

Characterization of the crude extract of *Portulaca oleracea* and the determination of the polyphenol oxidase kinetics in the presence of Cu and Zn

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ABSTRACT

The aqueous crude extract of purslane (*Portulaca oleracea* L.) was characterized for its antioxidant activity, total amounts of proteins, and phenol content. Furthermore, the kinetic parameters (V_{max} and K_m) of the enzyme polyphenol oxidase (PPO) in the crude extract of purslane were analyzed in the presence of the heavy metals (HM) Cu and Zn. Results indicated that the crude extract of purslane contains 0.847 mg/ml amount of proteins and 107.88 mg/ml amount of phenolic compounds. The extract's ability to scavenge free radicals was demonstrated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, results, which showed 48.8% improvement over the control gallic acid. The optimal temperature for PPO activity was 40°C, and the optimal pH was 6.0. Moreover, results showed a high browning intensity after incubation for 100 minutes. Kinetic studies illustrated the marked effects of the HM Cu and Zn on the studied kinetic parameters of PPO by performing changes in the values of V_{max} and K_m . These HM can act as noncompetitive or uncompetitive inhibitors on enzyme kinetics. In conclusion, depending on the type and concentration of Cu and Zn, the presence of these HM reduces PPO action. Further investigation is needed to show the effects of other HM on PPO activity in purslane extracts.

1. INTRODUCTION

The demand for medicinal plants has increased recently because they play an important role in ecosystems. Medicinal plants contain different compounds such as phenols, antioxidants, and other secondary metabolites, and therefore have long been used in alternative treatments for modern medicines [1]. Antioxidants are chemicals or substances that scavenge free radicals and protect cells from their harmful effects [2]. Superoxide radicals, hydrogen peroxide, and hydroxyl radicals are examples of free radicals that have adverse effects on almost all biochemical compounds such as proteins, DNA, and carbohydrates [2,3]. Antioxidant compounds are becoming increasingly important in medicinal chemistry because they perform a protective role by scavenging free radicals and inhibiting the oxidation of molecules [4]. Some fruits and vegetables contain antioxidant compounds, vitamin E,

vitamin C, and carotenoid [5]. Phenols and flavonoids are the most common antioxidant compounds found in plants [6]. Phenolic compounds are biological molecules that have an essential role in plant growth, development, and defense. Structurally, it consists of a hydroxyl functional group with a ring structure like benzene [7]. The redox properties of phenolic molecules enhance the antioxidant activity and can play a crucial role in neutralizing free radicals and decomposing peroxides [8].

Heavy metals (HM) are divided into two categories: essential and nonessential. Fe, Mo, and Mn are critical micronutrients among these metals. While Hg, Ag, Sb, Pb, and others have no known nutritional value and may be hazardous to plants and microorganisms [9], they may be toxic to plants and microorganisms. Most HM, especially Cu and Zn, either serve as cofactor and enzyme reaction activators or exert catalytic properties such as the prosthetic group in metalloproteinase [10]. Zn is a vital micronutrient affecting several plant metabolic processes. Zn's phytotoxicity manifests as a restriction in growth, development, and metabolism, as well as increase in oxidative damage in a variety of plants [11]. Cu is an essential micronutrient that plays a vital role in CO₂ assimilation and ATP synthesis. Cu can

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inhibit respiration, adversely affects nitrogen and protein metabolism, decreases chlorophyll content, and inhibits photosynthetic activity in leaves [12]. Cu can catalyze the production of hydroxyl radicals from nonenzymatic reactions and alleviate oxidation stress and may enhance the antioxidant response in plants [12].

