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Effect of pH extraction buffer on antioxidant enzymes activities in water lily's leaves and petioles

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Abstract

Water lily (*Nymphaea antares*) is one of the most valuable aquatic ornamental plants which has bright potential in the floriculture industry. It may be useful as an urban ecosystem and as a source of medicinal compounds. Due to its potential to become a new value-added product in the food industry, water lily (*N. antares*) was investigated in this study. Therefore, the goal of this study was to determine the nutritional content and antioxidant activity in water lily leaves and petioles with different pH extraction buffers. Water lily extract was obtained using three different pH extraction buffers, Tris buffer at pH 6.8, pH 7.1 and pH 7.8. The heat capacity of the extract was analysed using differential scanning calorimetry (DSC) and different functional groups were identified using Fourier transform infrared spectroscopy (FTIR). Thermal denaturation of the leaves sample was detected at 81.84°C. The antioxidant enzymes activities including catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO), and superoxide dismutase (SOD) were determined in the leaves and petioles of water lily. In leaves, PPO activity was found to be higher in samples with pH 7.1 of the extraction buffer while there were no significant differences for activities of CAT, POX and SOD in all samples. In petioles, PPO and POX activity were found to be higher in samples with pH 7.8 and 7.1 of extraction buffer, respectively. Thus, the study found that a pH range of 7 to 9 extraction buffers did not greatly affect most of the analysis performed.

1. Introduction

Native aquatic plants generally grow and adapt to a particular environment that is at least frequently filled with water, such as in a wetland, as these aquatic plants grow in permanently or seasonally wet environments (Knight *et al*., 2014). One of the species included in this group is water lily (*Nymphaeceae*) (Lacoul and Freedman, 2006). Water lily (*Nymphaea antares*) is one of the most valuable aquatic ornamental plants which have a bright potential in the floriculture industry (Pareek and Kumar, 2016). Water lily is an aquatic herb of genus *Nymphaea,* from family *Nymphaeaceae* (Chen *et al*., 2017)*,* a precious perennial aquatic flower plant that is divided into two ecological classes, namely tropical and hardy water lily (Zhu *et al*., 2012). Water lily is, frequently found along the edges of ponds, lakes, and streams (Crane and Friis, 2020). This plant has various functions for example the whole flowers and roots can be made into tea while the alkaloid-rich leaves have been used medicinally since ancient times (Les, 2002). The whole plant aqueous decoction is used by 'Hausas' in Northern Nigeria for the treatment of Guinea worm infection and 'Yoruba' in Southern West Nigeria for the treatment of rheumatic pain and as an antitumor agent (Ogbadoyi *et al*., 2007). The antibacterial (Akinjogunla *et al*., 2010), antidiabetic (Dodamani *et al*.,

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2012) and antioxidant (Afolayan *et al*., 2013) effects of this plant have also been documented.

Antioxidants are compounds that act as the first defence for our body against free radical damage and are vital in sustaining optimum health and well-being (Zainol *et al*., 2018). Antioxidant compounds act as a major function in our body due to favourable effects on our health (Dian-Nashiela *et al*., 2015). Free radicals or reactive oxygen species (ROS) are compounds formed when oxygen combines with other molecules (Chong *et al*., 2018). There are two groups of ROS that can be found that are oxygen-centred radical and oxygen-centred non-radicals. Oxygen-centred radicals are superoxide ion ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$), alkoxyl radical ($RO^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$). Oxygen-centred nonradicals are hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) (Michalak, 2006). Some of the vegetable and fruit phenols appear to be bound by tight hydrophilic and hydrophobic bonds to the polysaccharides of the plant cell wall (Koley *et al*., 2010). The degradation of the cell-wall polysaccharides can stimulate the release of these phenolics by enzyme-catalysed extraction (Khairil Anuar *et al*., 2020). Supporting enzymes that have often been used as biocatalysts to obtain target compounds from various plants could increase the efficiency of the extraction, yield and biological activities of polysaccharides (Karaki *et al*., 2016).

The functions and advantages of natural antioxidants in food and biological systems have received much public concern. This is due to the benefits of natural antioxidants; besides it is safer and healthier than synthetic antioxidant (Chong *et al*., 2020). Hence, this study aimed to investigate the effects of pH extraction buffers on antioxidant enzyme activities as well the protein characteristic in water lily. Consequently, the information and data obtained from this study can be a reference for further studies and contribute to the development of new value-added products in the food industry as food ingredients or food supplements.

