

Determination of the activity and kinetics parameters of proteases in the crude plant extracts of *Mentha piperita* L. and *Thymus capitatus* L.

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ABSTRACT

The present study provides plant-derived extracts to characterize the activity and kinetics of protease enzymes of *Mentha piperita* L and *Thymus capitatus* L. The results illustrated that these crude extracts have a proteolytic activity within optimum pH (3–9) and optimum temperature (35–50°C). The study of the ionizable groups in or around the active site of these proteases by Dixon-Webb's plot reveals the occurrences or the presence of aspartic acid with pKa₁ at pH 2.9 and pKa₂ at pH 3.2, and cysteine amino acids in or around the active site with pKa₁ at pH 6.7 and pKa₂ at pH 7.2; interestingly, these proteases maybe belonged to the acid proteases and neutral proteases. Enzymatic kinetics studies (k_m , V_{max} , V_{max}/k_m) indicated that egg albumin is a good substrate for proteases of *M. piperita* L. and *T. capitatus* L.

1. INTRODUCTION

In most biological and industrial fields, protease enzymes play a crucial role and responsible for catalyzing the hydrolysis of peptide bonds in proteins and small peptides [1,2]. They participate in metabolism and regulation reactions such as the complement system, blood clotting cascade, apoptosis pathways, and other reactions [3]. There are many classifications for these proteases. They classified as two major groups: Exopeptidases and endopeptidases. In addition, they are classified according to the character of their catalytic active site and action as serine, threonine, cysteine, aspartate, and glutamic acid proteases [4]. Alternatively, proteases may be classified according to the optimal pH as basic proteases, neutral proteases, and acid proteases [5].

Proteases play a role in most physiological processes such as cell maintenance, cell signaling, food digestion, wound healing, and cell differentiation and growth [6]. Deficiency in some proteases may result in many serious disorders and abnormal tissue destruction such as cataracts, cancer, stroke, viral infections, and Alzheimer's disease. Recently, some proteases inhibitors used for the treatment of many diseases [7].

Proteases are found in animals, fungi, and microorganisms [8,9]. They catalyze the peptide bonds and are characterized as small sized molecules, compact, and spherical structures [2].

Proteases are most industrial enzymes in the world [10]; their proteolytic action makes changes in the physicochemical, biological, and immunological properties of proteins. Modification of protein properties such as solubility, coagulation, emulsification, and foaming is examples on these changes. Improvement of the nutritional characteristics or change of flavors, taste and odors are examples on the hydrolysis of food proteins [11].

In industrial fields, proteases used in food industries, leather, milk clotting, food complements, production of emulsifiers, cheese making, meat processing, detergent, and in the treatment of many human diseases [12]. Proteases can catalyze different pathways as mediators in many cellular events such as blood clotting, apoptosis, inflammation, and other pathways [13].

In addition, to their applications in industry, and biotechnology, they have a very important role in the pharmaceutical industry. Some crude plant extracts with a high content of protease enzymes have been used in traditional medicine. Some plant extracts used in the treatment of cancer, anti-tumors, digestive disorders, and immune modulation problems [14,15].

Proteases occur naturally in all organisms, especially in microbes (bacteria, fungi, and yeast) [16]. Nevertheless, plant proteases

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participate in some pharmaceutical and industrial applications, and found to play an important role in plant metabolism and in some biochemical mechanisms such as plant germination, breakdown of protein, leaf senescence, and storage [17,18].

The current study was to investigate the protease activity and kinetics in two plants that used widely in folk medicine: *Mentha piperita* L. and *Thymus capitatus* L. *Mentha piperita* L. (Mentha) is a highly aromatic perennial plant, one of the most widely consumed herbs belonging to family Lamiaceae. Mentha has an astringent, antiseptic, antipyretic, antispasmodic, anticatarrhal, rubefacient, stimulant, emmenagogue, antiproliferative [19,20], and antimicrobial [21].

