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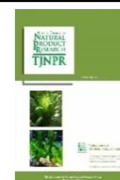

Evaluation of the Anti-inflammatory, Antioxidant, and Protease Inhibitory Activity of the Crude Methanol Extract of *Portulaca oleracea*.

Amasee A. Sohemat[1], Omar M. Atrooz[2], Husni S. Farah[1]*

[1]*Pharmacological and Diagnostic Research Centre, Department of Laboratories Medical Sciences, Faculty of Allied Medical Sciences, Al-Ahliyya Amman University, Amman, Jordan. Post code: 19328*
[2]*Department of Biological Sciences, Mutah University, P.O. Box (7), Zip code 61710. Mutah-Jordan.*

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ABSTRACT

Protease inhibitors have considerable applications in biomedicine, biotechnology, and therapy. The aim of the study was to evaluate the methanol crude extracts of aerial portions of *Portulaca oleracea* (*P. oleracea*) for anti-inflammatory, antioxidant, and protease inhibitor activities, and to evaluate their inhibitory action against various therapeutically significant proteases. A modified colorimetric assay was used to analyze the protease inhibitors on trypsin and other therapeutic enzymes. *P. oleracea* crude leaf extract exhibited 79.92% antioxidant and 75% anti-inflammatory activity. Analysis of the effects of crude *P. oleracea* extract on the values of enzyme kinetics ($K_{m,}$ V_{max}) was determined by Lineweaver-Burk reciprocal plot. It was found that the extract exhibited a potent inhibitory effect on the V_{max} (µmole/min) of the chymotrypsin 26.0, trypsin 18.0, papain 8.33, and elastase 10.0, when compared to control 41.0, 73.0, 11.11, and 20.3, respectively. While no effect on K_m except for elastase which was decreased from 0.026 mM for the control to 0.01 mM. It was found that this effect showed a powerful inhibition in a different way. There is a mixed non-competitive inhibition on elastase as well as pure non-competitive inhibition on chymotrypsin, trypsin, and papain. The crude extract contained 53.3%, 55.7%, 14.9%, and 26.9%, respectively, of protease inhibitors for the therapeutic protease enzymes trypsin, papain, chymotrypsin, and elastase. Therefore, trypsin, papain, chymotrypsin, and elastase have been inhibited by the crude extract of *P. oleracea*. Characterization of the crude extract indicated potent anti-inflammatory and antioxidant effects.

Keywords: Protease inhibitors, *Portulaca oleracea*, Anti-inflammatory, Antioxidant, Plant extracts.

Introduction

Proteases play critical roles in a variety of biological processes, including cell growth and death, signal transmission, and homeostasis, due to their numerous roles in protein maturation, degradation, and post-translational modification.[1] The majority of proteases are produced as inactive zymogens and turn active by a variety of processes, including proteolysis, cofactor binding, posttranslational modification, conformational alteration, or localization modifications, and regulated by the presence of internal protein degradation and inhibitors.[2,3] Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins. The chemical composition of the catalytic groups is the basis for a generally recognized taxonomy of proteolytic enzymes. There are five kinds of proteases: serine, threonine, cysteine, aspartic, and Metallo catalytic forms.[4]
Enzyme inhibitors (EIs) are substances that bind with an enzyme's active site in a permanent (irreversible), temporary (reversible), competitive, or non- competitive way to impede or change the enzyme's normal catalytic action.[5]

