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Research Article

DETERMINATION OF BIOACTIVITIES OF *Convallaria Majalis* L. (LILY OF THE VALLEY), ISOLATING PHARMACEUTICAL ACTIVE INGREDIENTS AND INVESTIGATION ITS INDUSTRIAL USAGE

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Abstract: *In this study, some biological activities of Convallaria majalis L. (Asparagaceae), which attracts attention with its pleasant smell, were determined, and the isolation of drug active substances and industrial usability were investigated. For this purpose, the protease enzyme that catalyzes the hydrolysis of proteins, which is one of the most important enzyme groups in both industrial and biochemical applications, into peptides and amino acids was purified from C. majalis. The protease enzyme was purified using Three phase partitioning (TPP) method. Optimum pH and temperature for the enzyme, K_m, and V_{max} values for casein, azocasein, gelatin, hemoglobin, and azoalbumin substrates were determined. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) was used to check the purity of the purified protease enzyme. The molecular weight of the enzyme was calculated as 54.347 kDa using gel filtration chromatography. The effects of SDS (Sodium dodecyl sulfate), EDTA (Ethylenediaminetetraacetic acid), β-mercaptoethanol compounds and Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Fe^{2+}, and Fe^{3+} cations (10 mM, 1 mM, and 0.1 mM) concentrations on enzyme activity were investigated. Volatile and aromatic components analyzed with Headspace gas chromatography/mass spectroscopy (GC/MSD). It was determined that C.majalis flowers contain volatile organic compounds, citronellol (9.6 %), geraniol (8.4 %), benzyl alcohol (35 %), phenylacetonitrile (3.0 %), farnesol (1.9 %), 2,3-dihydrofarnesol (0.88 %), green grassy notes; (Z)-3-hexen-1-ol (11 %), and (Z)-3-hexenyl acetate (7.8 %). As a result of the work, it was determined that the aromatic of C. majalis can be used as an active ingredient and it has been concluded that there is industrial use.*

Keywords: *Asparagaceae, Convallaria majalis, Muğla, Protease Enzyme, Volatile and Aromatic Components*

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1. Introduction

Medicines are used by all healthcare professionals today to improve health and treat diseases. The use of drugs began in BC when it was realized that some parts of plants and animals healed diseases or healed injured people. Many countries around the world such as Egypt, India, Iran, and China have been developing traditional medicines and medical practices for hundreds of years [1]. Even today, where technology is developing rapidly, herbal medicines are still an important part of the pharmaceutical market [2]. However, this plant richness cannot be utilized sufficiently. The reason for this is that even a single plant has a wide variety of phytochemicals and the effect of using a whole plant as a medicine

is often not known precisely, and the therapeutic efficacy of herbal medicines is due to the assembled efficacy of different biological active substances in the plant raw material. [2-3]. The properties of plants that are important for human health have been investigated in laboratories since 1926 [4].

Considering these studies, Turkey is a natural laboratory with its rich flora and many medicinal and aromatic plants. The *Asparagaceae* family (*Puschkinia* Adams, *Chionodoxa* Boiss., *Scilla* L., *Prospero* Salisb., *Muscari* Mill., *Bellevalia* Lapeyr. and *Convallaria* L.), which contains many medicinal and aromatic plants, plants have been researched recently. Turkey is the "diversity center" of these breeds. These genus, which are discussed under the *Liliaceae* family in the 8th volume of Flora of Turkey and the East Aegean Islands [5], are currently evaluated under the *Asparagaceae* family [6]. The *Asparagaceae* family includes 128 genera and 2929 species that are naturally distributed in temperate, subtropical, and tropical regions around the world. In Turkey, this family is represented by 182 species belonging to 19 genus [7-8-9]. The genus *Convallaria* L. is represented in Turkey by the taxa *C. majalis* L. var. *transcaucasica* (Utkin ex Grossh.) Knorr. and *C. majalis* L. var. *majalis* L. [5]. *C. majalis* has many uses in pharmaceutical fields. It is collected in the spring and dried in the shade. Drog is odorless and has a very bitter taste. In its composition, saponins and glycosides (convallatoxin) carries. It has diuretic and heart-strengthening effects of fresh flowers effects are greater. It is used successfully in mild heart weakening [10]. Again, according to the literature data of the study of Chushenko et al. [11], the dry air parts of the plant contain between 0.1 % and 0.5 % CG (cardiac glycosides). The primary glycosides of the remarkably varied group it contains are convalloside (from 4 % to 24 %), convallatoxol (from 10 % to 20 %), convallatoxin (from 4% to 40%), lokundjoside (from 1 % to 25 %) and desglucocheirotoxin (from 3 % to 15 %) [12]. The raw material of the plant has been used for a long period and a number of drugs have been improved on its basis, one of which is the drug "Corglycon" produced by LLC "Pilot" [2].

To the best of our knowledge, as in this study, there is no study in which the parameters presented in the study of *C. majalis* are combined. Therefore, in this study, it was aimed to purify and characterize the protease enzyme and to investigate the potential of this flower to be used in the cosmetic, food, and pharmaceutical industries.

