Advances in Pharmacology and Therapeutics Journal
Shahid Sadoughi University of Medical Sciences, Yazd, Iran

SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES

Original Article

Almond Oil-based Violet Flower Extract: General Characteristics of Violet-Almond Oil

Running Title: General characteristics of violet-almond oil

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ARTICLEINFO

Received: 12/26/2022
Accepted: 02/12/2023

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Abstract

Introduction: Violet-almond oil (VAO) as a widely used remedy with various healing properties in traditional Persian Medicine (PM), suffers from a lack of standardization parameters. This study intended to determine some physicochemical and phytochemical characteristics of VAO.

Methods: Organoleptic properties (including color, smell, turbidity, appearance, and viscosity) were determined by sensory evaluation. Specific gravity, refractive index, acid value, saponification value, fatty acid profile, and amino acid profile were determined by appropriate methods. The total phenolic content and microbial load also were measured.

Results: It was characterized as a light yellow and thin oil with a mild odor, density of 0.91 g/ml, a refractive index of 1.470, acidity index of 1.66, and saponification value of 189.61. Total phenol and flavonoid contents based on Gallic acid and Routine were 0.014 and 0.048 mg/ml, respectively. Its main fatty acids were linoleic acid, palmitic acid, stearic acid, and oleic acid. Glutamine and ornithine were the most abundant amino acids, respectively.

Conclusion: The general characteristics obtained may provide preliminary data for the standardization of this valuable traditional remedy.

Keywords: *Viola odorata* L., Sweet almond oil, Persian medicine, Amino acids, Standardization

Citation: Alijaniha F, Emadi F, Kamalinejad M, Naseri M, Bahaedin Z. Almond Oil-based Violet Flower Extract: General Characteristics of Violet-Almond Oil. Adv Pharmacol Ther J. 2023;3(1): 29-36.

Introduction

Viola odorata L. (VO) belongs to the Violaceae family, commonly called sweet violet, is a perennial herb native to Asia, Europe, and North Africa. It is widely grown in the northern regions of Iran and is called Bānāfsāj in Persian Medicine (Iranian Traditional Medicine) (PM) textbooks (1). It has a long history of medicinal use in folk medicine as well as many therapeutic effects that are shown in recent studies. In PM, it is used as an antipyretic, thirst quencher, expectorant, laxative in bilious affections, and as a remedy for cough, sore throat, catarrh, headache, and scabies (2). Recent clinical studies have also shown its effects as an antimicrobial, antioxidant, hypnotic, anti-cancer, anti-cough, anti-asthmatic, and beneficial for benign prostatic hyperplasia (1,3). Active constituents of *V. odorata* L. have been identified as alkaloids, tannins, phenolic, coumarins, flavonoids, glycoside, saponins, methyl salicylate, mucilage, and vitamin C (4). Various cyclotides with promising therapeutic effects also have been identified in this medicinal herb (5).

Herbal oils in PM are a group of medicinal preparations with various applications which are commonly used topically or orally in a single form or in combination with other herbal medicine (6). One of the most widely used herbal oils in PM is violet-almond oil (VAO), which is prepared by extracting violet flowers with sweet almond oil as a base. Two methods for preparing this oil are mentioned in Qarabadins (traditional pharmacy textbooks). In the first method, the peeled almond kernels are placed next to fresh violet flowers for a while, until the almonds take on the color and smell of the flowers. These almonds are then squeezed to make oil. In the second method, violet flowers are poured into almond oil and gently heated. Finally, the mixture is filtered and the oil is obtained.

In addition to *V.odorata* L., sweet almond oil has many useful properties that have been mentioned in both traditional and modern medicine (7,8), and in combination with the active compounds of violet flower, valuable healing properties are obtained for VAO. It has a cold and wet temperament according to PM literature and some properties such as tonic of hair and nail, treatment of some skin disorders as well as insomnia, coughing, and asthma have been contributed to it (8). Due to the wide spectrum of therapeutic applications of VAO, as well as its relatively high price, adulteration, impurities, and substitution are very common concerns in its preparation process. Therefore, determining criteria for identification and quality control to ensure the effectiveness and safety of this product is important. According to the loss of data for the characterization of violet-almond oil, the current study was designed. The aim was to provide characteristic data including some physicochemical parameters for identification and qualification of this traditional herbal oil as well as providing an overview of its therapeutic indications proposed by PM scholars along with evidence from new clinical studies.

