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FULL PAPER

Effect of pH and temperature on antioxidant enzymes activities in *Morinda citrifolia* L. (Mengkudu) leaves extract

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Abstract

Morinda citrifolia L. (Mengkudu) leaf is not well-known for its benefits compared to *M. citrifolia* fruit. It can be considered a good source in healing disease and anti-cancer properties due to the high content of antioxidant enzymes. The objective of this study was to determine the antioxidative activities of *M. citrifolia* leaves extracted at different pH (pH 3 to pH 9) and temperatures (20°C to 80°C), based on four types of antioxidant enzymes test, namely catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO) and superoxide dismutase (SOD). Another analytical test is the protein determination of *M. citrifolia* leaves using Bovine Serum Albumin as standard. All of the tests were conducted using spectroscopy methods. Catalase (CAT) activity was monitored by reduction of absorbance due to the decomposition of hydrogen peroxide and peroxidase (POD) activity was observed by an increment of absorbance caused by the oxidation of 4-methylcatechol by hydrogen peroxide. Polyphenol oxidase (PPO) was monitored by an increment of absorbance due to the oxidation of 4-methylcatechol and superoxide dismutase (SOD) activity was determined using the NBT-based method, which monitors the amount of enzyme causing 50% inhibition of photochemical reduction of NBT. Results indicated that CAT activity and POD activity were significantly highest ($p<0.05$) at pH6, 0.51 U/mg for CAT, 2.58 U/mg for POD, while SOD activity was significantly higher at pH7, 0.47 U/mg. However, no significant difference ($p>0.05$) was observed for PPO activity in pH treatment. For different temperature treatments, CAT activity was significantly highest ($p<0.05$) at 50°C, 0.36 U/mg, while PPO activity and SOD activity were found to be significantly highest ($p<0.05$) at 30°C, 0.64 U/mg for PPO and 0.43 U/mg for SOD. However, all-temperature treatments given did not significantly affect POD activity. *Morinda citrifolia* leaves have a good antioxidant potential and can be practised in the treatment of diseases associated with oxidative stress.

1. Introduction

Morinda citrifolia L. (Mengkudu) has been used as a medicine for centuries in every corner of the globe due to its therapeutic capabilities, which include the treatment of rheumatic and other pain and healing benefits (Chong *et al*., 2018). *Morinda citrifolia* is also reported to exhibit a natural cure for lowering blood pressure, reducing joint swelling, stopping internal and external infections, clearing out congestion, and even preventing the growth of pre-cancer cells (West *et al.,* 2018). The beneficial health effects observed might result from specific compounds extracted from the roots, leaves, peel and fruits such as nitric oxide, alkaloids and sterols with antioxidant potential (Chan-Blanco *et al.,* 2006; Mohd Zin *et al.*, 2007). *M. citrifolia* L. has been the focus of many recent studies due to its potential effects on the treatment and prevention of several diseases. However, previous studies commonly focus on antioxidant content in *M. citrifolia* L. fruit extract instead of leaf.

Plants are typically endowed with free radical scavenging compounds such as vitamins, terpenoids, phenolic acids, lignins, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other antioxidant-rich metabolites (Srinivasahan and Durairaj, 2014). Antioxidants are vital in blocking and scavenging free radicals, which protects humans from infections and

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degenerative disorders (Ng *et al.,* 2020). Furthermore, current research is now focusing on natural antioxidants derived from plants due to safe therapies (Santhoshkumar *et al.,* 2013), since herbal and natural goods are thought to function better for humans than synthetics. Natural antioxidants can protect the human body from free radicals by retarding the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods (Gulcin, 2020).

Activated oxygen or oxygen-free radical-mediated damage to plants has been established or implicated in many plant stress situations (Chong *et al.,* 2020). Many free radicals are chemically reactive and can cause the oxidative breakage of double bonds in membrane lipid fatty acids (Endo *et al.,* 2019). Several enzymes are involved in the production and scavenging of free radicals in plants, especially catalase (CAT) and superoxide dismutase (SOD), while Catalase enhanced the removal of hydrogen peroxide (H_2O_2) (Kurutas, 2016). Catalase is one of the major antioxidant enzymes that efficiently scavenges H2O2 and does not require a reducing substrate to perform the task (Kumari *et al*., 2006). One unit (U) of catalase activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under assay conditions. The damaging effects of superoxide can be modulated if the radicals are scavenged by reacting with superoxide dismutase (Ighodaro *et al*., 2018). The combined action of SOD and CAT converts potentially dangerous superoxide radicals and hydrogen peroxide molecular oxygen and water, thus averting cellular damage (Jahan *et al*., 2014). As a result, the goal of this study was to assess the antioxidative activity of *M. citrifolia* leaves extracted at various pH and temperature conditions using four different antioxidant enzyme tests: catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), and superoxide dismutase (SOD).