*Corresponding author. E mail: h.farah@ammanu.edu.jo
Tel: 00962796535979

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EIs have grown to be a particularly appealing target for drug development and discovery since they are now useful for treating a wide variety of disorders, the majority of which are in clinical use. Examples involve protease inhibitors (PIs) for the treatments of AIDS, monoamines oxidases-A for anxiety, tyrosinases inhibitors for hyperpigmentation, and glycosidase inhibitors for diabetic, amongst many others[6]. Cholinesterase inhibitors are utilized to handle Alzheimer's disease and myasthenia gravis.[6] The ability of natural EIs to specifically bind and block the active sites or modulatory sites of enzymes allows them to self-correct metabolic imbalance or protect against infections and other dangerous organisms.[7] Protease inhibitor activities, or their combinations, are independent prognostic indicators for complex wound healing.[8] Plant substances, such as secondary metabolites, small molecules, and gene-derived proteins, are commonly used in these protective mechanisms. The majority of plant-derived PIs are organic substances such as amino acids or their derivatives, which are proteases inhibitors that rely on proteins or peptides. There are around 6700 plant-derived proteinaceous PIs, which may be divided into at least 12 groups based on structural similarity or sequence homology.[9] Few of these have been studied at the protein level, and many PIs have been discovered by in silico study of nucleic acid sequences using homology.[9] A few PIs have been found to have roles other than inhibiting proteases, such as growth factor activity, receptor clearance signaling, or participation in carcinogenesis.[10]
One of the most important defense techniques utilized by plants to resist predators is the usage of PIs, which are particularly effective against phytophagous insects and microorganisms. The defensive capabilities of plant protease inhibitors (PPIs)are based on the inhibition of proteases found in insect stomachs or generated by microorganisms, resulting in a reduction in the availability of amino acids essential for growth and development.[11] PPIs perform functions such as preventing

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proteolytic degradation of storage proteins in seeds or kernels, allowing for the controlled mobilization of nutrients in the form of amino acids or small peptides, and protecting the plant from herbivores by inhibiting digestive enzymes or hemolysis of the gastrointestinal tract, causing these pests to starve.[12] PIs that are specific and selective can inactivate target proteases in the pathogenic process of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer, and AIDS.[13,14] In addition, they can be used in treatment against SARS-CoV-2 and other coronaviruses[15]. Several studies have found links between PI use and the development of Type 2 diabetes and metabolic syndrome. PIs interact with adipose tissue, causing oxidative stress, which affects adipocytokine production, differentiation, and autophagy.[16]

Medicinal plants have long been utilized as an alternative to contemporary medications because they contain various chemicals including phenols, antioxidants, anti-inflammatory, and other secondary metabolites.[17] Free radicals can be scavenged by antioxidants, which shield cells from their damaging effects.[18] The oxidative stress caused by an imbalance in the generation of reactive oxygen species (ROS) and the ability of cell enzymes to act as antioxidants can be managed by the bioactive components of plants.[19] Polyphenols are the most prevalent antioxidants in human nutrition. They are a group of bioactive substances that can treat and fend off diseases associated with stress. Salvia miltiorrhiza extracts have medicinal benefits for treating inflammation, fibrosis, oxidative stress, and apoptosis. [20,21]

Purslane (global panacea) belongs to the Portulacaceae family. It is a perennial herbaceous weed that grows in both temperate and tropical climates. It has been used as a food and medicine in China for countless years.[22] High in proteins, carbohydrates, calcium, potassium, zinc, and other bioactive compounds, P. oleracea L. is a form of Chinese traditional medicine with a special medicinal purpose. [6] It has been used to treat dysentery with bloody stools, as well as boils and ulcers, dermatitis, erysipelas, and insect and snake bites topically, and other diseases. [22-24]

This study investigated the anti-inflammatory and antioxidant activities and assessed the protease inhibitors found in the methanolic crude extracts of *Portulaca oleracea* and to ascertain how these protease inhibitors affect therapeutic proteases.

Materials and Methods

Experimental procedure and study design

The plant samples were obtained from local markets in Jordan (Al-Karak) from March to May, 2022. The plant was classified by Prof. Saleh Al-Quran (Department of Biological Sciences, Mutah University, Jordan). Thirty-five grams of dry aerial parts of plant were homogenized in an electronics blinder machine for 3-5 minutes by adding 150 mL of methanol solution (80%, v/v). The resultant solution was filtered using cloth sheets and filtering papers. Ultrasonication and centrifugation was used to homogenize the solution for 15-20 minutes at 1500 rpm (Manufacturer: B. Herml AGD-7209 Gosheim) until it was completely clear and sediment-free. The top layer, known as the supernatant, was then collected and evaporated by rotary evaporator to dryness (5.8 g).