2. Materials and Methods

2.1. Chemicals and Reagents

Casein, azocasein, azoalbumin, hemoglobin, standard serum albumin, ethanol, gelatin, methanol, ammonium persulfate, acrylamide, N, N'-methylene bisacrylamide, bromphenol blue, glycine, N, N, N', N' tetramethyl ethylene diamine, *n*-butanol, hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate, acetic acid, glycerine, 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, trichloro acetic acid (TCA) chemicals were obtained from Sigma-Aldrich Chemia GmbH Steinheim Germany.

2.2. Plant Material

C. majalis was harvested from Akyaka, Muğla, and its surroundings during the maximum flowering period (in April and May 2017). The plant was identified by one of the authors (Dr. Alevcan Kaplan from Batman University) and given a voucher specimens Muğla/2016/02 before being deposited at the Muğla Sıtkı Koçman University. *C. majalis* flowers were kept in deep freezing at -80 °C until used in the experiments

2.3. Purification of Protease Enzyme from *C. majalis* Flowers (TPP method)

2.3.1 Preparation of Homogenate

C. majalis flower (10 g) was weighed, crushed in a mortar until the surface area was thoroughly reduced, and homogenized by adding 150 mL of PBS (sodium phosphate buffer; pH 7, 0.05 M). It was placed in a -80 ^{o}C cooler in a suitable beaker, and thereafter a few hours it was removed and left to dissolve at room temperature. This process was performed three times. The homogenate, which was removed from -80 ^{o}C and dissolved at room temperature was filtered and kept for 25 min centrifuged at 6.000 rpm. Protein content determination was made in the supernatant after centrifugation of the homogenate [13].

2.4. Determination of Protease Enzyme Activity

The PA (proteolytic activity) of the protease enzyme purified from the lily of the valley was determined by the casein digestion method in the presence of 1 % casein. 1 g of casein was dissolved in 95 mL of 0.05 M phosphate buffer (pH 7) and the volume was made up to 100 mL with the same buffer. The prepared solution was kept in a 95 °C water bath for 30 min and was ready to be used. In the proteolytic activity measurement, 1 mL of casein, and 0.5 mL of enzyme solution were added and the total volume was completed to 2.5 mL with buffer solution. Enzyme-added tubes were incubated in a water bath at 40 °C for 20 min. Thereafter, 3 mL of 5 % TCA (trichloroacetic acid) was added to the reaction stopped. It was incubated for 30 min for complete precipitation to occur and after this time it was centrifuged at 6.000 rpm for 20 min. After the supernatant was filtered, the amount of fragmented products in the supernatant was assigned by the Bradford method. The PA was calculated as μg protein/mL that the enzyme breaks down per min [14].

2.5. SDS-PAGE analysis of Enzyme

It was controlled whether the protease enzyme purified by Laemmli was purified and subunit by SDS-PAGE [15].

2.6. Kinetic Studies on Protease Enzyme Purified from *C. majalis*

2.6.1 Assignment of Optimum pH

To define the optimum pH of the protease enzyme purified from *C. majalis* flower, its PA against casein was used. To define the optimum pH, acetate buffer was used for pH 4-5, phosphate buffer was used for pH 6-7, Tris-HCl buffer was used for pH 8-9, and borate buffer was used for pH 10. The pH of the buffer solutions was adjusted using 1 M HCl and 1 M NaOH solutions. 1 mL of casein solution, 500 μL of enzyme solution, and 1 mL of buffer solution at appropriate pH were added to each sample tube. 1 blank solution was prepared for all samples as blank; buffer was used in lieu of enzyme in blank solution.

2.6.2 Assignment of Optimum Temperature

In order to define the optimum temperature of the protease enzyme purified from *C. majalis* flower, the activity was defined by increasing 10 °C in the range of 0 to - 90 °C. For each temperature experiment, 2 tubes, 1 blank, and 1 sample were prepared. 1 mL of casein solution and 500 μL of enzyme solution were added to the sample tubes and the final volume was made up to 2.5 mL with buffer solution. Blind, tube, and sample; It was incubated for 20 min at 10 to - 90 °C. Then, the reaction was stopped by adding 3 mL of 5 % TCA. It was incubated for 30 min for complete precipitation to occur

and after this time it was centrifuged at 6.000 rpm for 15 min. After the supernatant was filtered, the amount of fragmented products in the supernatant was defined by the Bradford Method [16].

2.6.3 Molecular Mass Determination of Protease Enzyme Purified from *C.bmajalis* by Gel Filtration Chromatography Method

After purifying the protease enzyme from *C. majalis* flower, its molecular mass was defined using GFC (gel filtration chromatography) method. Suspension Sepharose 4B (140 mL) was dissolved in distilled water and allowed to swell overnight at room temperature. The gel (1x30) was then loaded onto the column. Equilibration was performed with 0.05 M Na_3PO_4/1 mM DTT buffer until no absorbance was observed in the column at 280 nm. BSA (66 kDa), Albumin EGG (45 kDa), ß- Amylase (200 kDa), ß-lactalbumin (18.4 kDa), and Lysozyme (14.3 kDa) solutions to be 0.2 mg/mL after column equilibration loaded and eluted with 0.05 M Na_3PO_4/1 mM DTT buffer and a standard graph was created. After the column was re-equilibrated, the purified protease enzyme was loaded onto the column and eluted from the column using the same buffer. The flow rate of the column was adjusted to 20 mL/h with the help of a peristaltic pump. Eluates were collected at 4 mL in each tube. The molecular mass of the enzyme was defined with the help of the standard graph created.