2. Materials and methods

2.1 Raw materials

Fresh *M. citrifolia* L. leaves (MCL) (DINO 04-1425) were obtained from MARDI (Jerangau Station), Terengganu, Malaysia. The samples were washed with running tap water, separated, and air dried (until 6% moisture content) on the surface before being cut into pieces and stored at 4°C prior to the extraction process.

2.2 Extraction

The extraction procedure was divided into two stages, different pH extraction methods with 7 different pH values namely pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9, and with 7 different temperature points in the range of 20°C to 80°C.

2.3 pH dependant extraction

Approximately 10 g of frozen *M. citrifolia* L. leaves was homogenized in 50 mL of 100 mM buffer (varies from pH 3 until pH 9) containing 0.016 g of ascorbic acid and 0.5 g of polyvinylpyrrolidone and was rested for 5 mins at 5°C. The homogenate was filtered through three layers of cheesecloth and then the filtrate was centrifuged at 4000 rpm for 30 mins, and finally, the supernatant was collected (Alici and Arabaci, 2016). The optimal pH value for *M. citrifolia* L. activity was determined in buffers of pH 3.0 to 9.0. The buffer systems used were citrate buffer for pH 3.0-5.0, phosphate buffer for pH 6.0-7.5 and Tris-HCl buffer for 7.5-9.0.

2.4 Temperature dependant extraction

The crude extract was prepared by homogenization of 10 g frozen *M. citrifolia* L. leaves in 50 mL of 100 mM sodium phosphate buffer (pH 7.0) containing 0.016 g of ascorbic acid and 0.5 g of polyvinylpyrrolidone and was rest for 5 mins at the certain temperature set of 20°C to 80°C. The homogenate was filtered through three layers of cheesecloth and then the filtrate was centrifuged at 4000 rpm for 30 mins, and the supernatant was collected (Alici and Arabaci, 2016).

2.5 Determination of antioxidant enzymes activities

2.5.1 Determination of catalase activity

Catalase (CAT) activity was measured spectrophotometrically at room temperature by monitoring the decrease in absorbance at 240 nm (each 10 s for 1 min) resulting from the decomposition of H_2O_2 (Khairil Anuar *et al.,* 2020). One unit (U) of catalase activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under assay conditions. The reaction mixture contained 1.9 mL of 100 mM sodium phosphate buffer (pH 7.0), 1 mL of 30 mM H_2O_2 and 100 µl of crude extract in a total volume of 3.0 ml.

2.5.2 Determination of peroxidase activity

The peroxidase (POD) activity was determined using 4-methylcatechol as substrate. The increase in the absorption caused by oxidation of 4-methylcatechol by H_2O_2 was measured at 420 nm spectrophotometrically each 10 s for 1 min. The reaction mixture contained 2.4 ml of 100 mM sodium phosphate buffer (pH 7.0), 50 µL of 5 mM 4-methylcatechol, 50 µL of 5 mM H_2O_2 and 500 µL of crude extract in a total volume of 3.0 mL at room temperature. One unit of enzyme activity was defined as a 0.001 change in absorbance per min, under assay conditions (Onsa *et al.,* 2004).

2.5.3 Determination of polyphenol oxidase activity

Polyphenol oxidase (PPO) activity assay was carried out by measuring the increase in absorbance at 420 nm (each 10 s for 1 min) for 4-methylcatechol spectrophotometrically. The assay was performed with 2 ml of 100 mM sodium phosphate buffer (pH 7.0), 0.5ml of 5 mM 4-methylcatechol and 0.5 ml of crude extract at room temperature. The total volume of the reaction mixture was 3.0 ml. One unit (U) of enzyme activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per min (Can *et al.,* 2013).