Determination of Anti-inflammatory activity

The anti-inflammatory activity was determined using previously reported method. [25] The testing solution (0.5 ml) contained 0.05 ml of sample extracts of *P. oleracea* and 0.45 ml of bovine serums albumins (0.5% w/v aqueous solutions). The samples were then incubated for a total of 20 minutes at 37 °C, then 2.5 ml of phosphates buffers (pH 6.3) was added. A spectrophotometer ((Biotech Engineering Management Co. Ltd., UK) was used to detect the absorbance at 660 nm. The reference was a representation of 100% denaturation of proteins. 0.05 ml of distilled water was utilized in place of the plant extract samples in the testing control solution (0.5 ml), whereas 0.45 ml of BSA and the testing solution (0.05 ml) were used in the final control solution (0.5 ml). The following equation was used to calculate the percentage inhibition of protein denaturation [26].

Percentage of anti-inflammatory=100-(A_T - A_P /A_c) *100%

Where A_T is the test solution, A_P is the product control and A_c is the test control.

Determination of the Antioxidant Activity

The colorimetric method was used to explore the potential of the *P. oleracea* extract to reduce the radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) [25]. The reaction mixture consists of 1.0 ml of DPPH solution (60 µM in methanol) and 50 µl of the *P. oleracea* extract. After incubation at room temperature for 30 minutes, the optical densities were read against methanol solution as a blank sample at 517 nm. The positive control used was Gallic acid. The percentage of antioxidant activity = (Ac –As/ Ac) *100%, where Ac is the control and As is the sample.

IC_{50} Calculation

IC_{50} represents the concentration at which the crude extract of *P. oleracea* (g/mL) exerts half of its maximal inhibitory effect as anti-inflammatory and antioxidant activity. The IC_{50} was calculated by IC_{50} calculator (AAT Bioquest).

Enzyme inhibitory assay

The protease activity was measured using 1% (w/v) casein as substrate with slight modifications.[26] The proteolytic reaction mixture contained 1.0 mL of enzyme solution (chymotrypsin, trypsin, papain, elastase), 1 ml of 0.1 M phosphate buffer (pH 7), and 2 ml of 1% casein, and 1 mL of the methanolic extract of *P. oleracea*. The reaction was initiated by adding the enzyme solution to the reaction mixture. Then mixed and incubated at 37˚C for 30 minutes in an agitated water bath. The addition of 5.0 ml of trichloroacetic acid (0.4 M), and a 10-minute centrifugation at 10,000 rpm stopped the process. Following that, 2.0 ml of sodium carbonate (0.4M), 1.0 ml of supernatant, and 1.0 ml of Folin-phenol Ciocalteu's reagent were combined. For the creation of the standard curve, several tyrosine concentrations were used (0.1mg/mL to 1.0 mg/mL). and the supernatant's absorbance was measured at 750 nm.[26]

The inhibitory activity was calculated using the following formula: Inhibitory activity (%) = $[(Ac - As)\ /Ac] \times 100$, where Ac is the absorbance of control without inhibitor, and As is the absorbance of the test sample with inhibitor.

Enzyme Kinetics

The effect of crude extract on the enzyme kinetic parameters values (Km and Vmax) of therapeutic enzymes were analyzed and determined at different concentrations of casein (0.025- 2.0%). The Lineweaver-Burk reciprocal plot was used to determine the Km and V max values. [27]

Statistical Analysis

The experimental results were obtained in triplicate. Microsoft Excel 2016 software was used to express the data as mean ± SD, n = 3.