2.6.4 Assignment of Kinetic Parameters (V_{max} and K_m values) of Protease Enzyme Purified from *C. majalis*

The PA of the enzyme was used to define the V_{max} and K_m values of the protease enzyme purified from the *C. majalis* flower. 100 µL, 200 µL, 400 µL, 600 µL, and 800 µL were added to all the tubes from the solution containing 1 g casein in 100 mL. 500 µL of enzyme solution and buffer solution were added to the tubes with a final volume of 2.5 mL. The tubes were incubated at 40 °C for 20 min, after which 3 mL of 5 % TCA solution was added to the tubes. After 30 min, the precipitated proteins were centrifuged at 6.000 rpm for 15 min and then filtered and the amount of protein in the supernatant was defined by the Bradford method. Blanks were prepared at the same substrate concentrations without the enzyme, buffer solution was used instead of the enzyme. 1/V versus 1/[S] Lineweaver Burk plotted. Kinetic parameters (V_{max} and K_m values) were calculated from the results we obtained.

2.7. Assignment of Substrate Specificity of Protease Enzyme Purified from *C. majalis*

The substrate specificity of the protease enzyme purified from *C. majalis* flower against casein, hemoglobin, gelatin, azoalbumin, azocasein substrates was determined by utilizing the proteolytic activity of the enzyme. In order to determine the enzyme's activity, 100 µL, 200 µL, 400 µL, 600 µL, and 800 µL of 1 % hemoglobin, gelatin, azoalbumin, and azocasein solutions were taken and the volume was completed to 1 mL using distilled water. 0.5 mL of enzyme solution was added to each prepared tube, and buffer solution was added so that the final volume was 2.5 mL. It was incubated at 40 °C for 20 min. Then, the reaction was stopped by adding 3 mL of 5 % TCA. It was incubated for 30 min for complete precipitation to occur and after this time it was centrifuged at 6.000 rpm for 15 min. After the supernatant was filtered, the number of fragmented products in the supernatant was defined by the Bradford Method [16]. The PA for the enzyme breaks down µg protein/mL. calculated in min.

2.8. Assignment of the Effect of Some Cations on Protease Enzyme Activity Purified from *C. majalis*

To determine the effect of some cations on protease enzyme activity purified from *C. majalis*, a concentration of 10 mM; $HgCl_2$, $CaCl_2$, $FeCl_2$, and $MgCl_2$ solutions were prepared. 100 µL, 200 µL, 300 µL, 400 µL, and 500 µL were taken from these solutions with final concentrations of 0.4 mM, 0.8 mM, 1.2 mM, 1.6 mM, and 2 mM. Concentration of 0.1 mM; $HgCl_2$, $CaCl_2$, $FeCl_2$, $MgCl_2$ solutions were prepared. 100 µL, 200 µL, 300 µL, 400 µL, and 500 µL were taken from these solutions with final concentrations of 0.004 mM, 0.008 mM, 0.012 mM, 0.016 mM, and 0.02 mM, and their effect on the purified protease enzyme was determined. Proteolytic activity was determined by the casein digestion method in the presence of 1 % casein. 1000 µL of the substrate (1 % casein), 500 µL of enzyme solution, 100 µL, 200 µL, 300 µL, 400 µL, and 500 µL of 10 mM, 1 mM, and 0.1 mM cation solutions were added to each tube and the final volume was 2, made up to 5 mL. After mixing, the tubes were incubated in a water bath at 40 °C for 20 min. After 20 min, 3 mL of 5 % TCA solution was added to each tube and left to stand for 30 min. It was centrifuged at 6.000 rpm for 15 min and the supernatant was filtered. The amount of disintegrated product in the supernatant was defined by the Bradford method [16].

2.9. Assignment of the Effect of Some Chemicals on the Protease Enzyme Activity Purified from *C. majalis*

The effects of SDS, EDTA and *β*-mercapto ethanol on the protease enzyme activity purified from *C. majalis* flower was investigated. For this, the proteolytic activity of the purified enzyme against the casein substrate was used. 100 µL, 200 µL, 300 µL, 400 µL, and 500 µL were taken from 0.1 mM, 1 mM and 10 mM prepared SDS, EDTA, and *β*-mercaptoethanol solutions, 1 mL of 1 % casein solution and 0, 5 mL of enzyme solution was added. The volume was made up to 2.5 mL using a buffer solution. The amount of protein that the enzyme breaks down per min was calculated by the Bradford Method [16].