Thymus capitatus L. (Thymus) is a genus containing about 350 species belongs to the family Lamiaceae. Thyme leaves have been used in foods for the flavor, aroma, and preservation of meat, fish, and food products [22]. It possesses biological properties: Antimicrobial activity, antifungal activity, and antiseptic and antioxidant activity, and regulate gene expression [23,24].

In the current study, crude plant extracts of *Mentha piperita* L. (Mentha) and *Thymus capitatus* L. (Thymus) used to characterize the activity and kinetic parameters of protease enzymes. A literature review survey indicates that there are no studies or researches about the proteases activity or kinetics in these plant extracts.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

Sodium-potassium Tartrate, casein, albumin, and cupric sulfate pentahydrate (Sigma-Aldrich, Germany). Sodium carbonate and egg albumin (BDH .GPR. England). Phenol reagents (Folin-Ciocalteu) (s.d. fine-CHEM Ltd). Sodium phosphate-3-hydrate and sodium acetate-3-hydrate (DAB, PHEUR, B.P., PH. Franc). Acetic acid glacial (NenTech Ltd., UK). Trichloroacetic acid (TEDIA Company, Inc., USA).

Mentha piperita L. (Mentha) and *Thymus capitatus* L. (Thymus) freshly collected from local markets (once) in September 2014–2015. The plants' aerial parts (leaves) thoroughly washed with distilled water and refrigerated at 4°C until used. The research was done in the biochemistry lab at the Department of Biological Sciences, Mutah University.

2.2. Crude Plant Extract

Forty grams of plant leaves were ground by an electric homogenizer. The suspensions were finely powdered and mixed with phosphate-buffered (0.1 M, pH 7.0). Then, the homogenized mixture was filtered and centrifuged for 10–15 min. The collected supernatant was considered as the crude plant extract (enzyme solution) [1].

2.3. Protein Measurement

Protein concentration determined by the Lowery colorimetric method [25]. The bovine serum albumin was the standard used. The standard curve used to determine the protein amount in the extracts (mg/ml).

2.4. Protease Assay

The protease activity is determined by Fahmy method [17]. Briefly, to an Eppendorf tube, 50 µl of the crude plant extract (enzyme solution) was added to 650 µl of 50 mM phosphate-buffered (pH 6.5) and 100 µl

of egg albumin (as a substrate) at 37°C for 1 h. Then, 200 µl of 10% trichloroacetic acid was added to stop the reaction. After centrifugation, the absorbance of the supernatant measured at wavelength 360 nm using UV-Vis spectrophotometer (Biotech Engineering Management Co. LTD, UK). The activity of protease was defined as the amount of enzyme that hydrolyzes 1 µmol of amino acids (in terms of tyrosine) from egg albumin per minute under the standard assay conditions.

2.5. Influence of pH and Temperature on Enzyme Activity

The acetate buffer (50 mM, pH 2.0–5.0) and phosphate-buffered (50 mM, pH 6.0–10.0) were used to determine the effects of pH on the enzyme activity [3]. The initial velocity data obtained at different pH values used to indicate and determine the possible ionizable groups in the active site of the protease enzyme by using Dixon–Webb's plot [26]. The temperature range 25–60°C was used to perform the effects of temperature on the enzyme activity [3].

2.6. Substrate Specificity and Kinetic Studies

Substrate specificity was determined as a relative proteolytic activity of crude plant extracts of proteases by the Patil method using egg albumin as a substrate with minor modifications. Kinetic experiments performed using varying concentrations of substrate (1–3%) and Line weaver–Burk plot used to determine the K_m , and the V_{max} [27].

2.7. Statistical Analysis

The assays were performed in triplicate and expressed as mean ± standard deviation (SD). The statistical analysis was performed by *t*-test, if *P* < 0.05 was statistically significant.

3. RESULTS AND DISCUSSION

Protease plays a key role in physiological as well as in many pathological processes. Many diseases are linked with deficiency or abnormality of protease enzymes such as Arthritis, cancer, viral infections, and some degenerative diseases. They have found extensive applications in bioremediation processes.

The present study investigated the activity and kinetic parameters of protease enzymes obtained from green leaves of Mentha and Thymus. Egg albumin was used for substrate specificity.

