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Research Article

BIOCHEMICAL INVESTIGATION OF THE PHARMACEUTICAL AND COSMETIC USE OF *NARCISSUS* (*Narcissus tazetta* L. subsp. *tazetta* L.) GROWING NATURALLY AROUND IN MUĞLA, TURKEY

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Abstract: *The history of cosmetic products is as old as human history, and these products have played a wide variety of important roles in people's daily lives. Today, within creasing levels of well-being leading to greater consumer demand, the cosmetics sector has developed considerably and has become one of the most significant sectors in the global economy. In line with the ever-increasing and changing demand, consumers have often come to prefer products of herbal origin which may also be beneficial for their health. In this study, the TPP method was used to purify the protease enzyme, which is one of the most important enzyme groups in industrial and biochemical applications, from the flowers of the Narcissus (Narcissus tazetta L. subsp. tazetta L.) plant, which grows naturally in the Muğla region. In addition to examining some of the bioactivities of the Narcissus flower, the aim was also to obtain the essential oil of the Narcissus flower and to combine it with the essential oils of other flowers based on the concept of notes, and to thereby design new perfume combinations.*

Keywords: *Narcissus flower; perfume; phenolic compound; protease enzyme; three phases partitioning*

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1.Introduction

Amaryllidaceae is a monocot plant family with approximately 85 genera and 1100 species world-wide; it has a great economic value as an ornamental plant and is also widely used for the treatment of medical conditions [1,2]. Narcissus L. species, which are members of the Amaryllidaceae family, are perennial, herbaceous plants with bulbs that bear fragrant flowers. Up to 30 different species grow naturally in Western Europe, the Mediterranean region, China, and Japan [3,4].

Amaryllidaceous plants have biological activities which allow them to be used in a medical context, and their alkaloids have wide applications worldwide [5, 6, 7]. These alkaloids are a group of secondary metabolites on which many studies have been carried out due to their biogenetic, pharmacological, and physiological activities. To the present time, more than 500 Amaryllidaceae alkaloids have been isolated

from different plants of the family, and modern phytochemical studies have shown that these alkaloids are responsible for many pharmacological activities [5, 8, 9, 10]. Due to their similarity to morphine and codeine skeletons, they have strong analgesic effects. Their pharmacological activities include anticholinesterasic, antifungal, anti-inflammatory, cytotoxic, anticancer, antiplatelet, antifeedant, antiparasitic, and antiviral activities [5, 11]. In the *Narcissus* genus, the alkaloids are divided into eight groups according to their skeleton type. These groups are classified according to the name of the representing alkaloid: norbelladine, lycorine, homolikorin, haemantamine, narciclasine, tazettine, pancracine and galantamine [5]. Among these alkaloids, in particular with regard to galantamine, analgesic [12], antiviral, cytotoxic [13], antimicrobial and antioxidant [14] activities have been reported, and the plant is known to be used in the treatment of Alzheimer's due to its effects in inhibiting acetylcholinesterase [15]. Apart from these known therapeutic effects of the plant, it is also a valuable product in the cosmetic industry. It has been grown in the Aegean region since ancient times, and essence has been obtained from its flowers [4].

The aim of this study was to collect the *Narcissus* flower, which grows naturally in Muğla and its surroundings between February and April and is easily recognized by its scent, and to thereafter determine some of its bioactivities and conduct research on the purification of the protease enzyme for pharmaceuticaleffects. The study also aimed to research the use of the plant by using it to design a perfume for the cosmetic industry.

2. Materials and Methods

2.1. Chemicals

Azocasein, casein, azoalbumin, hemoglobin, standard serum albumin, ethanol, gelatin, methanol, ammonium persulfate, acrylamide, N, N'-methylene bisacrylamide, bromphenol blue, glycine, N, N, N ', N' tetramethyl ethylenediamine, n -butanol, hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate, acetic acid, Sephadex G-100, CM-Sephadex, glycerin, sodium chloride, sodium acetate, sodium phosphate, phosphoric acid, sulfuric acid, Coomassie brillant blue G-250, Coomassie brillant blue R-250, protein standards, t-butanol, hexane, trichloracetic acid (TCA) chemicals were purchased from Sigma-Aldrich Chemia GmbH Steinheim Germany.