2.10. Assignment of Aromatic Volatile Organic Compounds of *C.majalis* Flowers by Headspace GC/MSD

The fresh *C. majalis* flower, which was cut into pieces, was weighed as 5.00 g into a 20 mL headspace vial. Then, anhydrous $MgSO_4$ was added to it and it was mixed completely with the magnetic. The vial was placed in the headspace sampler and the extraction process was started, which will take 30 min at 90 °C. After 30 min, the volatile components at the top of the vial were transferred with helium gas for 1 min by the headspace sampler with a GC Split/Splitless inlet transferline. Headspace GC/MSD instrument analysis parameters are given in Table 1.

Table 1. Headspace GC/MSD instrument analysis parameters

Device Parameters	
Balancing Time	2 min
Maximum Temperature	300 °C
Device Program	60 °C for 1min for 10 °C /min; 100 °C for 1 min 10 °C/min; 260 °C for 8 min
Operation time	30 min
MMI Input Parameters	
Method	Divide
Heater	250 °C

Table 1. Continued.

Thermal Aux (Tranfer Line)	
Heater	On
Temperature	250 °C
Column	
Name	Agilent J&W 19091S-431UI HP-5MS UI (15µmx250µmx0.25µm)
Pressure	21.801 psi
Flow	1.8 mL/min
MS Acquire Parameter	
Acquisition Mode	View
EM voltage	1200
Low mass	35.0
High mass	400.0
Threshold	150
MS Source	230 °C max 250 °C
MS quadrupole	150 °C max 200 °C
GC-MSD Parameters	
Device Temperature	95 °C
Cycle Temperature	110 °C
Transfer Line Temperature	120 °C
Bottle Balance	30 min
Injection Time	1 min
GC Turnover Time	40 min
Bottle Size	20 mL
Fill Mode and Pressure	Pressure / 14psi
Cooldown	0.5 min
Extraction Method	Multiple extractions

3. Results and Discussion

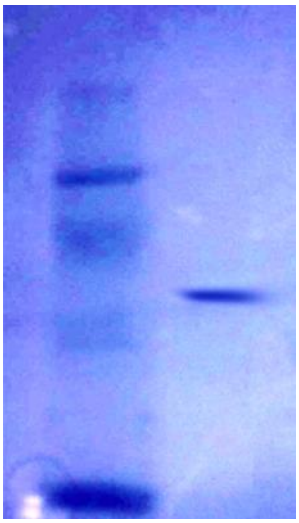
In recent years, scientists have been increasingly interested in natural compounds that can act as therapeutics or preventatives against diseases [17]. It has been stated by the World Health Organization that 21.000 plant species are suitable for the preparation of medicines [18]. In industrial production processes, it is possible to use environmentally friendly biological methods by using plants instead of chemical methods. The environmental friendliness of many industrial processes is increased by using enzymes. Processes using enzymes are cleaner, safer, and often more economical. New products developed with biotechnological methods have less negative effects on the environment [19]. And, it is known that proteolytic enzymes obtained from plants are very interesting because they can be active in a wide temperature and pH range [20]. Here, we isolated and purified the protease from *C. majalis* flowers and performed enzyme kinetics study and industrial usability potential was revealed.

In the first step of the study, the protease enzyme was purified by the TPP method, which is a practical and usually one-step process that can be used quite successfully and widely in recent years, especially in the separation of enzymes and proteins [1]. The choice of organic solvent and phase-forming salt is a very important step in the TPP system. Ammonium sulfate, which is an effective cosmotropic agent, was preferred as the phase-forming salt, and t-butanol, which is a cosmotropic and clumping agent at room temperature, was preferred as the organic solvent. In this process, while the ammonium sulfate saturation was 30 % (w/v) and the homogenate: the t-butanol ratio was 1:1.5, the enzyme preferred to stay in the middle phase predominantly. Thus, characterization processes were

performed using these ratios. The results are depicted in Table 2. As seen in Table 2, it was observed that the activity and protein amount were higher with the TPP method, a purification fold of 1.04, and a yield of 77.7 % was obtained. The purity and subunit of the protease enzyme purified by SDS-PAGE developed by Laemmli [15] were checked (Figure 1). There are many studies reporting a significant increase in enzyme activity and yield with the TPP method [21-22]. Uçkaya [23] found the protease enzyme from the *C. sinensis* plant at 40 % (w/v) ammonium sulfate saturation and 1:1.5 (v/v) homogenate:t-butanol ratio with an activity yield of 677.51 % and a purification fold of 9.04 and purified from the middle phase of the TPP system with a purification fold. Gul et al. [24] bromelain protease enzyme from the crown waste of pineapple by TPP method (1.0:0.5) with a crude ratio of 70 % $(NH_4)_2SO_4$ saturation t-butanol extract at pH 7.0, with purification fold of 3.4 with a recovery of 244 %. We can suggest that TPP is an effective non-chromatographic method for the extraction of proteases from crude extracts and that the method can be developed and used commercially in implementation in different industrial sectors such as food and medicine.