Portulaca oleracea L. is a herbaceous succulent plant that grows in temperate and tropical climates around the world and is used to treat bug bites, dermatitis, and ulcers in traditional Chinese medicine [11,13]. It has the advantage of containing a high amount of ascorbic acid, α -linolenic acid, β -carotene, proteins, carbohydrates, and minerals such as Na, Ca, Zn, and K [13,14]. The leaves and stems are among the edible parts, as they have a slightly sour taste similar to the taste of spinach. *Portulaca oleracea* is an abundant source of omega-3 fatty acids and antioxidant compounds, particularly flavonoids [15]. This plant is used in many countries as a diuretic, febrifuge, antiseptic, antispasmodic, and vermifuge [11,14].

The enzyme polyphenol oxidase (PPO) is considered a copper-containing enzyme that oxidizes phenolic molecules to o-quinone [16,17]. Tyrosinase, catechol oxidase, and laccase are the three primary forms of PPO found in plants, and they are categorized based on their method of action and substrate specificity [17].

Because of contamination of the environment by some HM and their accumulation in all parts of plants and the cause of harmful effects on plant growth and development, it is necessary to characterize some plant extracts and study the effects of some HMs on some enzyme kinetic parameters. Therefore, the main aim of this study was to measure and analyze the total amounts of phenols and proteins in the crude purslane extract and evaluate its antioxidant activity and to investigate the browning intensity and Zn and Cu's effects on the enzyme kinetics (V_{\max} and K_m) of PPO in purslane extract.

2. MATERIALS AND METHODS

2.1. Preparation of Crude Plant Extract

The plant samples of purslane were obtained from Mutah city markets (Al-Karak, Jordan) between February and July 2020. Fifty grams of fresh aerial parts of the plant was homogenized with the addition of phosphate buffer (pH 7.0) solution in an electronic blender machine for 3–5 minutes. The obtained solution was primarily purified by filtration using cloth sheets and filter papers. The solution was homogenized by centrifugation at 1,500 rpm for 10–15 minutes (Jepson Bolton Co. Ltd., UK). Then, the upper layer (supernatant) was collected and kept at 4°C as crude purslane extract [18].

2.2. Total Proteins

The total proteins in the crude extract of purslane were determined using the Lowry method. The calibration standard curve was created using gradient concentrations (0.07–2.4 mg/ml) of the standard bovine serum albumin [19].

2.3. Determination of Total Phenols

The total amount of phenols was analyzed by using the Folin–Ciocalteu method [20]. Gradient concentrations of the gallic acid (as standard) from 0.5 to 4.0 mM were used to obtain the calibration

standard curve. Briefly, the mixture contains 200 μ l of 10% of Folin–Ciocalteu reagent, 100 ml of crude extract of purslane in phosphate buffer (pH 7.0), and 800 ml of 700 mM Na_2CO_3 . The mixture was incubated for 30 minutes at room temperature. Then, the optical densities were read at 760 nm (Biotech Engineering Management Co. Ltd., UK). The total phenolic amounts were expressed as equivalents of the gallic acid in milligram per gram of the sample extract.

2.4. Determination of the Antioxidant Activity

The colorimetric method of Bal *et al.* [21] was used to explore the efficiency of the purslane extract to reduce the radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The reaction mixture consists of 2.0 ml of DPPH solution (60 μ M in methanol) and 50 μ l of purslane extract. After incubation at room temperature for 30 minutes, the optical densities were read against methanol solution as a blank sample at 520 nm. The positive control used was gallic acid. Antioxidant activity (%) = $(A_c - A_s) \times 100\%$, where A_c is the control and A_s is the sample.

2.5. Browning Intensities

For each sample, a cuvette was filled with 1.5 ml of freshly prepared enzyme solution. The optical density (absorbance) was measured at a wavelength of 410 nm by a spectrophotometer at intervals of 20 for 100 minutes. The absorbance was taken as the browning intensity [18].