2.2. Plant material

The flowering specimens of *Narcissus tazetta* subsp. *tazetta* L. plant were collected in February and April around Muğla. It was also purchased from local marketers in the Muğla market and kept in a deep freezer at -18 °C until used in our work. *Narcissus* flowers were kept in deep freezing at -80 °C until used in our experiment.

2.3. Purification of protease enzyme from *Narcissus* flowers using the three-phase partitioning method

2.3.1. Preparation of protease homogenate

The *Narcissus* flowers were weighed 10 g, crushed well in a mortar, and thoroughly homogenized by adding 150 mL of sodium phosphate (PH: 7 0.05M) buffer. It was placed in a -80 ^{o}C refrigerator in a container and allowed to dissolve after a few hours. This process was carried out in three replicates. The homogenate, which was dissolved by extracting from -80 ^{o}C, was separated from the pulp by filtration and

was dissolved for 25 min. centrifuged at 6.000 rpm. Protein content was determined in the supernatant after centrifugation [16]. Protein concentration was determined by the method of Bradford using Coomassie Brilliant Blue G-250 [17].

2.4. Determination for protease enzyme activity

The proteolytic activity of protease enzyme purified from *Narcissus* flowers was determined according to the method of casein digestion in the presence of 1 % casein. To measure proteolytic activity, 1 mL substrate (casein), 0.5 mL enzyme solution was added and the total volume was 2.5 mL with buffer solution. The tube containing the enzyme was incubated for 20 minutes in a water bath at 40 °C. The reaction was then stopped by the addition of 3 mL of 5 % TCA. It was waited for 30 minutes for the decay to take place completely, and this was then centrifuged for 20 minutes at 6000 rpm. After the supernatant was filtered, the amount of the degraded products in the supernatant was determined by the Bradford method. Proteolytic activity was calculated as micrograms per minute protein / mL of the enzyme [18].

2.5. Preparation of extracts

2.5.1. Essential oil production by hydrodistillation

The hydrodistillation method was applied for 3 hours using the Clevenger apparatus. On average, 150 g of herb flowers were used in each experiment. The water and essential oil mixture collected at the end of 3 hours was separated by liquid-liquid extraction using hexane. Some Na_2SO_4 was added to remove any water that might have remained on the essential oil. Finally, the essential oil obtained by removing the solvent under pressurized nitrogen gas was used in perfume formulations.

2.5.2. Essential oil production with hexane

For the hexane extract, an average of 200g of fresh plant sample was crushed with a mixer, placed in a 2.5 liter glass flask with a lid, and 2L of hexane was added to it and left to stand for two weeks. The hexane extract obtained was filtered. The filtered extracts were combined and the hexane was removed at 35 °C in the evaporator to obtain its essential oil [19].

2.5.3. Essential oil production with ethanol

An average of 200 g plant sample was thoroughly disintegrated with a mixer. 2 L of ethanol was added to the sample in a 2.5 liter closed flask and left to stand for two weeks. The resulting ethanol extract was filtered. The filtered extract was removed from the ethanol at 60 °C in the evaporator.

2.6. Developing cosmetic product formulation related to essential oil, extract, and enzyme

In order to evaluate the use of *Narcissus* flowers in the field of cosmetics, perfume formulations were created with essential oils obtained by hydrodistillation method, hexane, and ethanol extraction, and cosmetic product formulations containing protease enzymes were developed.

2.6.1. Perfume formulation studies

Perfume is a fragrant liquid obtained by mixing natural or synthetic scented oils (raw materials), water, and alcohol in specific proportions (Table 1). By making changes to the proportions of this mixture, the liquid, which we generally call perfume, is transformed into various forms. It takes various names according to these proportions. For example; Perfume, Eau De Parfum, Eau De Toillet etc. as. In short, all

these products that you see similar to each other in the market express different versions of this fragrant liquid that we call perfume.

Table1. Perfume formulations

Formulation	
Ethyl alcohol	%85
Plant Extract	%15
Distilled water	%5

2.7. Extraction conditions of plant samples

For plant sample analysis, methanolic extracts were prepared for 24 hours at room temperature using a magnetic stirrer, the solutions were filtered with blue band filter paper to get rid of possible solid particles, impurities and provide advanced homogeneity. After determining the final concentration of the extracts obtained, the extracting solvent was removed in a rotary evaporator at 60°C and the residue was dissolved in 10 mL of pure water with a pH of 2. Then, firstly diethyl ether and then ethyl acetate extraction was performed 3 times for 5 mL. At the end of the extraction process, evaporator bubbles were removed and their solvents were removed in a rotary evaporator at 60°C. The extracts whose flask content was dissolved with 2 mL of methanol were analyzed by HPLC-UV.