Table 2. Purification results of protease enzyme purified from homogenate obtained from *C. majalis* flower by TPP method

Samples	Volume (mL)	Activity (EU/mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (folds)	Yield (%)
Homogenate	100	0.894	89.4	107.75	0.82	1	100
Medium phase	100	0.695	69.5	82.87	0.84	1.024	77.7



I II

Figure 1. SDS-PAGE image of the purified protease enzyme (I, standard protein mix: BSA (66 kDa), Albumin EGG (45 kDa), ß-Amylase (200 kDa), ß-lactalbumin (18.4 kDa), Lysozyme (14.3 kDa); II, Protease enzyme purified from *C. majalis* flower)

To define the optimum pH value of protease enzyme purified from *C. majalis* flowers, activity measurements were made at a pH 4-5, phosphate buffer for pH 6-7, Tris-HCl buffer for pH 8-9, and borate buffer for pH 10 and the activity-pH graph was plotted. The amount of PA versus pH change is shown in Figure 2. It was defined that the optimum pH of the enzyme was 5 and it showed activity in the pH 4-9 range. The skin pH is between 4.0 and 6.0. Considering this result, it is thought that the use of *C. majalis* in cosmetic products and perfumes may be suitable for skin health. Moreover, the fact that the enzyme remains active in a wide temperature and pH range indicates that it can be used in different

areas of industrial processes. Similarly, similar, or different findings have been reported in various studies on different plant proteases [25-26-24]. The difference in the findings may be due to the different plant materials. And, in order to define the optimum temperature of the *C. majalis* purified protease enzyme, the activity was defined by increasing 10°C in the range of 0 to -90 °C. The amount of PA versus temperature change is shown in Figure 3. It was defined that the optimum temperature of the enzyme was 30 °C and the enzyme demonstrated activity in the range of 20 to -60 °C. The optimum temperature of the protease enzyme that purifies from the *C. majalis* flower is at the appropriate temperature for enzymes. This shows that the enzyme will not lose its activity under room conditions in cosmetic products. Gul et al. [24] reported the optimum temperature of bromelain protease as 35 °C. Many studies reporting different or similar optimum temperatures have been reported by different researchers [27-28-29-30].

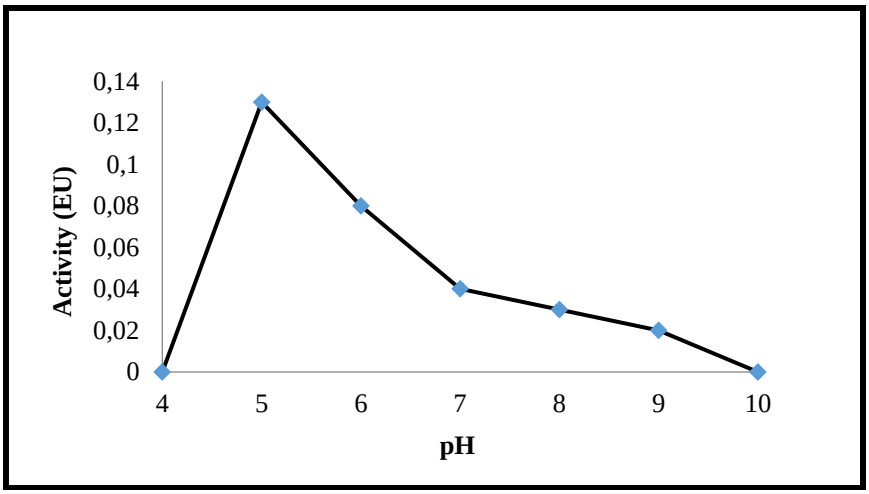


Figure 2. The efficacy of pH on the activity of protease enzyme purified from *C. majalis* flowers

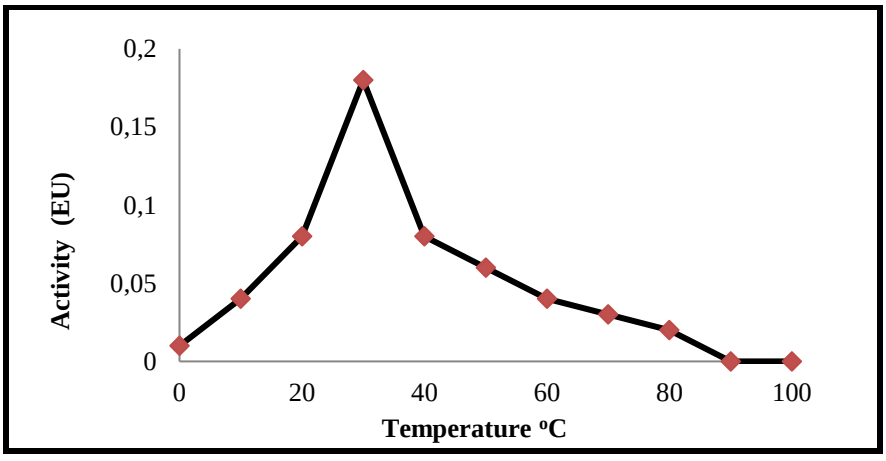


Figure 3. The efficacy of temperature on the activity of protease enzyme purified from *C. majalis* flowers