2.6. The Effects of Substrate Concentrations on PPO Activity

The PPO activity was measured using different concentrations (0.01–0.2 M) of the substrate catechol at pH 6.0 and temperature 40°C. The sample tube contained 2.0 ml of desired concentration of catechol (0.01–0.2 M), 0.1 ml of enzyme solution, and 0.9 ml of 0.2 M phosphate buffer. The absorbance was read at 410 nm against a blank tube containing only the catechol and buffer. One unit of PPO activity was defined as the change in absorbance of 0.001 minute^{-1} [22].

2.7. The pH and Temperature Effect of pH on PPO Activity

The influence of pH on PPO activity was tested using acetate and phosphate buffer at varying pH values (3, 4, 5, 6, 7, 8, and 9). The effects of temperature in the range of 25°C to 100°C (25°C, 40°C, 60°C, 80°C, and 100°C) were utilized to establish the optimum temperature under standard conditions of catechol concentration and optimum pH value [18,22]. These enzymes' reaction rates were measured, and the relative activity (%) was calculated.

2.8. Enzyme Kinetics

The kinetic parameters values (K_m and V_{\max}) of PPO were analyzed and determined at different concentrations of catechol. The PPO activity was monitored at 410 nm, and the K_m and V_{\max} values were obtained from the Lineweaver-Burk plot.

Fresh stock solutions of Cu sulfate and Zn sulfate (0.1 and 0.3 M) were prepared. The reaction mixture contained 2.0 ml of catechol

(20 mM), 0.4 ml of phosphate buffer, 0.5 ml of heavy metal solution, and 0.1 ml of enzyme solution [23].

2.9. Statistical Analysis

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

3. RESULTS

3.1. Total Proteins and Total Phenolic Compounds

Purslane's crude leaves extract was tested to determine the amount of proteins in the sample. The crude extract has a concentration of 0.847 ± 0.021 mg/ml, according to the data. The phenol content of the crude extracts of leaves of purslane was measured using gallic acid as the standard. The results showed that the crude extract of purslane has a considerable amount of phenols compared to the gallic acid as the control (107.88 mg/ml).

3.2. DPPH Radical Scavenging

The crude leaves extract of purslane was found to possess 48.8% of antioxidant power by using the DPPH radical scavenging method.

3.3. Browning Intensity

This study showed a relationship between the time and browning intensity of the crude extract of purslane. High browning intensities were found (3.0 ± 0.1) after 60 minutes of incubation, and it was turned out that the browning intensities increased over time, as illustrated in Figure 1.

3.4. Temperature and pH Effects on PPO Activity

The influence of pH on PPO activity was investigated by measuring its activity at various pH levels (3.0 to 9.0). The enzyme's optimum activity of pH in the crude extract of purslane was seen at pH 4.0 and 6.0 (Fig. 2). The relative activities of PPO at pH 4.0 and 6.0 were

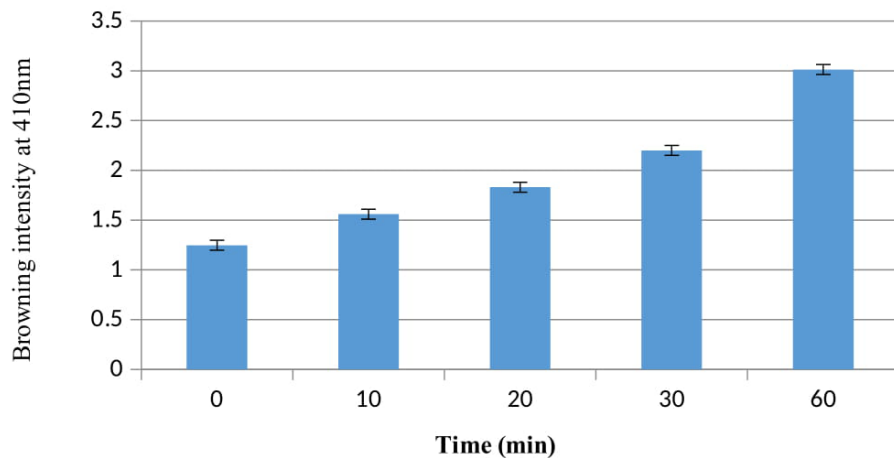


Figure 1: Browning intensity (optical density) of crude extract of *P. oleracea* with time (minute) at 410 nm. Mean \pm SD, $n = 3$.