Material and Methods

Determination of VAO characteristics

Preparation of traditional VAO

Dried *V.odorata* L. flowers were purchased from a valid local supplier in Tehran. Identification and authentication performed in the pharmacognosy

 DOI: https://doi.org/10.18502/aptj.v3i1.12489

department of the school of pharmacy, Tehran University of medical sciences. Also, Voucher specimens of *V.odorata* L. (No. PMP-542) were deposited in the herbarium of the pharmacy school of Tehran University of Medical Sciences, Tehran, Iran. Pure and standard sweet almond oil was obtained from Barij-essence Pharmaceutical Co. with its analysis report.

According to one of the methods mentioned in a credible pharmaceutical text of PM (8), 40 g of VO flower powder was macerated in 400 ml of sweet almond oil, and the mixture was kept in a water bath (38˚C) for 72 hours and was stirred every 12 hours. Then the VO powder was removed by a paper filter (Whatman No.1) and the obtained VAO was kept in a dark, well-closed glass container.

Determination of organoleptic properties

Organoleptic properties including color, smell, turbidity, appearance, and viscosity were evaluated by sensory examination.

Determination of physicochemical parameters

Physicochemical parameters including specific gravity, refractive index, acid value, and saponification value were determined according to the methods of USP (9).

Determination of total phenolic contents

The total phenolic content of VAO was analyzed based on Gallic acid content by Folin Ciocalteu reagent according to the method described by Zargaran et al. (10). The Gallic acid (Sigma) was used as standard. The sample of VAO was extracted by methanol then 5 ml of Folin Ciocalteu reagent (diluted with distilled water 1:10) and 4 ml of Na2CO3 1M, were added to both sample and standard solutions. The total phenol content was determined by colorimetric assay at 765 nm after 15 minutes. A standard curve was prepared by measuring the absorption of Gallic acid solution in 0, 50, 100, 200, and 250 mg/ml concentration in methanol: water (50:50 v/v). The tests were repeated three times and their mean was presented as Gallic acid equivalent (mg/ml of VAO oil)

Determination of total flavonoid contents

Quantification of the total flavonoid content of VAO was performed according to the method described before (11). A mixture of 5 ml of 2% aluminum trichloride in methanol and 5 ml of VAO hydro-alcoholic extract was centrifuged at 4000 rpm for 8 minutes. The clear phase was separated and the total flavonoid content was determined via its calorimetric assay at 415 nm with a blank sample of VAO and methanol. The standard curve by Rutin (Sigma, Aldrich) solution was used and related concentrations were: 0, 5, 20, 50, and 80 mg/ml). Measurement was repeated three times and the mean of them reported as the total flavonoid content as mg of Rutin equivalent per milliliter of VAO.

Determination of amino acid profile

Amino acid analysis of reverse-phase high-performance liquid chromatography (RP-HPLC) was performed by the SHIMATZU instrument. Derivatization was done by o-phthaldialdehyde (OPA)/N-acetylcysteine, and a fluorescent detection method was used (12).

Fatty acids analysis

The fatty acid profile of the oil was determined for both base sweet almond oil and VAO. Preparation of the fatty acid methyl esters was

done using the IUPAC method (13). The separation was carried out on a 50% poly cyano. The separation was carried out on an Agilent 6890 column (30 m× 0.25 mm ×0.25µm BPX5) at 50 ˚C for 1 min, then 3˚C per min to 133˚C, then 2 ˚C per min to 199 ˚C, which was maintained for 0.2 min, 1.5˚C per min to 295˚C, which was maintained for 0.2 min. The injector temperature was kept at 290 ˚C and injected as split-less to a flow of 1 ml per min linear velocity. The mass spectra interface was kept at 220 ˚C and signals were measured at m/z in 40 ms intervals, using scan mode (GC-6980 and MS-5971, Agilent Technologies, USA).

Microbiological quality control

Determinations of the total viable aerobic count, as well as specific pathogens examination, were carried out to assess the quality of VAO. The tests were carried out according to the methods and limits of WHO (14). For initial preparation, 10 g or 10 ml of the VAO was mixed with 5 g of 20 R polysorbate, heated to 40 ° C, and stirred while heating in a water bath. 85 ml of broth lactose was added and heated to about 40 ° C to form an emulsion while pH was adjusted to about 7. To determine the total viable aerobic count, 1 ml of the sample was added to a test tube containing 5 ml of sterile Tryptic Soy Broth (TSB) medium. Also, 1 ml of it was poured into the first test tube of the dilution tube series. For dilution, 6 tubes, each containing 9 ml of TSB, were pre-prepared and sterilized. In the same way, one ml was transferred from the first pipe to the second pipe and after stirring, one ml was transferred from the second pipe to the third pipe, and so on until the end. To determine the colony count, four solid culture mediums were prepared for every dilution, two containing Caso Agar (TSA) for bacteria and others containing Saburated Dextrose Agar (SDA) for fungi. 1 ml of every dilution was poured into each plate and mixed with a sterile melted medium. TSA plates were incubated at 36 ° C and SDA plates at 25 ° C. The resulting colonies were counted in TSA plates after 24 hours and in SDA plates after 72 hours.