Afterward, the molecular mass of protease enzyme purified from *C. majalis* flower was determined by GFC method. The molecular mass of the protease enzyme purified from the *C. majalis* was defined as 54.347 kDa by GFC method. The standard graph prepared for molecular weight determination is given in Figure 4 and the calculations for the standard protein graph are given in Table 3. The absorbance graph prepared for the molecular weight determination of the protease enzyme from

C. majalis flowers by gel filtration chromatography is shown in Figure 4, and the calculations for the molecular weight determination are shown in Table 4. Banik et al. [31] obtained purified of the protease having a molecular mass of 51 kDa. Khan et al. [32] calculated the molecular weight of a new cysteine protease enzyme purified from *Juglans regia* L. as 11.2 kDa. Demir et al. [27], calculated the molecular weight of cysteine protease isolated from *Capparis spinosa* L. capsules as 46 kDa. In the other literature research, *Viola odorata* L. 25 kDa [33], *Pinus brutia* Ten. 28.2 kDa [34], *Chrysanthemum coronarium* L. 33.2 kDa [35], *Lilium candidum* L. 29.2 kDa [36], *Citrus limon* (L.) Burm.F. 12 kDa, *Citrus sinensis* (L.) Osbeck 30 kDa [23], *Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier [37] protease enzyme as a result of gel filtration chromatography molecular weights were determined. It was observed that the molecular weight of *C. majalis* was twice that of other plants.

Table 3. Calculations for the standard protein chart

Standard protein mixture	MW(Dalton)	Tube sequence	Ve/Vo	LnMW
Lysozyme	14300	44	13.681	9.568
ß-Lactoglobuline	18400	35	10.882	9.820
Albumin, EGG	45000	23	7.151	10.714
Albumin, Bovine	66000	18	5.597	11.097
ß- Amylase	200000	13	4.042	12.206

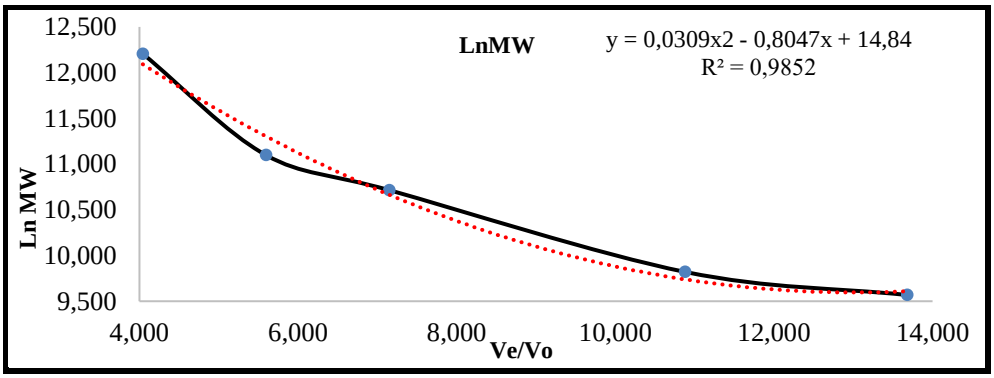


Figure 4. Gel filtration chromatography standard curve

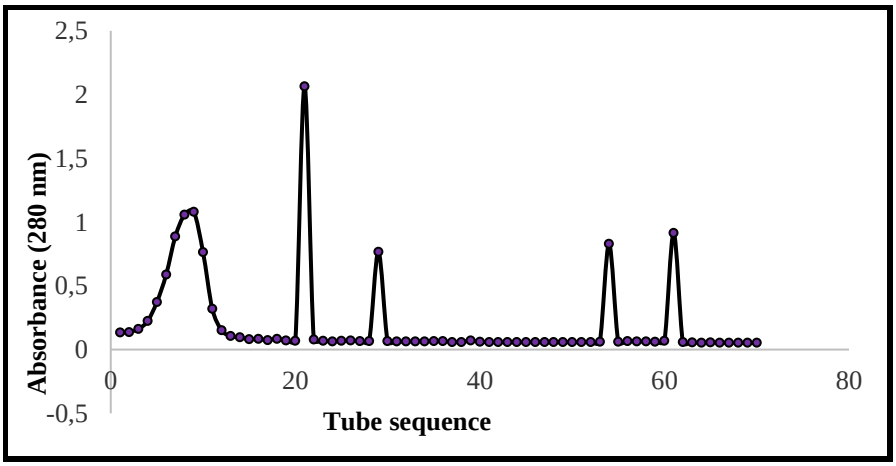


Figure 4. Absorbance graph prepared for molecular mass determination of protease enzyme from *C. majalis* flowers by CFG method

Table 4. Calculations for molecular mass determination of protease enzyme purified from *C. majalis* flowers by CFG method

Proteins	MW (Dalton)	Tube sequence	Ve/Vo	LnMW
1. Protein	373304	9	2.798	12.830
2. Protein	54347	21	6.529	10.903
3. Protein	24256	29	9.017	10.096
4. Protein	22924	54	16.790	10.040
5. Protein	44057	61	18.966	10.693