3.1. Estimation of Protein Concentration

The protein concentrations in crude plant extract estimated by the Lowry method. Table 1 shows the protein concentration in the crude extracts of the selected plants. From the standard curve, the protein concentration in the extracts calculated. The highest protein concentration found to be in the Mentha (58.16 µg/ml) and low concentration in Thymus (5.67 µg/ml), relatively.

3.2. Estimation of the Optimum pH and the Ionizable Groups

For Thymus, the profile of pH relative activity ranged between pH 2.0 and 3.5 with maximum relative activity 65% and at pH 6.0–8.0 with maximum relative activity 100% [Figure 1]. Therefore, Thymus has two optimum pH at 3.0 and 6.5. On the other hand, Mentha has two optimum pH at 3.0 and 9.0 with relative activity 100% and 70%, respectively [Figure 1]. In general, the presence of two optimum pH may be due to the presence of isoenzymes for the particular protease.

In the literature, it was reported that the optimum pH for most plants is different and it was ranged from 3.0 to 10.0. For example, Ali *et al.* [28]

reported that *Carum copticum* has two peaks at pH 3.0 and pH 7.0 while for *Allium cepa* has one optimum pH at 10.0 (alkaline). In comparison with fungal proteases, the majority of the pH optimum ranged between 8.0 and 12.0, while for bacteria are varied from one strain to another and ranged between 3.0 and 11.0 [29].

Dixon–Webb’s plot used to indicate and determine the ionization groups in the active site of the protease enzymes. According to the Dixon and Webb’s “protein conformation can be maintained within 4-5 pH units but activity different,” it means that the ionizable groups of amino acids in the active site are affected by pH. The analysis of the Dixon–Webb’s plot of the Thymus and Mentha is shown in Figures 2 and 3, respectively, these figures illustrate the presence of aspartic acid and cysteine amino acids in both of the selected plants, which indicate that proteases in the crude

plant extracts of these plants may belong to the acidic and neutral proteases.

In both Thymus and Mentha, the ionizable groups found to exhibit two types. One type with pKa₁ at pH 6.7 and pKa₂ at pH 7.2 which suggests the presence of the amino acid cysteine in the active site of the enzyme and therefore maybe belong to the neutral proteases group (Cysteine protease), while the other ionizable groups have pKa₁ at pH 3.2, and therefore maybe belong to the acidic proteases group (Aspartic protease) [Figures 2 and 3]. The results of pKas, suggest the presence of aspartic acid and cysteine amino acids in and around the active sites of these protease enzymes [30,31].

Aspartic proteases (EC 3.4.23) are that proteolytic enzymes have two aspartic acids in their active sites. On the other hand, the catalytic mechanisms of the cysteine proteases, it involves the nucleophilic cysteine sulfur group in the catalytic dyad. In general, the cysteine proteases such as Actinidain, Bromelain, Calpains, Caspases, Papain, and others are found in fruits, leaves of different plant families.

Table 1: Protein concentration (µg/ml) in the crude plant extracts of the selected plants. Data presented are Means±SD of n=3 experiments

Sample	Protein concentration (µg/ml)
<i>Mentha piperita</i> L. (Mentha)	58.16±4.5 µg/ml
<i>Thymus capitatus</i> L. (Thymus)	5.67±1.6 µg/ml

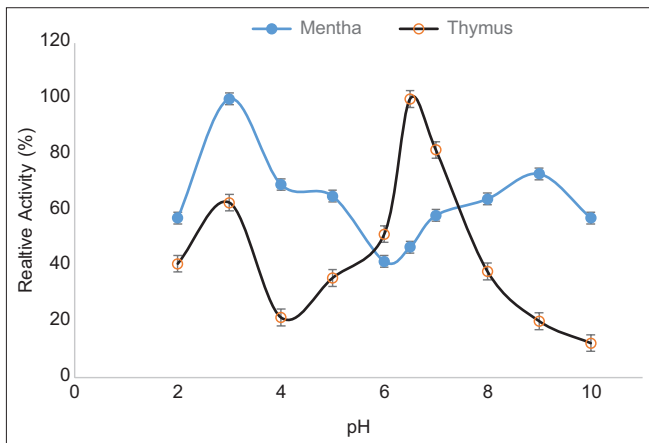


Figure 1: The relative activity (%) of the pH profile of the protease enzymes in crude plant extracts of Thymus and Mentha. Data presented are means±SD of n = 3 experiments

3.3. Estimation of Optimum Temperature

The optimum temperature of an enzyme is considered a very important factor especially for those proteases used in the industry. In general, most industrial enzymes require a higher temperature than physiological enzymes.