2. Materials and methods

2.1 Sample preparation

Water lily (*Nymphaeaceae antares*) leaves and petiole were harvested from a pond of a private residence situated in Kampung Lorong Bukit, Kuala Terengganu, Malaysia. The plant was identified by Tuan Hj. Mohamad Razali Salam, a plant botanist in the Faculty of Science and Marine Environment (FSSM), Universiti Malaysia Terengganu (Herbarium UNISZA, No: UNISZA0001135). All samples of leaves and petioles of water lily were thoroughly cleaned under running tap water and then drained off on a piece of tissue paper. The cleaned samples were then cut into smaller pieces before undergoing the further process. The chemical substances for analysis purposes are analytical grades such as Tris-HCl, Bovine serum albumin, pyrogallol, benzidine, sodium phosphate and standard protein marker were purchased from Sigma-Aldrich, MO, USA.

2.2 Proximate analysis

Proximate analysis of the leaves and petioles of water lily was conducted based on the standard procedure of the Association of Official Analytical Chemists (AOAC, 2007). The percentage of moisture, ash, crude protein, crude fat, and crude fibre was evaluated. The percentage of carbohydrates = 100-(percentage [protein + moisture + crude fat + ash + crude fibre]) was determined as the carbohydrate content. All the analysis was carried out in triplicate.

2.3 Water lily extraction

Eight grams of water lily leaves and petioles were washed, cleaned and cut into small cubes separately. Samples were thoroughly homogenized at 4°C using a mortar in 70 mL 1M Tris-HCl buffer with 3 different pH treatments (pH 6.8, 7.1 and 7.8). The mortar was surrounded by ice cubes to keep it at a sufficiently low temperature. The homogenates were then centrifuged for 30 mins at 13000×*g* at 4°C (Gyrozen, Korea). The supernatants were kept at -40°C and used for protein determination, protein characterization and antioxidant enzyme assay.

2.4 Protein quantification

The concentration of protein in both leaves and water lily petioles was measured using Bradford (1976) method with bovine serum albumin (BSA) was assigned as standard. The approach is based on a colourimetric assay proportional to the protein binding of the Coomassie blue dye. Each sample was allowed to incubate at room temperature for 20 mins. The absorbance of each sample was measured at 595 nm. The absorbance of each BSA standard was plotted as a function of its theoretical concentration.

2.5 Thermal characteristics

Thermal characteristics of protein in water lily's leaves and petioles were examined by differential scanning calorimeter (DSC Q2000, Research Instrument, Switzerland), following the method as described by Mariod *et al*. (2010) with slight modification. Three microliters of sample were placed in sealed aluminium pans. The scanning temperature was assigned at 30 to 120°C with a heating rate of 10°C/min. The Indium

standards were used for temperature and energy calibration. The denaturation temperature and denaturation enthalpy value was determined from each thermogram (Gorinstein *et al*., 1996).

2.6 Functional groups

The functional groups of water lily samples were determined using Fourier-transform infrared spectroscopy (FTIR) (Nicolet iS10, Thermo Scientific, US) in which 5 mg of water lily dried powder was mixed with 250 mg of KBr (1:50). The mixture was homogenized using an agate mortar and pestle and then was pressed into a pellet (1-2 mm thick films) with a 15-ton hydraulic press. The FTIR spectra were then obtained from a wavenumber of 600 to 4000 cm^{-1} during 64 scans with 2 cm^{-1} resolutions. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample (Zainol *et al*., 2017).

2.7 Antioxidant enzyme assays

2.7.1 Catalase

The catalase activity of water lily's leaves and petioles were analysed followed the method by Khairil Anuar *et al*. (2020). The reaction mixture contains 1.25 mL of 50 mM sodium phosphate buffer (pH 7), 0.15 mL of H_2O_2 (0.5 mL) and 0.1 mL enzyme extract. Blanks contain the same reaction mixture except for enzyme extract. The absorbance of blank was read at 240 nm followed by samples read for every 5 mins. The catalase activity was measured by decomposition of hydrogen peroxide (H_2O_2) as determined by a decrease of absorbance at 240 nm.