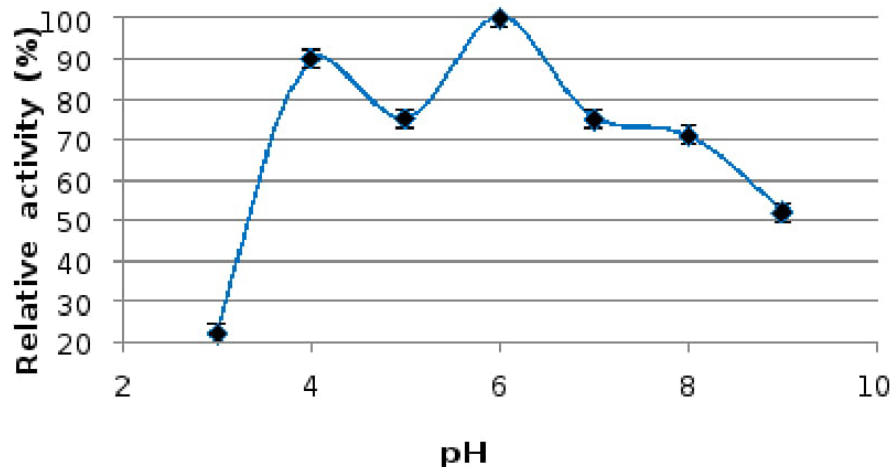


Figure 2: Relative activity (%) of PPO in the crude extract of *P. oleracea* at different pH values. Mean \pm SD, $n = 3$.

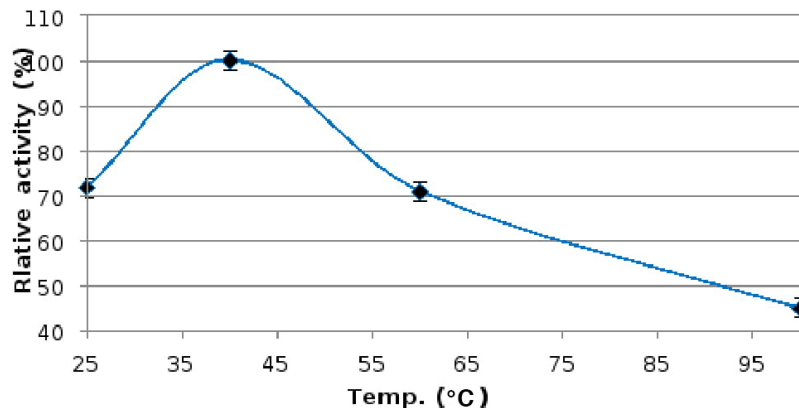


Figure 3: Relative activity (%) of PPO in the crude extract of *P. oleracea* at different temperature values (°C). Mean \pm SD; $n = 3$.