Figure 4 shows that maximum relative activity (100%) for proteases of Thymus at temperature 40°C. The activity profile of the temperature for Mentha was different. The range of the activity from 25 to 60°C with the lowest relative activity 60% at temperature 45°C and high activity at temperature 35°C (100%) and relative activity 90% at temperature 50°C [Figure 4]. This thermal stability of proteases may be due to the presence of some metal ions needed for their biological activities [4]. The activity profile for elevation temperature above 50°C for all plants shows a sharp decrease in the activity. The explanation for these results depends on the protein nature of enzymes that are the elevation of temperature above 60°C leads to protein denaturation.

3.4. Determination of K_m and V_{max}

In addition to pH and temperature, substrate concentrations considered as one of the main factors for determining the activity of the enzymes.

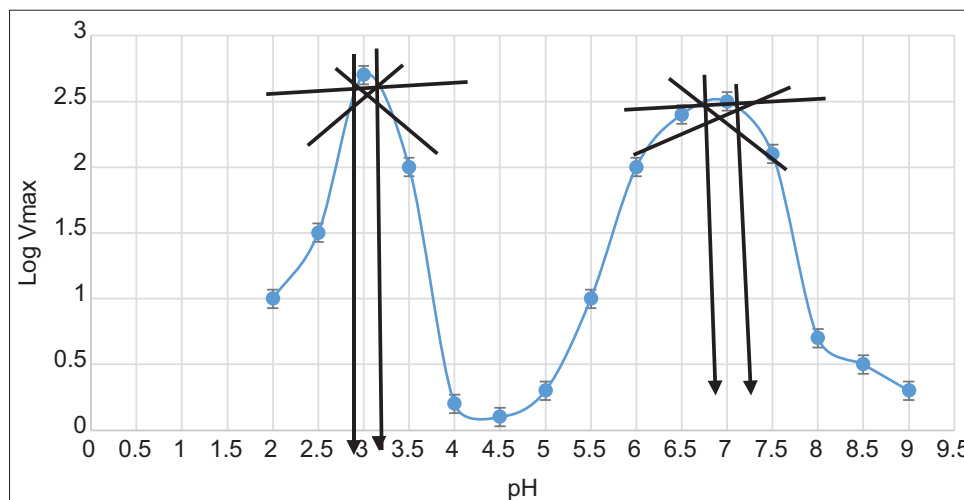


Figure 2: Dixon–Webb’s plot for the determination of possible ionizable groups in the active site of proteases of Thymus using egg albumin as a substrate. Data presented are means ± SD of n = 3 experiments