In order to define the substrate specificity of the protease enzyme purified from *C. majalis* flowers, proteolytic activity measurements were made against hemoglobin, gelatin, azoalbumin, azocasein substrates, and Linewear-Burk plots were drawn. By using these graphs, K_m and V_{max} values were determined for each substrate and shown in Table 5. As shown in Table 5 the most interest was against the casein (K_m and V_{max} values; 1.20 µM and 1.295 µg/mL.min, respectively), and no activity in azocasein substrate. Demir et al. [27] reported that cysteine protease from *Capparis spinosa* showed an exhibited the greatest protease activity with gelatin (K_m and V_{max} values;0,96 mg/L and 2.17 mg/L·min) and no activity toward hemoglobin and azoalbumin. Demir et al. [29] calculated the K_m and V_{max} values of white oleander, pink oleander, and red oleander plants against casein substrate, 1.187 µM and 1.303 µg/mL.min; 1.230 µM and 1.315 µg/mL.min; 1.229 µM and 1.309 µg/mL.min, respectively. Banik et al. [31] noted that the K_m and V_{max} values of the protease enzyme isolated from the leaves of *Moringa oleifera* Lam. as 5.47 mg/mL and V_{max} as 588.23 µM/min. Yıldırım Çelik [38] determined the Km and V*max* values of the protease enzyme purified from *Crocus biflorus* Mill. tubers as 0.9 g/L and 47 mg/L.min, respectively. Additionally, they noted that the enzyme has also been found to break down hemoglobin, albumin, and gelatin. Alıcı [37] tried to define the substrate specificity by comparing the activity values of pure strawberry protease in the presence of azocasein-modified substrate and natural protein substrates such as casein, BSA, gelatin, and hemoglobin. It was determined that the enzyme replied with all tested substrates, showing the highest specificity against hemoglobin and the lowest specificity against casein.

Table 5. Substrate specificity results of protease enzyme purified from *C. majalis* flowers

Substrate	K_m (µM)	V_{max} (µg/mL.min)
Casein	1.20	1.295
Hemoglobin	2.24	1.441
Azoalbumin	1.84	0.705
Gelatin	2.88	1.041
Azocasein	nd*	nd*

*nd: not determined

The effect of Hg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mg^{2+} (10 mM, 1 mM, 0.1 mM) cations on the activity of the protease enzyme purified from *C. majalis* flowers was also defined. Chlorinated salts of all cations were used. Results are demonstrated in Table 6. It was determined that Mg^{2+}, Fe^{2+}, Fe^{3+} and Ca^{2+} ions activate the enzyme and Hg^{2+}, Mn^{2+}, and Ni^{2+} cations inhibited the enzyme. Demir et al. **[**27] reported that all Hg^{2+} concentrations on the enzyme (cysteine protease from *Capparis spinosa*) activity of metal ions inhibited the protease activity, Ca^{2+}, $Mg^{2+,}$ and Zn^{2+} activate the enzyme, especially at low concentrations, while Co^{2+} inhibited the enzyme, albeit less than Hg^{2+}, at high concentrations. Yıldırım

Çelik [38] reported that upon the activity of the protease enzyme purified from *Crocus* tubers, Ca^{2+} and Mg^{2+} metal ions with concentrations of 10^{-1} and 10^{-2} M increased the activity of the enzyme in both concentrations, Fe^{3+} completely inhibits the enzyme at 0.1 M concentration, Fe^{3+} ions increased the activity of the enzyme at 0.01 M concentration, while Zn^{2+} ions did not affect it at both concentrations. Alıcı [37] as a result of the study, examining the efficacy of metal ions on the enzyme activity (protease enzyme purified from strawberries) shows that all metal ions, except Co^{2+} ions, inhibit the enzyme. Especially Cu^{2+}, Hg^{2+}, Cd^{2+}, and Mn^{2+} ions caused the strawberry protease enzyme to lose most of its activity and even Cu^{2+} and Mn^{2+} ions (5 mM) completely inhibited the enzyme. The activation effect of Co^{2+} ions on strawberry protease is extremely impressive. When Co^{2+} ions were used at the level of 2 mM, it increased the enzyme activity by 172 %, and when used at the level of 5 mM, it increased by 237%.