2.7.2 Peroxidase activity (POX)

Peroxidase activity (POX) was determined using the method by Khairil Anuar *et al*. (2020). The blank consisted of 4 mL of 0.2 M acetate buffer (pH 4.8), 0.4 mL H_2O_2 (3%), and 0.2 mL 20 mM benzidine. The assay mixture consisted of 4 mL of 0.2 M acetate buffer (pH 4.8), 0.4 mL of H_2O_2 (3%), 0.2 mL of 20 mM of benzidine and 0.2 mL of enzyme extract. The increase in absorbance was recorded at 530 nm. POX activity was described as 1 µM benzidine oxidised per min per mg protein (Unit mg^{-1}) (protein).

2.7.3 Polyphenol oxidase activity

Polyphenol oxidase activity was measured based on the method of Kim and Kim (2013) at 40°C. The reaction mixture consisted of 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 mL of 20 mM pyrogallol and 0.2 mL of enzyme extract. The absorbance was recorded at 430 and PPO activity was described as 1 µM pyrogallol oxidised per min per mg protein (Unit mg-1 (protein)).

2.7.4 Superoxide dismutase (SOD)

Superoxide dismutase SOD activity in water lily's leaves and petioles was determined using the method of Kanungo *et al*. (2015). A reaction mixture consisted of 1 mL of a formulation (27 mL 50 mM sodium phosphate buffer (pH 7.8), 20 µL ethylene diamine tetraacetic acid (EDTA), and 1.5 mL L-methionine), 1 mL 75 µM nitroblue tetrazolium (NBT), 50 µL enzyme extract, and 10 µL 2 mM riboflavin. Blanks consisted of the same reaction mixtures as the sample and the tube was wrapped with aluminium foil to indicate the non-irradiated blank. The negative control reaction mixture was prepared in the same way as the blank except for the omission of enzyme extract and is wrapped with aluminium foil. The reaction mixture was irradiated with light for 10 mins and the absorbance against the non-irradiated blank was recorded at 560 nm. One unit of SOD activity is defined as the amount of enzyme that inhibits 50% of NBT. SOD activity was calculated by evaluating its ability to inhibit NBT photochemical reduction at 560 nm.

2.8 Statistical analysis

Results obtained from the antioxidant enzyme assays were analysed by using the Minitab 14.12.0.0 Statistical Software. The results were expressed as mean±standard deviation. The significant difference at ($p<0.05$) was performed by one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) test.

3. Results and discussion

3.1 Proximate composition

Proximate analysis results of leaves and petioles of water lily are summarised in Table 1. Petioles showed higher moisture content that is 95.27±0.35% and significantly different to leaves which contain 88.31±0.35% of moisture content. The result is in accordance with the findings by Okoye *et al*. (2002) who reported that the leaves of water hyacinth (*Wichhornia crassipes*) had the least moisture content of 85.15% while the petioles which are constantly in contact with water had the higher water content than leaves that is 91.78%. Since water lily is an aquatic plant, it is expected to contain more water than plants that grow on land. Similarly, the dried leaves showed higher moisture content (7.74±0.76%) than dried petioles (6.87±0.57%). A range of 4 - 14% of moisture content in the dried African leafy vegetables was an effective preservation technique to prevent deterioration (Van't Hag *et al*., 2020). Furthermore, Odewo *et al*. (2014) quoted that the

Table 1. Proximate composition of Water lily (*N. antares*) leaves and petiole

	Moisture content (%)		Fat (%)	Fibre (%)	Crude Protein (%)	Ash (%)	Carbohydrate (%)
	Fresh	Dried					
Leaves	88.31±0.35[b]	7.74±0.76[a]	5.24±0.77[a]	13.14±0.79[a]	19.54±0.72[a]	10.05±1.17[b]	44.29±2.45[a]
Petioles	95.27±0.35[a]	6.87±0.57[a]	4.18±0.73[a]	12.15±0.69[a]	3.37±0.14[b]	17.26±1.02[a]	56.17±4.51[a]

Values represent the mean±standard deviation. Values with the same superscript within the column are not significantly different ($P<0.05$).