2.5.4 Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by measuring the inhibition in the photoreduction of nitroblue tetrazolium (NBT) by the SOD enzyme (Kumar *et al.,* 2012). The reaction mixture contained 2.3 mL of 50 mM sodium phosphate buffer (pH 7.6), 0.1 mL of 0.1 mM EDTA, 0.1 mL of 50 mM sodium carbonate, 0.2 mL of 12 mM L-methionine, 0.1 mL of 50 µM NBT, 0.1 mL of 10 µM riboflavin and 0.1 mL of crude extract in a final volume of 3.0 mL. The SOD reaction was carried out by exposing the reaction mixture to white light for 15 mins at 25°C. After 15 mins of incubation, absorbance was recorded at 560 nm using a spectrophotometer (Shimadzu, Japan). One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

2.6 Determination of protein concentration

Approximately 2 g of sample was ground in 10 mL of 0.1M NaOH at 25°C using mortar and pestle. The homogenate was then transferred into 2.5 mL Eppendorf tubes and incubated at 25°C for 12 hrs (Barbarino and Lourenco, 2005). After incubation, the homogenate was then centrifuged at 4000 rpm for 45 mins. The resulting supernatant was collected and stored at -20°C for further analysis. The procedure used for UV absorption spectrum analysis of both protein extract and protein standards was carried out according to Onasanya *et al*. (2015) and Grimsley and Pace (2003). An aliquot of 0.1 ml crude protein extract was added to 1.9 mL of 0.1 M NaOH, and the resulting absorbance was measured at 280 nm. For standard, 0.1 mL each of 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and 250 µg/mL of the protein standard Bovine Serum Albumin (BSA) was added to 1.9 mL of 0.1 M NaOH, mixed well absorbance was recorded at 280 nm. A standard curve was prepared by plotting the absorbance values at 280 nm against the concentration of protein standard (BSA). Table 1 show the absorbance of standard Bovine Serum Albumin (BSA) and the absorbance of recorded unknown sample concentration. Absorbance for unknown sample concentration was 0.02 at 280 nm. The absorbance values of standard BSA were plotted in a graph. The unknown sample concentration was found to be 0.04 mg/mL.

2.7 Statistical analysis

The statistical analysis used in this study is a One-way analysis of variance (ANOVA). The triplicate data obtained were analysed using a Completely Randomized Design (CRD) by Minitab 14.12.0.0 Statistical Software. The results were expressed as mean and standard deviation, with the independent variables being the type of extraction treatments, different pH extraction, and different temperature extraction, with the factor levels consisting of 7 different pH (pH 3, pH 4, pH 5, pH 6, pH 7, pH 8, pH 9) and 7 different temperatures (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C).

Table 1. Absorbance of standard and unknown sample concentration

BSA (mg/mL)	Absorbance at 280 nm
0	0
0.05	0.02
0.1	0.04
0.15	0.06
0.2	0.07
0.25	0.09
Unknown concentration	0.02

3. Results and discussion

3.1 Effect of different pH extraction on antioxidant enzymes activities

3.1.1 Catalase activity

Table 2 shows that all samples (treated with pH 3 until pH 9) undergo a reduction of absorbance every 10 s for 1 min resulting from the decomposition of hydrogen peroxide (H_2O_2). Samples of pH 5, pH 4, pH 3 (acidic group) exhibited somewhat lower absorbance values at 240 nm. Meanwhile, pH 9, pH 8 (alkali group), pH 7 (neutral) and pH 6 (slightly acidic) exhibited higher activities. The pH 7 sample has the highest antioxidant enzyme activity while the pH 5 sample exhibited the least activity. Catalase activity was significantly highest ($p<0.05$) at pH 6 which is 0.5125 U/mg. Other samples except the pH 6 sample showed no significant difference ($p>0.05$) from each other. Alici and Arabaci (2016) reported that the optimum pH value for antioxidant enzymes in plant samples ranges from pH 6 to pH 8. Most enzymes are active in the pH range of 6 to 8, but some enzymes can function in extreme pH conditions as