2.7.1. Determination of phenolic compounds by HPLC-UV

2.7.1.1. RP-HPLC-UV conditions

RP- HPLC-UV analysis was performed on an HPLC system equipped with a UV-Vis detector (Elite LaChrom Hitachi, Japan) at 280 nm wavelength (Table 2). Analyzes were carried out using a reverse-phase C18 column (150 mm x 4.6 mm, 5 µm; Fortis) and applying for a gradient program with acetonitrile, water, and acetic acid. The gradient program containing 2 % acetic acid (pure water) in reservoir A and 70-30 % acetonitrile-pure water in reservoir B is given in Table 2. In addition, the injection volume of the samples and standards was adjusted to 25 µL, the mobile phase flow rate to 1.2 mL min^{-1}, and the column temperature to 30 °C in the column furnace, thus optimizing the operation [20].

Table 2. RP-HPLC-UV gradient program

Time (min)	**A**	**B**
	2 % Acetic Acid (in distilled water)	**70-30 % Acetonitrile (in distilled water)**
0,01	95,00	5,00
3,00	95,00	5,00
8,00	85,00	15,00
10,00	80,00	20,00
12,00	75,00	25,00
20,00	60,00	40,00
30,00	20,00	80,00
35,00	95,00	5,00
50,00	95,00	5,00

2.8. Determination of the IR spectrum

The sample was taken and a drop of liquid was dropped onto a suitable disc, the other disc was pressed onto it to form a thin liquid film. It was placed in a disc carrier and placed in the sample compartment of the instrument. The spectrum was recorded by placing it on the IR spectrometer.

3.Results and discussion

Cosmetic and pharmaceutical preparations produced today using only plants, herbal medicines, and/or herbal ingredients are called "phytocosmetics". These should be designed with an awareness of the proper use of herbal ingredients in cosmetic products. In order to do this, it is important to go through a process that includes consideration for human health, as well as the product's reliability, quality, and sustainability. The sustainability of the intended and expected effects of herbal ingredients should be determined during the preparation/production phase of such phytocosmetics, and after they become a finished product [21]. Since herbal formulae are mixtures of more than one active ingredient, care should be taken to determine the stability profile of these phytocosmetics. Starting from this basis, this study was carried out in order to characterize and design a new, reliable, and prestigious product from the *Narcissus* flower.

A literature review [22] found that the protease enzyme has a better efficiency with the triple-phase method, which is a new method. For this reason, the TPP method was used to purify the protease enzyme from the flowers of the *Narcissus* plant. It was observed that the amount of protease enzyme activity and protein purified from *Narcissus* flowers was good with the triple-phase method. The *Narcissus* flowers yielded 59.84 % in the medium phase and 4,7 %, in the upper phase, respectively. Table 3 gives the purification results.

Table 3. The enzyme unit in protease enzyme homogenate obtained from *Narcissus* flower by triple-phase method, specific activity, and enzyme unit in homogenate purified protease enzyme, specific activity, and purification results

Samples	Activity EU/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (folds)	Yield (%)
Homogenate	0,787	78,7	94,38	0,83	1	100
Medium Phase	0,471	47,1	54,875	0,86	1,036	59,84
Upper phase	0,037	3,7	0,625	5,92	7,13	4,7

A standard chromatogram was produced by standard analysis before analyzing the phenolic compounds in the *Narcissus* flowers with HPLC-UV (Figure 1). Then, the phenolic components of *Narcissus* flowers of different colors were determined by HPLC-UV analysis, and the results are given in Table 4. As seen in Table 5, it was determined that the *Narcissus* flowers contained many bioactive molecules in different amounts and usefulness. Among their activities were antibacterial, antiviral, antifungal [23], carcinogenesis inhibition, apoptosis [24], antioxidant, anti-inflammatory, anti-atherosclerotic, immunostimulatory, antidiabetic, cardioprotective, antiproliferative, hepatoprotective, antihepatocellular carcinoma [25], cerebral ischemia, antiendotoxic, neuroprotective[26], anti-arrhythmic, and antithrombotic activities