moisture content in leaves of water lily's leaves and petal (% dry weight) was 1.88%, which was lower than the current study. However, it should be noted that moisture content would depend on the degree of drying for dry matter. It was clear that petioles are larger in density, weight and volume than leaves. A higher value of ash content was obtained in the petiole (17.26±1.02%) compared to the leaves (10.05±1.17%). Water lily leaves exhibited a higher crude fat content (5.24±0.77%) compared to petiole (4.18±0.73%) which might be due to the oily nature of the leaves (Tungmunnithum *et al*., 2020). Water lily leaves also exhibited significantly ($p<0.05$) higher crude protein content (19.54±0.72%) compared to the petioles (3.37±0.14%). This study also showed that water lily leaves yielded higher quantities of fibre (13.14±0.79%) than petioles (12.15±0.69%). High fibre in the leaves may be due to the rich in insoluble dietary fibre (Mohammed *et al*., 2013). Fascinatingly, this study shows that the leaves of the water lily and the petiole contain low fat but high in carbohydrates. Carbohydrate was found to be the greatest in both the leaves and the petiole. The amount of carbohydrate content was consistent with Odewo *et al*. (2014), which reported the highest components of the plant species.

3.2 Protein content in water lilies leaves and petioles

Table 2 shows the leaves with pH 7.8 of extraction buffer had the highest protein concentration that is 17.5±0.14 mg/mL while leaves with pH 7.1 of extraction buffer had the lowest amount of protein concentration that is only 8.2±0.42 mg/mL and all samples were significantly different ($p<0.05$) to each other. The yield and properties of protein content are influenced by the extraction process and various factors such as pH, salt concentration, medium ion strength, net charge and electrostatic repulsion (Tan *et al*., 2011).

Table 2 also reveals that the highest protein concentration was found in petioles subject to pH 7.1 treatment and that the protein concentration was not significantly different from that of petioles with pH 6.8 extraction buffer. In contrast, the lowest protein concentration was found in petioles with pH 7.8 treatment. Hence, both of them were significantly different ($p<0.05$) to petioles with pH 7.8 of extraction buffer. Based on this study, the protein concentration can be concluded as higher in leaves compared to petioles. The same result was found in water lily from Tatabu (Nigeria), where the crude protein in leaves was higher than petioles that are 19.54% and 9.04% respectively (Mohammed *et al*., 2013).

3.3 Protein thermal properties

Differential scanning calorimetry (DSC) determines the calorimetric changes in proteins as a function of temperature. Thermal protein denaturation is due to the rupture of intramolecular hydrogen bonds (Chen and Oakley, 1995; Cordella *et al*., 2003). Denaturation temperatures are measurements of protein thermal stability. Their determination under controlled conditions should provide a specific comparison of the different proteins' thermal stability. Data from DSC measurements of leaves at pH 6.8 extraction buffer is shown in Figure 1. The leaves extract had denaturation temperatures at 81.84°C. Mariod *et al*. (2010), reported that the thermal denaturation of V36 protein concentrates of defatted kenaf seed is at 81.8°C. This result is similar to the present study. Protein enthalpy modifications may be used to predict the degree of protein denaturation (Corkrey *et al*., 2014). The protein is heated at a constant rate in DSC and the thermal denaturation-related heat shift is evident. In the type of heat that determines an endothermic phase, the process absorbs energy from the surroundings. Due to heat denaturation, the amount of heat absorbed is determined by DSC in the form of enthalpy unfolding. The improvement in enthalpy was calculated when all of the protein crystallised (100% crystallization). Due to the unfolding of the protein, the property of crystallinity is linked to enthalpy. The protein will crystallize more rapidly when the protein absorbs more energy (Li *et al*., 2008). The present study shows the glass transition (T_g) of the leaves sample is at 81.84°C and the enthalpy (ΔH) value is very low that is

Table 2. Protein concentration (mg/mL) of water lily's leaves and petioles with different pH of extraction buffer.

pH of extraction buffer	Leaves pH 6.8	Leaves pH 7.1	Leaves pH 7.8	Petioles pH 6.8	Petioles pH 7.1	Petioles pH 7.8
Protein	12.5±0.42[b]	8.20±0.42[c]	17.50±0.14[a]	1.60±0.14[d]	2.10±0.07[d]	1.00±0.14[d]

Values represent the mean±standard deviation. Values with the same superscript within the row are not significantly different ($P<0.05$).

0.001426J/g. Ross and Karel (1991) suggested that glass transition temperature (Tg) is closely correlated with protein stability, which depends on storage conditions such as water activity, humidity and temperature. Hence, the T_g can be set as a reference parameter to characterize the properties, quality, stability and safety of the food systems. Figure 2 shows the comparison between thermograms of leaves and petioles samples.