Result and Discussion

The extraction yield of the crude methanol extract of *P. oleracea* was 16.6 % (w/w). The crude leaf extract of *P. oleracea* was found to possess 79.92% of antioxidant power by using the DPPH radical scavenging method, and the IC_{50} regression results were 1.5668 µg/mL (Figure 1). One of the studies indicated that the protective effects of *P. oleracea* extract could be through its antioxidant activity.[32] The phytochemical composition of *P. oleracea* of phenolic compounds and some vitamins were the main source for the antioxidant properties [33]. Furthermore, it was found that crude leaf extract of *P. oleracea* has 76.77% anti-inflammatory activity, with the IC_{50} value of 0.3506 µg /mL (Figure 1). Our results are consistent with those of a prior investigation that *P. oleracea* extracts exhibit a potent antioxidant and anti-inflammatory activity.[28] It was reported that the hydro-alcoholic extract of *P. oleracea* extract significantly decreased the concentration of both pro-inflammatory cytokines TNF-a and IL-6 in lipopolysaccharide-stimulated human peripheral blood mononuclear cells[28]. Moreover, similar results were obtained on lipopolysaccharide-stimulated macrophages.[29] In the anti-inflammatory experiment, bovine serum albumin was used for protein denaturation, which causes

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functionality loss[30]. In fact, denaturing proteins contribute significantly to inflammation, the primary source of health issues in humans. The exploration of anti-inflammatory effects usually uses protein denaturation assays as models. [31]

Analyzing the effects of *P. oleracea* crude extracts on some enzyme's kinetics reveal a change in the enzyme kinetics (Vmax and Km) as illustrated by Lineweaver-Burk reciprocal plot for the determination of K_m and V_{max} values. The chymotrypsin enzyme's kinetics in the presence and absence (control) of crude plant extract were found that the Vmax and Km displayed different values in the presence of *P. oleracea* crude extracts (26 µmol/min, 0.04 mM) than those observed in the control (41 µmol/min, 0.04 mM) (Figure 2). While different values of Vmax and Km were obtained in the presence of the trypsin enzyme and the crude extracts of *P. oleracea* (18 µmol/min, 0.01 mM) than those recorded for the control (73 µmol/min, 0.0105 mM) (Figure 3).

In addition, the Vmax and Km values were different in the presence of the enzyme's papain (Figure 4) and elastase (Figure 5) from control (8.33 µmol/min, 0.01 mM) and (10.0 µmol/min, 0.01 mM), respectively.

PIs are widely used in biomedicine, biotechnology, and therapy. Many protease inhibitors have recently been used in the treatment of infections, systemic, immune, inflammatory, respiratory, cancer, AIDS, covid-19, and other diseases. [34] This is the first study that screens crude extracts of *P. oleracea* for the protease inhibitors and to determine their effects on proteases with therapeutic importance.

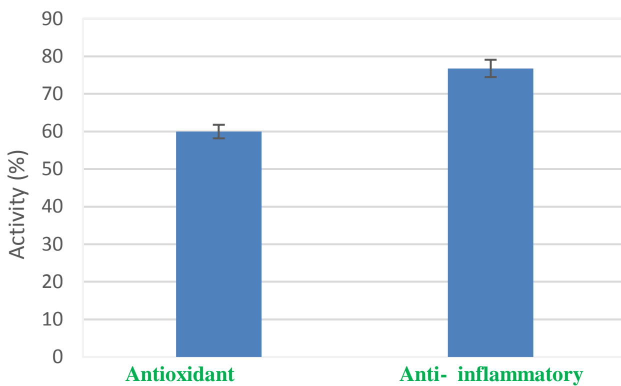

Figure 1: The percentage of the DPPH scavenging activity and anti-inflammatory activity of methanol crude extracts of *P. oleracea*. Mean± SD, n=3.