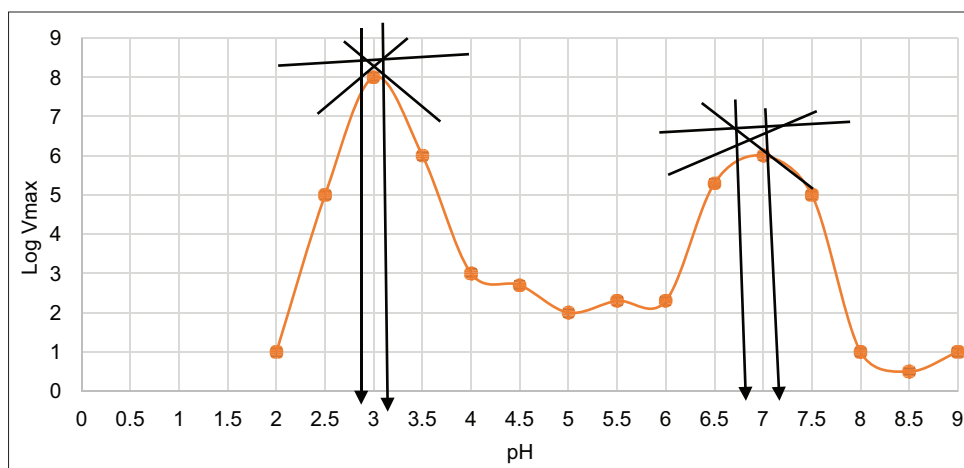


Figure 3: Dixon–Webb's plot for the determination of possible ionizable groups in the active site of proteases of *Mentha* using egg albumin as a substrate. Data presented are means \pm SD of $n = 3$ experiments

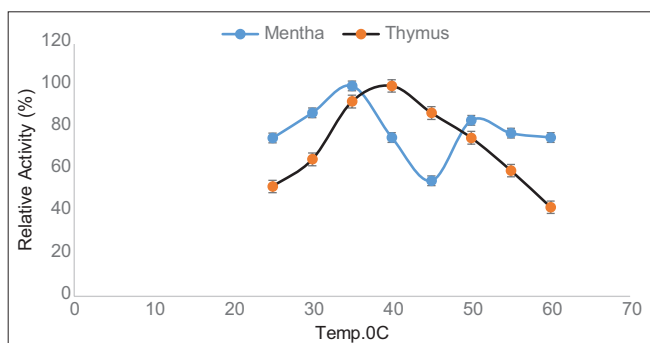


Figure 4: The relative activity (%) of the temperature profile of the protease enzymes in crude plant extracts of *Thymus* and *Mentha*. Data presented are means \pm SD of $n = 3$ experiments

In addition, it can be used for determining the enzyme kinetics (K_m and V_{max}) to illustrate the enzyme affinity and specificity. In the experiments, different concentrations of egg albumin (0.5–3.0%) were used. It was found that V_{max} was reached at a concentration of 3% for the egg albumin substrate.

Line weaver-Burk plots were used to calculate the K_m and V_{max} of proteases enzymes. Figure 5 illustrates the plots of K_m and V_{max} for the proteases of the *Thymus* and *Mentha* and all the results summarized in Table 2. Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) of the protease enzymes were determined using the above substrate.

Studying the kinetics of an enzyme considered a very important value in understanding the affinity and conditions suitable to obtain optimum activity, especially, if these enzymes used for industrial and biochemical applications. Proteases are one of the most groups of enzymes that play a role in many fields of industry.

According to the obtained data from Line weaver-Burk plots, substrate specificity (V_{max}/K_m) was calculated. Results in Table 2 showed different affinities toward egg albumin as a substrate. K_m value is a measure of the affinity of the proteases for the substrate, the smaller the value the higher the affinity of the enzyme for that substrate while V_{max} shows the catalytic efficiency, the higher the V_{max} the higher the efficiency.

In general, the best substrate for any enzyme depends on the strong binding of the substrate to the enzyme (low K_m) and high proteolytic

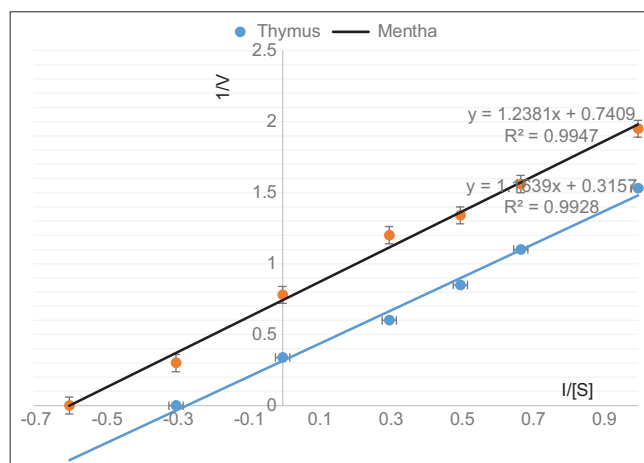


Figure 5: Determination of K_m and V_{max} values for protease enzyme in the crude plant extracts of the *Thymus* and *Mentha* using egg albumin as a substrate. Data presented are means \pm SD of $n = 3$ experiments