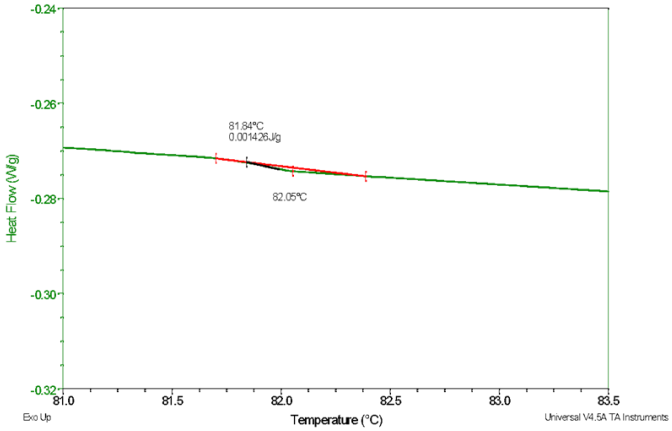

Figure 1. Differential scanning calorimetry (DSC) measurements for leaves extract at pH 6.8 of extraction buffer.

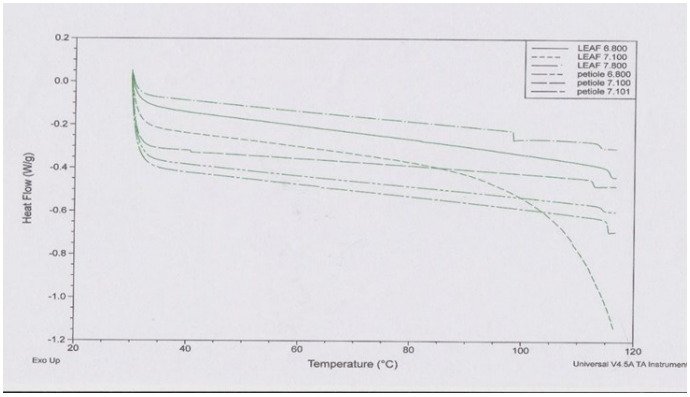

Figure 2. The comparison of thermograms of leaves and petioles samples.

3.4 Functional groups

Table 3 displays the effects of FTIR peak values and functional groups, and Figures 3 and 4 show the FTIR spectrum profile. The FTIR spectrum confirmed the presence of phenol and alcohol, alkyne, amide, ether and aromatic compounds in leaves and petioles of water lily. The peak values obtained for all leaves samples of the extraction buffer are somewhat similar. The more intense band occurring at 3385.95 cm^{-1}, 2112.42 cm^{-1}, 2357.38 cm^{-1}, 1644.52 cm^{1}, 1066.06 cm^{-1}, and 726.60 cm^{-1} corresponding to O-H/C≡C/C = O/C-O/C-H stretching and bending respectively indicate the presence of alcohol, alkyne, amide, ether and aromatic compounds in leaves of water lily (Figure 4). There was not much difference between the peak values obtained from leaves and petioles samples. The more intense band occurred at 3385.77 cm^{-1}, 2123.13 cm^{-1}, 1644.69 cm^{-1} and 726.55 cm^{-1} corresponding to O-H/C≡C/C = O//C-H stretching and bending respectively indicate the presence of alcohol, alkyne, amide, and aromatic compounds in petioles of water lily. The difference in functional groups present in petioles compared to leaves is the absence of ether in petioles. According to the match results for functional groups present in leaves samples by FTIR library, the highest match for leaves was 67.68% and 52.37% of water, deuterium-depleted and D-Ribulose respectively. A similar match is also found in petioles with the highest match as 68.07% water, deuterium-depleted. The phenol and alcohol group found in this study is according to the findings of Ashokkumar and Ramaswamy, (2014) which reported that methanol extract of *Phyllanthus amarus* leaves showed characteristic absorption bands at 3385 cm^{-1} for a hydroxyl (-OH) group. There is no absorbance in between the region 2220-2260 cm^{-1} indicating that there are no cyanide groups in all the extracts of the medicinal plants taken. This shows that samples taken for the study do not contain any toxic substances (Ragavendran *et al*., 2011). The very strong absorption band observed at approximately 3373-3422 cm^{-1} may be due to the presence of bonded N-H/C-H/O-H stretching of amines and amides (Liew *et al*., 2020).