Table 6. Results of the effect of some cations on protease enzyme activity purified from *C. majalis* flowers

Chemicals	Concentrations (mM)	Proteolytic activity (%)
Control	-	100
$MgCI_2$	0.1	107.28
	1	103.8
	10	70.15
$MnCI_2$	0.1	98.70
	1	95.18
	10	88.43
$FeCI_2$	0.1	108.31
	1	96.92
	10	72.06
$NiCI_2$	0.1	98.71
	1	87.05
	10	61.83
$FeCI_3$	0.1	113.34
	1	112.26
	10	104.21
$CaCI_2$	0.1	103.19
	1	98.20
	10	91.19
$HgCI_2$	0.1	99.03
	1	94.33
	10	80.26

The effects of SDS (Sodium dodecyl sulfate), EDTA (Ethylene diamine tetra acetic acid), and *β*-mercaptoethanol on the protease enzyme activity purified from *C. majalis* flower were investigated. For this, the proteolytic activity of the purified enzyme against the casein substrate was used. Results are depicted in Table 7. In the experiments, it was determined that the activity of the enzyme was partially inhibited at low concentrations of SDS and EDTA (known as active site-directed inhibitors) and that all chemicals used completely inhibited the enzyme at high concentrations. Inhibition of the enzyme in the presence of SDS indicates that it has more than one subunit. The decrease in activity in the presence of *β*-mercaptoethanol indicates the presence of disulfide bonds in the purified enzyme. By breaking the *β*-mercaptoethanol disulfide bonds, it changes the three-dimensional structure of the enzyme and causes it to lose its activity [39]. The decrease in activity in the presence of EDTA indicates that there is a metal

ion in the center of the enzyme as a cofactor. According to Demir et al. [27] reported that they observed that all compounds (PMSF, DIPF, *β*-mercaptoethanol, SDS, Phenanthroline, EDTA, and Iodoacetamide) tested on the activity of the enzyme (cysteine protease from *Capparis spinosa*) showed an inhibitory effect and that it was difficult to classify according to the catalytic type of this protease. Yıldırım Çelik [38] determined that SDS completely inhibited the enzyme at a concentration of 0.1 M, while *β*-mercaptoethanol did not affect the activity of the enzyme at a concentration of 0.01 M, but increased the activity at a concentration of 0.1 M.

Table 7. The results of the determination of the effect of some chemicals on the protease enzyme activity purified from *C. majalis* flowers

Chemicals	**Concentrations (mM)**	**Proteolytic activity (%)**
Control	-	100
EDTA	0.1	21.89
	1	9.40
	10	3.17
SDS	0.1	57.81
	1	32.25
	10	5.50
β-mercaptoethanol	0.1	12.40
	1	7.28
	10	0.52

Aroma substances of *C. majalis* flowers were determined using Headspace GC/MSD (Table 8). It was observed that the amount of benzyl alcohol (35%), Z)-3-hexen-1-ol (11%), citronellol (9.6%), and geraniol (8.4%) were high. Compounds with high protective properties are those that give off a nice smell. Generally considered a safe and effective solvent, benzyl alcohol is widely used in paints, adhesives, and curing inks. In addition, while it is widely used as a perfume as a floral fragrance, it is also preferred as a stabilizer and preservative in the food, cosmetic and pharmaceutical industries due to its antimicrobial activity [40]. Z)-3-hexen-1-ol (syn: cis-3-hexen-1-ol) can also play a decisive role in the grassy scent of green tea as "raw" due to its extremely strong and pungent green flavor. It is also a significantly important active ingredient found in vegetables such as grapes, passion fruit, and *Toona sinensis* (Juss.) M.Roem. [41]. Citronellol has some pharmacological effects such as antibacterial, antifungal, antispasmodic, hypotensive, vasorelaxant, and anticonvulsant activities [42]. In the literature review of Geraniol, this phytocomponent has anti-diabetic, cardioprotective, in *vivo* and *in vitro* antitumor activity; It is seen that it has antidepressant effect, insecticidal and/or repellent activity, antifungal, antioxidant, anti-inflammatory and antibacterial activity [43]. The inclusion of valuable flavoring substances leads us to a conclusion that is integrated with the other findings detected in the study. It is clearly seen that the *C. majalis* protease enzyme can be used effectively and healthily in various business fields such as cosmetics, textiles, and pharmaceutical industries. This study is also very valuable in terms of providing information to these sectors.

Table 8. Percentages of aroma components identified in *C. majalis* flowers

No	Name of Component	Percentage (%)
1	Benzyl Alcohol	35
2	Z)-3-hexene-1-ol	11
3	Citronellol	9.6
4	Geraniol	8.4
5	(Z)-3-hexenyl acetate	7.8
6	Geranyl acetate	3.3
7	(Z)-3-hexene-1-ol	3.0
8	Phenylacetonitrile	3.0
9	Famesol	1.9
10	Nerol	1.3
11	Geranial + benzyl acetate	0.96
12	2,3-dihydrofarnesol	0.88
13	Phenethyl alcohol	0.78
14	(E)-2-hexenal	0.18
15	Octanol	0.15
16	Nonanal	0.1
17	Decanal	0.07
18	Neral	0.02

4. Conclusion

As a result, each step of the purification, characterization, and investigation of different application areas of the protease enzyme from the *C. majalis* plant has been carried out successfully. *C. majalis* was used for the first time in our study as a source of protease enzyme. There are many ethnobotanical applications of the *C. majalis* plant, and it has been revealed in this study that it is important to integrate these data into ethnopharmacological use and to bring it into the scientific world as a source of protease enzymes for many industrial sectors such as cosmetics, textiles, and food.

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The Declaration of Ethics Committee Approval

The author declares that this document does not require 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 - Original draft preparation

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

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