Table 2: Comparison between the V_{max} , K_m , and the ratio of V_{max}/K_m for the crude plant extracts (proteases) of the selected plants in means of egg albumin as substrates

Sample	Egg albumin		
	V_{max} (U/mg)	K_m (mg/ml)	V_{max}/K_m
<i>Mentha piperita</i> L.	2.94	3.33	0.883
<i>Thymus capitatus</i> L.	1.3	1.7	0.764

activity (high V_{max}). It was found that the egg albumin has more affinity to toward *Mentha* proteases (K_m 1.7 mg/ml) than *Thymus* proteases (K_m 3.33 mg/ml), while *Thymus* proteases have higher catalytic efficiency (V_{max} 2.94 $\mu\text{mol}/\text{min}$) than *Mentha* proteases (V_{max} 1.3 $\mu\text{mol}/\text{min}$). By considering the ratio (V_{max}/K_m) [Table 2], the observed results indicated that egg albumin was a good substrate for proteases of *Mentha* (0.764) and *Thymus* (0.882).

4. CONCLUSION

The results showed that the egg albumin is a good substrate for the proteases enzyme in the crude plant extracts of *Mentha* and *Thymus*.

The activity and kinetics of these proteases have a strong binding to the substrate with high affinity and hydrolytic activity at 35°C and 40°C, and at pH 3.0 and 7.0, respectively. These results encourage the use of these enzymes in various industrial fields, especially food industries. It is the first study to determine the activity and kinetics of the proteases of these plants and the first to indicate that these proteases belong to aspartic and cysteine proteases groups (endopeptidases).

5. AUTHORS' CONTRIBUTIONS

Part of this research based on Fatmah Alomari's master thesis work. Prof. Omar M. Atrooz was the supervisor, writer, and editor of this paper.

6. CONFLICTS OF INTEREST

Authors declared that there are no conflicts of interest.

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None.