[27], as well as properties beneficial to cardiovascular disease, osteoporosis, skin disease, and neurodegenerative disease [28] and hypertension, and also anti-allergenic [29] properties. The quantities of epicatechin (223,22), vanillic acid (87,79), and rutin (60,18) were higher than those of other components. Vanillic acid and epicatechinhave become popular in the cosmetics industry because of their pleasant creamy fragrance [24]. Catechins in particular increase the penetration and absorption of healthy functional foods and biocosmetics into the body and skin, thus improving their effectiveness [30]. Moreover, epicatechin has a high prevalence of oxidative stress found in multiple conditions including Alzheimer's disease, muscular dystrophy, rheumatoid arthritis, diabetes, cancer, heart disease, and aging [31]. In line with the information obtained in this study, it has been shown that *Narcissus* flowers can be used in the cosmetics and pharmaceutical industries due to their high therapeutic value.

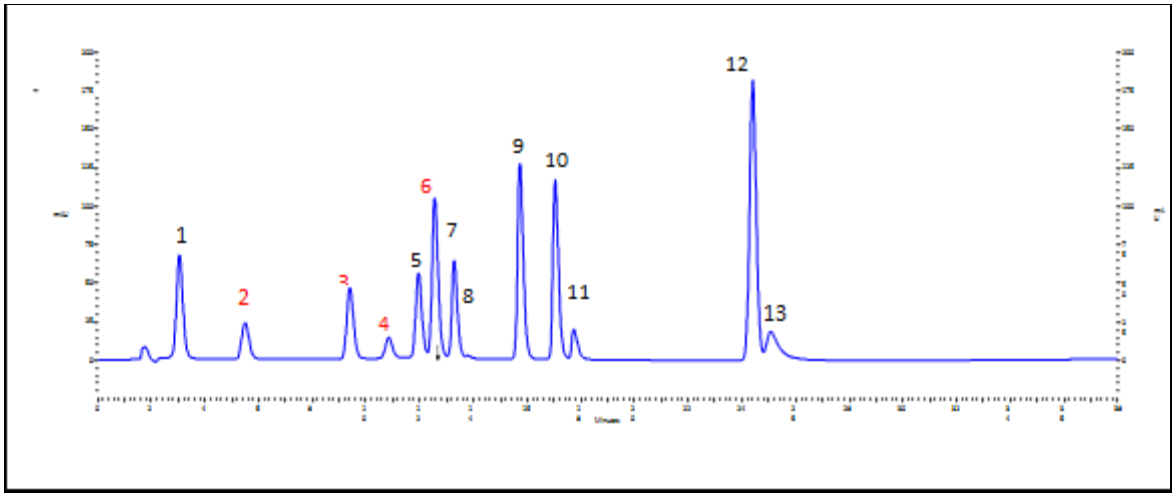

Figure 1. Phenolic acid standard chromatogram 1. Gallic acid, 2. Protocatechuic acid, 3. *p*-OH benzoic acid, 4. Catechin, 5. Vanillic acid, 6. Caffeic acid, 7. Syringic acid, 8. Epicatechin, 9. *p*-Cumaric acid, 10. Ferulic acid, 11. Rutin, 12. Daidzein, 13. *t*-cinnamic acid, 14. Luteolin

Table 4. Phenolic components of *Narcissus* flowers

No	Standards	Samples (µg extract/g sample)
1	Gallic Acid	n.d.
2	Protocatechuic Acid	n.d.
3	*p*-OH Benzoic Acid	41.83±0.11
4	Catechin	n.d.
5	**Vanillic Acid**	**87.79±0.12**
6	Caffeic Acid	23.52±0.14
7	Syringic Acid	23.39±0.07
8	**Epicatechin**	**223.22±0.16**
9	*p*-Coumaric Acid	22.09±0.07
10	Ferulic Acid	9.68±0.02
11	**Rutin**	**60.18±0.32**
12	Daizein	n.d.
13	*t*-Cinnamic Acid	n.d.
14	Luteolin	36.25±0.14

*nd: not determined

Additionally, IR spectrum measurements were taken as a result of the extraction of *Narcissus* (*Narcissus tazetta* L. subsp. *tazetta* L.) flowers. The results are given in Figures 2-3. It was observed that the natural *Narcissus* flower peaked at 2969,55. This peak indicates that there is a C-H bond in the flower. The peak at 2930,33 is an O-H bond. It was observed that natural *Narcissus* flowers gave similar peaks when the IR results were compared with commercial *Narcissus* essential oils. As a result of the data obtained from the studies, it was concluded that the *Narcissus* plant can be a very important raw material source for the cosmetic and pharmaceutical industries.