3.5 Effect of pH extraction buffer on enzymatic antioxidant activities

3.5.1 Catalase

Table 4 shows that the leaves sample with pH 6.8 and 7.1 of extraction buffer possessed similar catalase activity, which is 0.03 U/mg protein. However, all

Table 3. FTIR spectral peak values and functional groups obtained for the leaves and petioles

Sample	Peak no	Wavelength (cm^{-1})	Functional group	Mode of vibration
Leaves	1	3385.95	Phenol and alcohol O-H	stretch
	2	2112.42	Alkynes C ≡ C	stretch
	3	1644.52	Amide C = O	stretch
	4	1066.06	Ether C-O	stretch
	5	726.6	Aromatic C-H	Bend
Petioles	1	3385.75	Phenol and alcohol O-H	stretch
	2	2123.13	Alkynes C ≡ C	stretch
	3	1644.69	Amide C = O	stretch
	4	726.55	Aromatic C-H	Bend

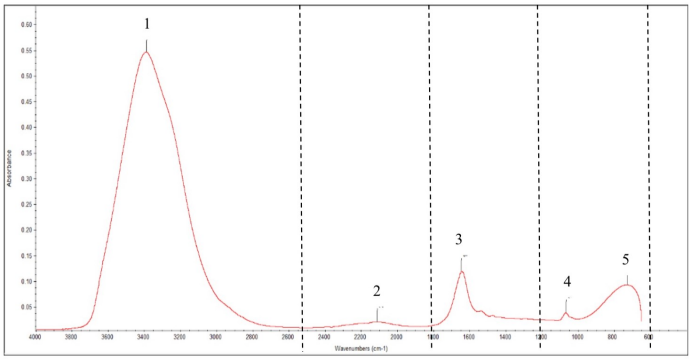

Figure 3. FTIR spectrum profile of water lily's leaves. 1 = Phenol and alcohol O-H, 2 = Alkynes C ≡ C, 3 = Amide C = O, 4 = Ether C-O, 5 = Aromatic C-H

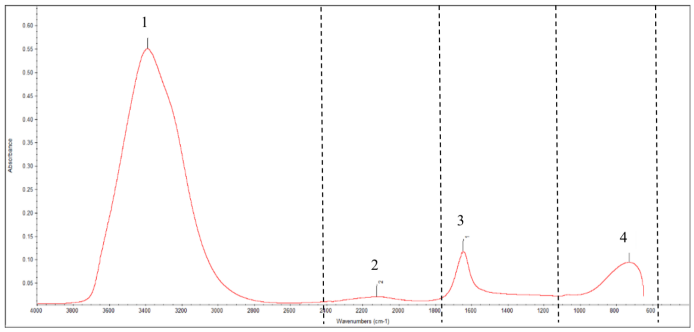

Figure 4. FTIR spectrum profile of water lily's petioles. 1 = Phenol and alcohol O-H, 2 = Alkynes C ≡ C, 3 = Amide C = O, 4 = Aromatic C-H.

Table 4. Superoxide dismutase activity, polyphenol oxidase activity, catalase H_2O_2 radical inhibition, and peroxidase activity water lily's leaves and petioles extraction at different pH conditions.

pH conditions	Sample part	Superoxide dismutase activity (m/mg)	Polyphenol oxidase activity (U/mg)	Catalase H_2O_2 radical inhibition (%)	Peroxidase activity (U/mg)
pH 6.8	Leaves	4.40±2.26^a	0.11±0.01^b	0.03±0.028^a	0.001±0.001^a
	Petioles	8.45±8.27^a	1.50±0.21^b	0.007±0.007^a	0.001±0.0003^b
pH 7.1	Leaves	2.65±0.92^a	0.30±0.01^a	0.03±0.014^a	0.001±0.0007^a
	Petioles	6.10±3.11^a	0.70±0.01^b	0.003±0.001^a	0.01±0.001^a
pH 7.8	Leaves	3.50±0.71^a	0.07±0.01^c	0.001±0.00^a	0.003±0.001^a
	Petioles	4.90±3.39^a	2.41±0.15^a	0.03±0.02^a	0.0003±0.000^b

Values represent the mean±standard deviation. Values with the same superscript within the column are not significantly different (P<0.05).

samples of leaves in pH 6.8, pH 7.1 and pH 7.8 of the extraction buffer showed no significant difference (p>0.05) among each other. There are many factors that can affect an enzyme's activity. These factors are generally referred to as effectors. Examples of effectors are temperature, pH, inhibitors, and activators. Effectors such as temperature and pH generally affect the non-covalent interactions that occur in an enzyme's structure. This result might be caused by the range of extraction pH used, which already falls in the optimum pH range for the enzyme. Most enzymes are active in the pH range of 6 to 8, yet some enzymes can function at a pH as low as 3 and as high as 10 (Robinson, 2015).