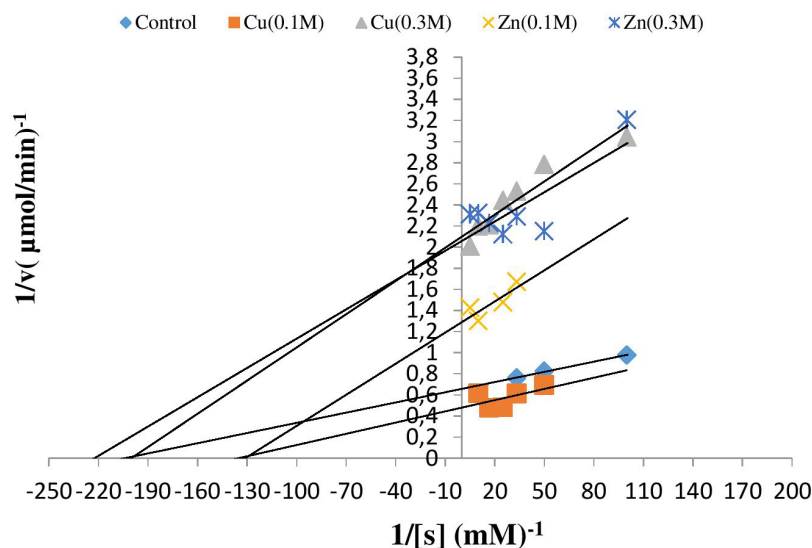


Figure 4: Lineweaver-Burk reciprocal plot for the determination of K_m and V_{max} values for PPO in the crude extract of *P. oleracea* in the presence and absence of HM (Cu 0.1 M, Cu 0.3 M, Zn 0.1 M, and Zn 0.3 M) using catechol as a substrate. Mean \pm SD, $n = 3$.

90% and 100%, respectively. The optimal temperature value of the PPO activity in the crude extract of purslane was 40°C (Fig. 3).

3.5. Analysis of Enzyme Kinetics

The effects of the presence and absence of Cu and Zn metals on PPO kinetic parameters were determined using the initial reaction rates at varying concentrations of the substrate catechol (0.01–0.2 M). The values of K_m and V_{max} were obtained by the Lineweaver-Burk reciprocal plot (Fig. 4). The results of K_m and V_{max} values of the enzyme are summarized in Table 1.

4. DISCUSSION

4.1. Characterization of the Crude Extract of Purslane

Characterization of the crude extract of purslane according to the amount of proteins was revealed to contain a low amount of

proteins. In the literature, the amount of protein varies from plant to plant, with Cowpea containing 0.23 to 0.28 mg/ml [24] and *Ocimum canum* containing 10.59 mg/ml [25]. In addition, the results showed that the crude extract of leaves of purslane has a considerable amount of phenols compared to other plant extracts. Previous studies have shown that the amount of phenols varied between plants, such as *Melilotus officinalis* 289.5 mg/ml, *Urtica dioica* 24.1 mg/ml, and green tea 31.95 mg/ml [25].

The DPPH technique was used to evaluate the antioxidant activity of the crude leaves extract of purslane. It was shown to exhibit a moderate percentage of DPPH radical scavenging activities compared to the gallic acid as the standard. DPPH assays are the traditional assays for evaluating the antioxidant activity of food products. Literature survey indicated that the DPPH radical scavenging activities varied between plant extracts; for example, it was 31.5% in *Styrax formosana* 31.5% and 54.74% in *Artocarpus*

Table 1: Kinetic values for PPO in the crude extract of *P. oleracea* in the presence and absence of Cu and Zn using catechol as a substrate at 40°C and pH 6.

Control/HM	K_m (mM)	V_{max} (μmol/minute)	Effects
Control	5.00	1.538	Normal
Cu (0.1 mM)	7.5	2.083	Activation
Cu (0.3 mM)	5.05	0.476	Noncompetitive
Zn (0.1 mM)	7.6	0.775	Mixed noncompetitive
Zn (0.3 mM)	4.00	0.476	Uncompetitive

lacucha Buch., while it was 33.03% in *Hopea odorata*. The amount of total phenolic compounds in the plant could explain the differential in antioxidant action. Flavor, color, and shape are three characteristics that consumers consider when purchasing vegetables and fruits. These characteristics are used to assess the quality and suitability of food. The color may be influenced by natural pigments, such as chlorophylls and carotenoids in food, or by pigments resulting from enzymatic or nonenzymatic reactions. Enzymatic browning results from PPO catalyzed oxidation of mono- and diphenols to o-quinines [4].