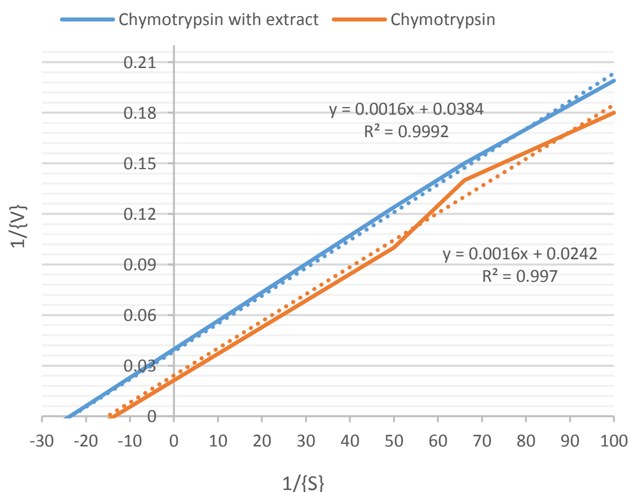

Figure 2: Lineweaver-Burk reciprocal plot for the determination of K_m and V_{max} values for chymotrypsin enzyme in the presence and absence of crude extract of *P. oleracea*.

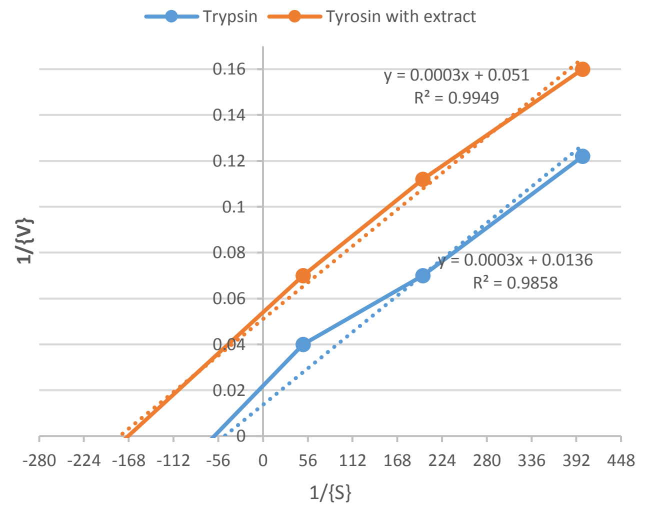

Figure 3: Lineweaver-Burk reciprocal plot for the determination of K_m and V_{max} values for trypsin enzyme in the presence and absence of crude extract of *P. oleracea*.

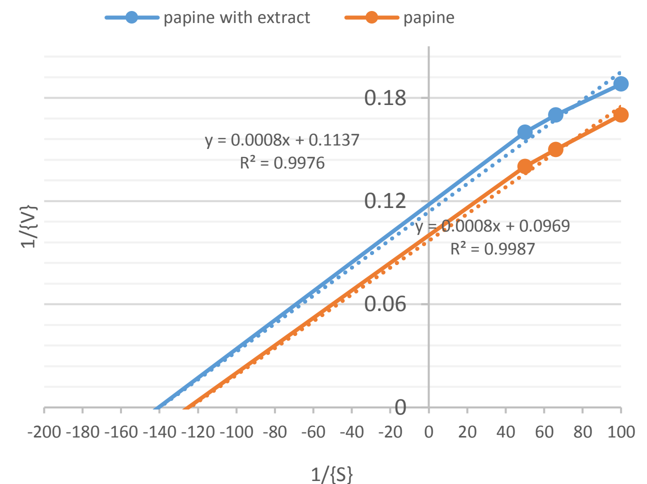

Figure 4: Reciprocal plot for the determination of K_m and V_{max} values for papain enzyme in the presence and absence of crude extract of *P. oleracea*.

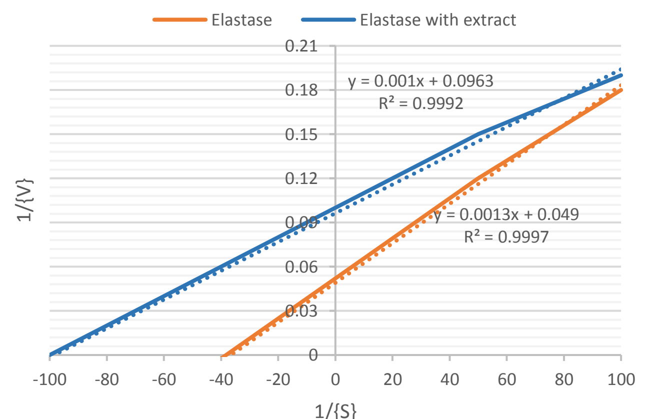

Figure 5: Reciprocal plot for the determination of K_m and V_{max} values for elastase enzyme in the presence and absence of crude extract of *P. oleracea*.