Catalase (CAT) is one of the major antioxidant enzymes and efficiently scavenges H_2O_2 and does not require a reducing substrate to perform the task (Kumari *et al*., 2006). The highest activity of catalase was found in petioles with pH 7.8 of extraction buffer that is 0.033 ±0.03 U/mg protein, as shown in Table 1. However, all samples of petioles with pH 6.8, pH 7.1 and pH 7.8 of extraction buffer showed no significant difference (p>0.05) among each other. The result from this present study can be supported by Arabaci and Usloqlu (2013), which stated that the effect of pH on the free and immobilized enzyme for H_2O_2 degradation was investigated in the pH range between 3 and 9 in acetate or/and phosphate buffers at 25°C. Finally, the activity measurements of the enzymes were made under the optimum assay conditions and resulted in similarities of pH stabilities for both enzymes. Both free and immobilized catalase gave an optimum at pH 7.5. However, the immobilized catalase showed much broader pH stability than the free enzyme. This suggested that immobilized enzyme was less sensitive to pH changes than free enzyme.

3.5.2 Peroxidase value (POX)

Peroxidase enzymes are widely distributed in plants and animals, including bacteria, to protect cells against the effects of oxidative stress and cell damage due to hydrogen peroxide. Table 4 shows the activity of peroxidase in water lily's leaves with different pH of extraction buffer. All samples of leaves with pH 6.8, pH 7.1 and pH 7.8 of the extraction buffer showed no significant difference (p>0.05) among each other. The highest peroxidase activity was possessed by leaves of pH 7.8 extraction buffer that is 0.003 U/mg. Saeidian and Ghasemifar, (2013) reported that pH is a determining factor in the expression of enzymatic activity in *Pyrus communis*. It alters the ionization states of amino acid side chains or substrates. For peroxidase activity, one pH optimum was observed at 6.5 and no activity was detectable at pH 3 and 9, regardless of the condition. Other than that, Kumar *et al*. (2008) reported that Ricinus peroxidases showed the maximum percent relative activity at pH 5 and it decreased when pH

increased. Dubey *et al*. (2007) has also shown a similar acidic pH ranging from pH 5 to pH 7 in four varieties of apple peroxidases. Optimal levels of peroxidase have also been reported from various vegetable sources (Vamos-Vigyazo and Haard 2009), apoplastic peroxidases from various plants (Hendriks *et al*., 1991; Nair and Showalter, 1996) at different acidic pH and *Cassia didymobotrya* peroxidase at pH 5.5 (Vitali *et al*., 1998). The estimation of secondary structural elements at different pH values suggested a maximum reduction of beta-strands and beta-turns at pH 5.5, which resulted in further exposure of the heme to the solvent and improved the overall conformational flexibility of the protein (Kamal *et al*., 2003). These findings were contrary to the present study due to the different samples and conditions used.

Peroxidase activity (POX) is considered a general response to oxidative stress, such as salinity stress in the examined plant species (Jaleel *et al*., 2009). POXs are involved in H_2O_2 scavenging, so their activity could decrease the oxidative stress caused by NaCl treatment. In addition, Yıldız (2019) reported that the rate of enzymatic browning in fruit and vegetables is dictated by the active polyphenol oxidase and peroxidase content of the tissues, the phenolic content of the tissues, pH, temperature, and the availability of oxygen to the tissues. Petioles with pH 7.1 of extraction buffer showed the highest peroxidase activity that is 0.006±0.00 U/mg and significantly different ($p<0.05$) to leaves with pH 6.8 and pH 7.8 of extraction buffer. This highest POX activity was linear to the highest protein concentration of petioles with a pH 7.1 extraction buffer. Khairil Anuar *et al*. (2020) reported that all peroxidase activities measured at their optimal pH showed a linear increase in activity with increasing protein concentration, suggesting that there were no effects of inhibitors or effectors within the supernatants.

