 3. Satisfaction evaluation by 20 volunteers for perilla serum (—■—) compared to base formulation (---●---)

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