This study showed a relationship between the time and browning intensity of the selected plant's crude extract. High browning intensities were found in purslane after 6.0 hours of incubation (data not shown). It was illustrated that the browning intensities increased over time (60 minutes). These results could be demonstrated by the level of PPO activity and the amount of phenolic compounds that can vary between plants. The findings were consistent with those by González *et al.* [26], Atrooz *et al.* [1], and Qi *et al.* [27], who reported that PPO activity and phenolic content are the most important determinants in browning reactions.

4.2. Enzyme Activity and Kinetics

The enzyme's optimum activity in the crude extract of purslane was seen at pH 4.0 and 6.0. The relative activities of PPO at pH 4.0 and 6.0 were 90% and 100%, respectively. The optimum pH depends on enzyme source and purity, substrate, and buffer system used.

In many vegetables and fruits, PPO enzyme has one optimum pH value, such as *Rumex obtusifolius* L. 7.0 [28] and Banana fruit 6.5 [29], but in some plants has two optimum pH values, such as lentil 4.5–5.5 [30]. Some plants have two optimum pH values due to the presence of isoenzymes [30].

It was illustrated that the optimal temperature for the PPO activity in the crude extract of purslane was 40°C. At 25°C, the relative activity was 71.8%. Raising the temperature above 45°C caused a remarkable decrease in the action due to the effect of denaturation. Thyme had an exact optimal temperature of 40°C [31], while apple juice had a higher optimum temperature of 50°C and 60°C [32], but raisins had a lower optimum temperature of 25°C [33], and eggplant fruit had a lower optimum temperature of 30°C [34]. The HM Cu and Zn's criteria on PPO kinetic parameters of V_{max} and K_m values in the crude plant extract of purslane (treated extract) illustrate different values from untreated extract (control). When compared to the control (V_{max} 1.538 mol/minute), only Cu at a low concentration (0.1 M) has an active effect on PPO activity (V_{max} 2.083 mol/minute). Other

HMs exhibited an inhibition effect on PPO activity (V_{max}) in the following order: Cu (0.3 M) = Zn (0.3 M) < Zn (0.1 M) < control. The type of inhibition is different. For example, Cu (0.3 M) exhibits a noncompetitive, and Zn (0.1 M) exhibits a mixed noncompetitive inhibition. Because V_{max} in both was decreased, K_m values may be the same or increased, respectively, while Zn (0.3 M) exhibited uncompetitive inhibition due to a decreased V_{max} (0.476 μmol/minute) and K_m (4.0 mM) in contrast with control 1.538 μmol/minute and 5.0 mM, respectively.

HM in high concentrations may exert toxicity in plant leaves or reduce metabolic activity or growth. For example, Zn toxicity causes chlorosis in younger leaves, inhibits plant growth, reduces metabolic activity, and causes oxidative damage to some plants [9]. Cu toxicity affects photosynthetic activity and nitrogen and protein metabolism [12].

5. CONCLUSION

The experiments were conducted to characterize the crude leaves extract of purslane and to analyze the Cu and Zn metals' effects on both PPO activity and kinetics in the extract. It was found that proteins' amounts were low, while the total phenols in purslane have a considerable amount, which would affect the antioxidant activity. When compared to the control (V_{max} 1.538 mol/minute), only Cu at a low concentration (0.1 M) has an active effect on PPO activity (V_{max} 2.083 mol/minute). Interestingly, the results of browning intensity and DPPH-scavenging activity in purslane correlated with total phenols. The presence of Cu and Zn metals causes inhibition effects (noncompetitive and uncompetitive) on the PPO activity depending on the type and concentration of Cu and Zn. Only Cu at a low concentration (0.1 M) has an activation effect. Therefore, further testing of the effects of other HMs on PPO kinetic parameters is recommended.

6. CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

7. AUTHORS' CONTRIBUTIONS

Part of this research was based on master thesis work of Shada Al-Maitah. Prof. Omar M. Atrooz was the supervisor, writer, and editor of this paper.

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9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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