REFERENCES

- Akhtaruzzaman S. Role of thiol proteases in tissue injury. *Am J Resp Crit Care Med* 2012;150:155-9.
- Tran TH, Ton TH, Nguyen PH, Tran TK, Vu TT, Bui LM, *et al.* Expression and Characterization of a new serine protease inhibitory protein in *Escherichia coli*. *Biomed Res Ther* 2020;7:3633-44.
- Ibrahim MA, Olatominwa JO, Aliyu AB, Bashir M, Sallau AB. Partial characterization of protease from the leaves of *Jatropha curcas*. *Int J Biol* 2012;4:79-85.
- Al-Sherri MA, Mostafa S. Products form and properties of protease produced by *Bacillus licheniformis*. *Biol Sci* 2004;7:1631-5.
- Sandhya C, Sumantha A, Pandey C, Webb CR, Soccol C. Proteases in Enzyme Technology. New Delhi, India: Asiatic Publishers Inc.; 2004. p. 312-25.
- Vanndita B, Parth K, Prasadhi T, Kameshwar SY. Enzymatic determination of catechol oxidase and protease from fruits (orange, apple) and vegetables (carrot, tomato). *IOSR J Pharm Biol Sci* 2013;5:29-35.
- Rawlings ND, Morton FR, Barrett T. MEROPS: The Peptidase Database. *Nucleic Acids J Res* 2006;34:D270-2.
- Jovanotti GA, Freitas SM, Silva LP. Proteinaceous protease inhibitors: Structural features and multiple functional faces. *Curr Enzyme Inhib* 2006;2:199-217.
- Christeller JT. Evolutionary mechanisms acting on proteinase inhibitor variability. *FEBS J* 2005;272:5710-22.
- Leary D, Vierros M, Hamon G, Arico S, Monagle C. Marine genetic resources: A review of scientific and commercial interest. *Marine Pol* 2009;33:183-94.
- Pardo MF, Lopez MI, Canals F, Zviles FX, Natalucci CL, Caffini N. Purification of balansain I, an endopeptidase from unripe fruits of *Bromelia balansae* Mez (*Bromeliaceae*). *J Agric Food Chem* 2000;48:3795-800.
- Cheng K, Lu FP, Li M, Liu LL, Liang XM. Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations. *Afr J Biotechnol* 2010;9:4942-53.
- Ivanova D, Emonet C, Foata F, Affolter M, Delly M, Fisseha M, *et al.* A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase like serine proteases. *J Biol Chem* 2006;281:17246-52.
- Otsuki N, Dang NH, Kumagai E, Kondo A, Iwata S, Morimoto C. Aqueous extract of *Carica papaya* leaves exhibits anti-tumor activity and immunomodulatory effects. *J Ethnopharmacol* 2010;127:760-7.
- Padul MV, Tak RD, Kachole MS. Protease inhibitor (PI) mediated defense in leaves and flowers of pigeon pea (protease inhibitor mediated defense in pigeon pea). *Plant Physiol Biochem* 2012;52:77-82.
- Tang J, Li HL, Shen YH, Jin HZ, Yan SK, Liu RH, *et al.* Antitumor activity of extracts and compounds from the rhizomes of *Veratrum dahuricum*. *Phytother Res* 2008;22:1093-96.
- Fahmy AS, Ali AA, Mohamed SA. Characterization of a cysteine protease from wheat *Triticum aestivum*. *Bioresour Technol* 2004;91:297-304.
- Krystyna O, Marlena S. Cellular recycling of proteins in seed dormancy alleviation and Germination. *Front Plant Sci* 2016;7:1128-36.
- Torres-Martínez Y, Arredondo-Espinoza E, Puente C, González-Santiago O, Pineda-Aguilar N, *et al.* Synthesis of silver nanoparticles using a *Mentha spicata* extract and evaluation of its anticancer and cytotoxic activity. *Peer J* 2019;7:e8142-55.
- Felicia P, Alessandro P, Sara V, Loris B, Marco R, Zorzan M, *et al.* Anticancer effects of wild mountain *Mentha longifolia* extract in adrenocortical tumor cell models. *J Front Pharmacol* 2020;10:1647-58.
- Chahrazed B, Amine B, Ryad D, Mahmoud R. The effects of *Mentha × piperita* essential oil on *C. albicans* growth, transition, biofilm formation, and the expression of secreted aspartyl proteinases genes. *Antibiotics* 2019;8:1-15.
- Gruenwald J, Brendler T, Jaenicke C. PDR for Herbal Medicines. 3rd ed. United States: Thomson PDR, Inc.; 2004. p. 815-6.
- Mostafa K, Pezhman M, Mohammad RD, Saeed DN, Pouran K. Effect of thyme extract supplementation on lipid peroxidation, antioxidant capacity, PGC-1 α content and endurance exercise performance in rats. *J Int Soc Sports Nutr* 2017;14:11-9.
- Khalifa FK, Alkhalaf MI. Effects of black seed and thyme leave dietary supplements against malathion insecticide-induced toxicity in the experimental rat model. *J King Saud Univ Sci* 2020;32:914-9.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- Patil UK. Studies on Novel Thermostable and Solvent-tolerant Alkaline Protease from *Bacteria*, Ph.D. Thesis. India: North Maharashtra University, Jalgaon (Maharashtra State); 2010.
- Weaver HL, Burk D. The determination of enzyme dissociation constants. *J Am Chem Soc* 1934;56:658-66.
- Ali S, Qazi A, Khan MR. Protease activity in seeds commonly used as a herbal medicine. *Pak J Med Res* 2003;42:2.
- Hajji M, Kanoun S, Nasri M, Gharshallah N. Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1. *Proc Biochem* 2007;42:791-7.
- Jakubowski H. Biochemistry Online: An Approach Based on Chemical Logic. New York: College of Saint Benedict, Saint John's University. 2013.
- Alfredo A. Screening of *Mentha Cordifolia Opiz* buffer crude extract as a putative HIV-1 translational protease inhibitor. *BMJ Open* 2015;5:A1-53.

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