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According to the current study, K_m and V_{max} of trypsin, papain, chymotrypsin, and elastase were higher in the presence of crude extract than in the absence of crude extract (control) (Figures 2-5).
Also, it was investigated whether the crude extract of P. *oleracea* might suppress the action of trypsin, papain, chymotrypsin, and elastase. Figure 6 illustrates how *P. oleracea* can block trypsin, papain, chymotrypsin, and elastase activity in various ways. Therefore, *P. oleracea* crude extract had a strong inhibitory effect on therapeutic enzymes. According to the findings of this study, *P. oleracea* has the ability to inhibit trypsin, papain, chymotrypsin, and elastase activity through non-competitive inhibition. So, protease inhibitors in *P. oleracea* crude extract inhibited the V_{max} of trypsin, papain, chymotrypsin, and elastase but had no effect on Km except for elastase. Furthermore, the PIs of *P. oleracea* prevent the proteolytic action of proteases in different inhibitory activity (Figure 6). It was illustrated that the extract has a potent high inhibitory effect on papain 55.7% and trypsin 53.3%, while it has a low effect on elastase 26.9% and chymotrypsin 14.9%. PIs have been considered to be the safest alternatively treatment for herbivorous pests [35]. Extracts from *L. sativus* and *V. faba* inhibited the proteolytic activities of *H. cunea* by 34.72% and 22.27%, respectively. *H. cunea* protease activities, on the other hand, were barely inhibited by inhibitors isolated from *P. farcta* and *P. miliaceum*. Extracts of inhibitors from *L. sativus* and *V. faba* significantly inhibited trypsin activity. Inhibitors isolated from various chickpea types reduced 66% of the trypsin activities in *Helicoverpa armigera* (Hübner) [36]. Transgenic plants containing PIs have been shown to slow the growth and development of *Heliothis virescens* (Fabricius) [37], *Pieris rapae* (Linnaeus) [38], *H. armigera* (Fernandes et al., 2019), and *Plutella xylostella* (Linnaeus). [39]

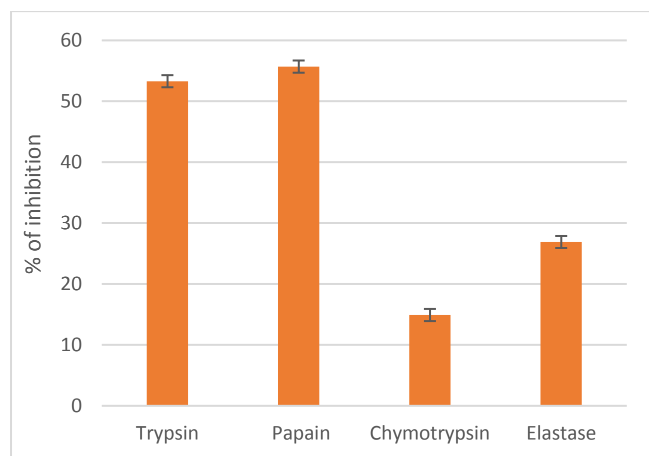

Figure 6: Inhibitor activity (%) of crude extracts of *P. oleracea* on different therapeutic protease enzyme. Mean ± SD, n = 3.

Conclusion

The results showed that crude extracts of *P. oleracea* have a potent antioxidant activity as demonstrated by the DPPH method and high efficient percentage of anti-inflammatory activity. The presence of PIs in plant crude extract inhibited a variety of therapeutic proteases, including trypsin, papain, chymotrypsin, and elastase, in various ways. Overall, our findings showed that the protease inhibitors found in *P. oleracea* crude extract have a non-competitive inhibitory effect.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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