3.5.3 Polyphenol oxidase (PPO)

Table 4 shows the activity of polyphenol oxidase activity (PPO) in water lily's leaves and petioles extracted in different pH of extraction buffers. Leaves extracted in pH 7.1 buffer exhibited significantly ($p<0.05$) the highest polyphenol oxidase activity than that of leaves with pH 6.8 and pH 7.8 extraction buffer. Table 4 also revealed that petioles extracted in pH 7.8 extraction buffer showed the highest PPO (2.41±0.15 U/mg) than that of petiole samples extracted in pH 6.8 and pH 7.1 of extraction buffer. Similarly, Derahman *et al*. (2017) reported that pH 8 (which was similar to pH 7.8) was suitable for extraction as the PPO showed the best results. Polyphenol oxidase catalyses two basic reactions that are o-position hydroxylation adjacent to the current phenolic substrate hydroxyl group (monophenol oxidase activity) and o-benzoquinone diphenol oxidation (diphenol oxidase activity). As a co-substrate, both reactions use molecular oxygen. It is still uncertain whether a single enzyme system experiences both mono- and di- phenol oxidase activities. However, the ratio of monophenol to diphenol oxidase activity is typically 1:10 or as low as 1:40 when both monophenol and diphenol oxidases are present in plants (Toledo and Aguirre, 2016). PPO optimum pH from some sources has also been reported to occur in the range of pH 6 to pH 8. Maximum PPO activity at pH 7 comparable to this study was observed in d'Anjou pears (Tuncay and Yagar (2011), cocoa beans (Lee *et al*., 1991), oil bean seeds (Onyebuchi, 2012). Other than that, a similar result was also found in the study done by Mizobutsi *et al*. (2010) which cited that the activity of polyphenol oxidase in litchi pericarp was maximum at pH 7.0. Sellés-Marchart *et al*. (2006) reported that differences in optimum pH for PPO activity depended on the plant sources, extraction methods, and purity of the enzyme, buffers, and substrates. Conformational changes in the enzyme under alkaline conditions have been due to the rapid deactivation of the enzyme at pH greater than 8 or the enzyme can react more quickly with o-quinone through the Maillard reaction and or Strecker degradation (converts alpha-amino acids into aldehydes) (Lee *et al*., 1991). Saeidian and Ghasemifar (2013) reported that polyphenol oxidase activity in *Solanum lycopersicum* extract was tested at different pHs ranging from pH 2 to pH 10, using cathecol and pyrogallol as a medium for green and ripe small cherry tomatoes.

3.5.4 Superoxide dismutase

Table 4 also illustrates the SOD activity in leaves and petioles of water lily with different extraction buffers respectively. The data clearly showed that leaves with pH 6.8 of extraction buffer showed the highest SOD activity that is 4.40±2.26 U/mL while leaves with pH 7.8 of extraction buffer showed the lowest SOD activity that is 2.70±0.92 U/mL. The highest SOD activity was found in petioles with pH 6.8 of extraction buffer and this result was linear to leaves sample that is 8.45±8.27 U/mL while petioles with pH 7.8 of extraction buffer showed the lowest SOD activity that is 4.9±3.39 U/mL. SOD activity was estimated by measuring its ability to inhibit the photochemical reduction of NBT at 560 nm.

4. Conclusion

The study revealed that the pH 7.1 extraction buffer showed a higher protein concentration of water lily's leaves and there was no significance in petioles. In determining the protein characterization, DSC showed

that the glass transition (T_g) of the leaves sample was at 81.84°C and the enthalpy (ΔH) value is very low that is 0.001426J/g. Other than that, the functional properties found by FTIR are O-H/C≡C/C = O/C-O/C-H stretching and bending respectively indicate the presence of alcohol, alkyne, amide, ether and aromatic compounds in leaves of water lily while O-H/C≡C/C = O//C-H stretching and bending respectively indicate the presence of alcohol, alkyne, amide, and aromatic compounds in petioles of water lily. Finally, in antioxidant enzyme assays, there was only PPO activity which was higher and significant in leaves with pH 7.1 of extraction buffer whereas, in petioles, POX and PPO activity were significant in petioles with pH 7.1 and 7.8 of extraction buffer respectively. From this research, it was found that pH 6.8, 7.1 and 7.8 of Tris buffer did not significantly affect antioxidant enzyme activities.

Conflict of interest

The authors declare that they have no conflict of interest.

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