Results

Organoleptic properties

The organoleptic properties of VAO were determined. It is characterized as a light yellow oil with a mild odor and thin consistency.

Physicochemical characteristics

Physicochemical parameters including specific gravity, refractive index, acid value, and saponification values were determined. The results are summarized in **Table 1**.

Table 1. Physicochemical specification of VAO

Method & Test Name	Test Results	Unit
Density	0.91±0.01	g/ml
Refractive Index	1.470±0.015	-
Acid Value	1.66±0.02	mEq* KOH/g
Saponification Value	189.61±0.01	mg KOH/g

* Miliequivalent

Total phenolic and total flavonoid contents

The total phenolic contents of VAO were measured by the folin ciocalteu method. The total content of phenolic compounds was found to be 0.014±0.001mg/ml in VAO.

The total flavonoid content of VAO was measured by the aluminum chloride colorimetric method.

 DOI: https://doi.org/10.18502/aptj.v3i1.12489

The total content of flavonoids was found to be 0.048±0.003 mg/ml in VAO.

Amino acid profile

The amino acid profile of the VAO was determined. The main amino acids were Glutamine, Ornithine, Serine, Isoleucine, Leucine, and Lysine. Their measured values were 234, 145, 120, 120, 115, and 90 µmol/ml respectively.

Fatty acid profile

The results of the Gas chromatography-mass spectroscopy (GC/MS) analysis of VAO are shown in **Table 2**. As it is obvious, Linoleic acid, Palmitic acid, Stearic acid, and Oleic acid were the most abundant fatty acids in AVO.

Table 2. Gas chromatography-mass spectroscopy (GC/MS) analysis of VAO

Gas chromatography-mass spectrometry (GC/MS) Analysis				
No	**RT**	**%**	**Components**	**KI**
2	13.78	0.03	Lauric acid, methyl ester	1530
4	16.13	0.61	Myristic acid, methyl ester	1731
8	17.21	0.18	Pentadecanoic acid, methyl ester	1831
9	18.01	4.54	*(Z)*-7-Hexadecenoic acid, methyl ester	1908
12	18.28	19.10	Palmitic acid methyl ester	1935
13	19.04	1.54	*Cis*-10-Heptadecenoic acid, methyl ester	2006
14	19.25	0.67	Hexadecanoic acid, 15-methyl-, methyl ester	2017
16	19.96	42.13	Linoleic acid, methyl ester	2055
17	20.41	2.56	Oleic acid, methyl ester	2177
18	20.45	1.53	8-Octadecenoic acid, methyl ester	2179
19	20.49	4.59	Stearic acid, methyl ester	2182
20	20.56	1.34	Oleic Acid	2186
21	20.66	0.33	Linoleic acid ethyl ester	2192
22	20.69	0.68	Oleic acid, methyl ester	2193
28	21.78	1.80	11-Eicosenoic acid, methyl ester	2257
30	21.97	1.06	methyl 18-methylnonadecanoate	2269
39	22.78	0.12	methyl ester-heneicosanoic acid	2418
43	23.25	2.40	*(z)*-9,17-Octadecadienal	2448
46	23.57	0.32	Behenic acid, methyl ester	2468
47	24.35	0.18	Tricosanoic acid, methyl ester	2616
53	26.01	0.13	Supraene	2807

85.84	**Total Identified**

Microbiological quality control

The results of the microbiological tests of the VAO sample in comparison to the limits mentioned in Pharmacopoeia are shown in **Table 3.**

Table 3. Results of the microbiological tests of VAO

Method & Test name	Test Reference	Specifications	Test Results	Unit
Total Plate Count	USP 40	Max 10^3	10<	CFU*/ml
Yeast & Mold	USP 40	Max 10^2	10<	CFU/ml
E. coli	USP 40	Negative	Negative	CFU/ml
Salmonella spp.	USP 40	Negative	Negative	CFU/ml
S. aureus	USP 40	Negative	Negative	CFU/ml
P. aeruginosa	USP 40	Negative	Negative	CFU/ml

*cfu: Colony Forming Unit

Discussion

Violet oil is one of the most widely used herbal oils in PM, which is usually prepared by extracting violet flowers with sesame oil or almond oil. An overview of PM textbooks on the clinical applications of VAO indicates a variety of effects.