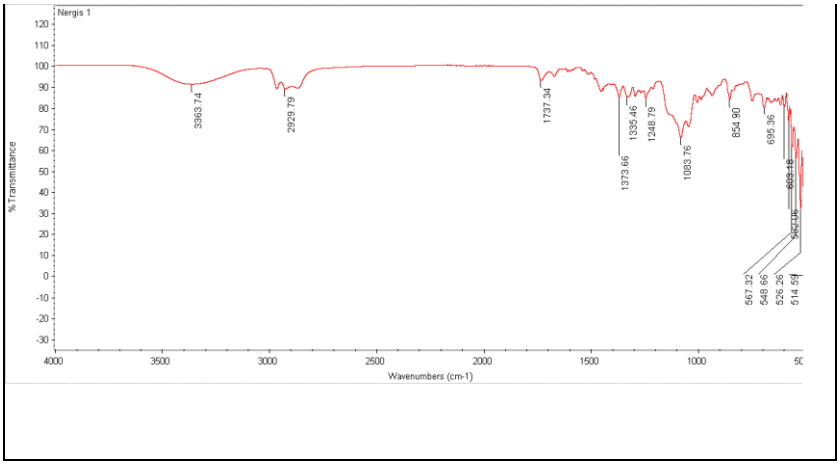

Figure 2. IR Spectrum of Commercial *Narcissus* essential oil

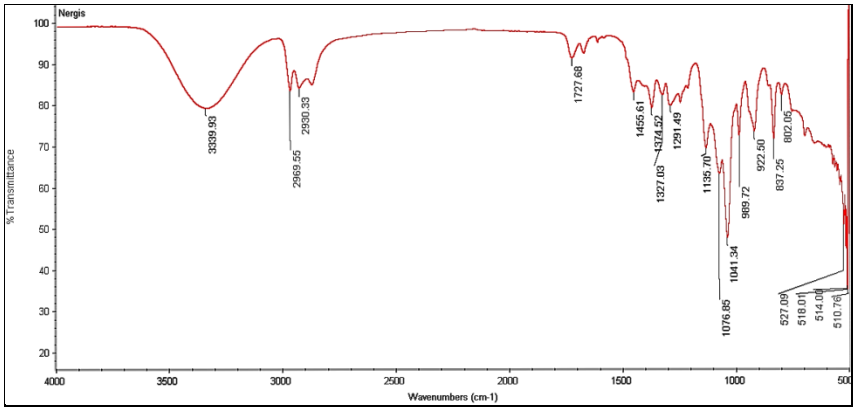

Figure 3. IR spectrum as a result of extraction of *Narcissus* flower

4. Conclusion

Humans have benefited from natural organic products for hundreds of thousands of years, and particularly from the healing and beautifying properties of herbs, oils, and potions. People suffering from various ailments were treated with these herbs and survived. The understanding of natural medicines has been passed down from generation to generation and has become part of many people's lifestyles today. Consuming natural products, using natural cosmetics, and taking vitamins and minerals to supplement our food are all examples of this. All of these are indispensable elements for natural and healthy life. This study determined some of the bioactivities of the *Narcissus* flower, investigated the purification of the protease enzyme and examined the industrial use of the plant for perfume design. This study has thus also increased

awareness of the use of the naturally growing *Narcissus* flower in Muğla, demonstrating that this plant can be cultivated and that employment opportunities can be created in this field. In addition, new products with a high added value can be designed in the cosmetics and perfume industry and offered to consumers.

The Declaration of Ethics Committee Approval

The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

Authors' Contributions

N. D: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation

S. N.D: Conceptualization, Methodology, Resources, Investigation

A.K: Methodology, Formal analysis, Writing

All authors read and approved the final manuscript.

The compliance to Research and Publication Ethics: This work was carried out by obeying research and ethics rules.

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