Like other herbal products, the proper quality of VAO is very important in its effectiveness and safety. In the current study, some physicochemical parameters commonly used for evaluating herbal oil quality were measured. Since most of the VAO is composed of sweet almond oil, the measured values in the oil were compared with similar physicochemical parameters in sweet almond oil. According to the sweet almond oil analysis certificate provided by the manufacturer, its specific gravity, refractive index, and acid volume were 0.916 (g/ml), 1.46, and 0.32 (meq KOH/g), respectively. As the results show, the acid value had been increased which could indicate the presence of higher amounts of free fatty acids. Fatty acids are oxidized or defeated due to environmental factors. Therefore, the observed difference can be attributed to these factors. The specific gravity and refractive index of the VAO are consistent with the values of base sweet almond oil.

Regarding the fatty acid profile, which was determined by derivation and then GC-MS analysis, there are differences in the values related to the base almond oil. For the sweet almond oil used in this study, according to the certificate of analysis issued by the manufacturer, oleic acid (62.25%), linoleic acid (27.71%), palmitic acid

 DOI: https://doi.org/10.18502/aptj.v3i1.12489

(6.91%) and stearic acid (1.53%) were its main fatty acids. The result of VAO analysis in the present study showed that the most abundant fatty acids are linoleic acid, palmitic acid, stearic acid, and oleic acid, respectively. The difference observed in the amount of fatty acids in VAO and its base (sweet almond oil), may be due to the breakdown of fatty acid molecules or other chemical reactions that cause them to convert to each other. Previously, only one study has been performed on the analysis of almond-violet oil by gas chromatography, which has reported oleic acid (70.54%), linoleic acid (18.22%), palmitic acid (8.51%), stearic acid (1.58%), and palmitoleic acid (0.69%) as its major fatty acids (15). These results are somewhat similar to the results of our study in terms of fatty acid composition, although there are differences in amounts.

Phenolic and flavonoid compounds are secondary metabolites in plants that play an important role in biological effects due to their antioxidant properties. Measuring the amount of these compounds can be a general criterion for evaluating the effectiveness of herbal medicine. In the current study, no comparison can be made regarding the amount of total phenol and total flavonoids in VAO, given that no previous study has been done. However, in a recent study, the total phenol measured in almond kernel samples was between 10.97 mg and 106.25 mg GAE/100 g, which can be considered for comparison (16).

Our results in determining the amino acid profiles of VAO showed that the most abundant amino acids were glutamine, ornithine, serine, isoleucine, leucine, and lysine, respectively. Previously, a study on sweet almonds reported the most amino acids available, glutamic acid, arginine, aspartic acid, leucine, and glycine, respectively (17). Also in another study, amino acid analysis by HPLC performed on almond aqueous extract showed that Alanine, Glutamine Glutamic acid, Glycine, Ornithine, Serine, and Aspartic acid had the highest concentrations, respectively (18).

The results of microbiological control tests showed that the studied VAO is of good quality and in addition to the absence of any of the studied pathogens, the number of viable microorganisms is also in the desired range.

Limitations and implications

One of the limitations of the current study includes not providing a fingerprint of the composition of this oil, which is suggested to be done in future studies by high-performance thin-layer chromatography (HPTLC).

Providing some characteristics of this traditional herbal oil in the form of several physicochemical parameters, the total phenolic, and flavonoid contents and the profiles of fatty acids and amino acids may somewhat pave the way for the standardization of traditional herbal oils).

Conclusion

The results of this study provided information for the initial evaluation of VAO quality. However, more comprehensive studies are recommended to accurately standardize it.

Conflict of interests: The authors declare no conflict of interest

Funding: No

Acknowledgments: The authors thank Prof. Suleiman Afsharypuor at Isfahan University of Medical Sciences for fruitful discussions.

Ethical considerations: Not applicable.

Authors' contribution: FA and FE and MK conceived the study, participated in the design, MN and ZB revised the final manuscript for important intellectual content.

All authors read and approved the final version of the manuscript.

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 DOI: https://doi.org/10.18502/aptj.v3i1.12489