Table 2. Antioxidant enzymes activities in different pH extraction

Extraction	CAT (U/mg)	POD (U/mg)	PPO (U/mg)	SOD (U/mg)
pH 3	0.33±0.04^{c}	1.47±0.16^{c}	0.15±0.00^{c}	0.17±0.02^{d}
pH 4	0.32±0.04^{cd}	1.70±0.27^{bc}	0.24±0.01^{b}	0.18±0.02^{d}
pH 5	0.35±0.00^{bc}	1.90±0.13^{b}	0.26±0.00^{b}	0.28±0.03^{c}
pH 6	0.51±0.02^{a}	2.58±0.27^{a}	0.31±0.04^{a}	0.38±0.01^{b}
pH 7	0.41±0.01^{b}	1.02 ±0.11^{d}	0.25±0.03^{b}	0.47±0.02^{a}
pH 8	0.31±0.03^{d}	1.18±0.22^{cd}	0.21±0.01^{bc}	0.35±0.003^{b}
pH 9	0.39±0.03^{b}	1.21±0.04^{cd}	0.22±0.01^{bc}	0.28±0.03^{c}

Values are presented as mean±SD, n = 3. Values with different superscript within the same column are significantly different ($p<0.05$).

low as pH 3 and as high as pH 10 (Robinson, 2015). It was found that the optimum pH value for catalase enzyme in *M. citrifolia* leaves extract was pH 6 which is in agreement with the study by Alici and Arabaci (2016). Catalase activity measurement involves the conversion of hydrogen peroxide to water and oxygen and purification from different natural sources including animal tissues, plants and microorganisms, which act as a defence mechanism by limiting the accumulation of H_2O_2 (Sofo *et al.,* 2015).

3.1.2 Peroxidase activity

The pH 6 sample exhibited significantly the highest ($p<0.05$) peroxidase activity (2.58 U/mg) (Table 2). The optimum pH value for antioxidant enzymes in plant samples is usually in the range of pH 6 to pH 8 as stated by Alici and Arabaci (2016). Peroxidase showed the maximum activity at a range of pH 5 to pH 6.5 and decreased as pH increased (Kumar *et al.,* 2008). Mizobutsi *et al.* (2010) quoted that the maximum activity of litchi peroxidase was observed at a slightly acidic pH and reached a maximum point at pH 6.5. The data is in accordance with the study by Alici and Arabaci (2016), and Kumar *et al.* (2008) who revealed that the optimum pH value for peroxidase enzyme in *M. citrifolia* L. leaves extract was pH 6.

3.1.3 Polyphenol oxidase activity

Table 2 also shows the effect of different pH extracts on polyphenol oxidase activity (PPO). All samples except pH 3 indicated no significant difference ($p>0.05$) among each other. The study revealed that pH 3 samples exhibited significantly the lowest polyphenol oxidase activity (0.15 U/mg) while the highest activity was expressed by pH 6 extraction (0.31 U/mg). The pH optimum of PPO activity from some sources also occurs in the range of pH 6 to pH 8 (Alici and Arabaci, 2016). Most of the polyphenol oxidase studies indicated an optimum activity between pH 4 and pH 7 (Sikora *et al.,* 2019). Mizobutsi *et al.* (2010) reported similar results as PPO reached a plateau of maximal activity at pH 6.5 and 7, which decreased when the pH was reduced or elevated and no activity was detected at pH 2.5 and 9.5. The results also indicated that polyphenol activity was significantly low in the pH 3 sample, in concert with the study by Mizobutsi *et al*. (2010) who indicated that polyphenol oxidase activity was lowest at pH 3 and completely inactive at pH 2.5. However, there are often discrepancies in the published values for polyphenol oxidase enzyme activity from the same source and differences in pH optimum depending on cultivars and maturity of plant samples. Besides, 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.

3.1.4 Superoxide dismutase activity

The data (Table 2) also revealed that superoxide dismutase (SOD) was significantly high ($p<0.05$) in the pH 7 sample (0.47 U/mg). Antioxidant enzymes in plant samples usually have optimum pH in the range of pH 6 to pH 8 (Alici and Arabaci, 2016). Zelko *et al.* (2002) stated that SOD is a closely related enzyme that catalyses the breakdown of the superoxide anion into oxygen and hydrogen peroxide. Superoxide dismutase or superoxide reductase is a primary oxygen radical scavenging enzyme for eukaryotic, aerobic cells and tissues. This makes superoxide dismutase an important antioxidant enzyme that protects tissues against oxidative injury from free oxygen radicals generated by various metabolic processes (Wang *et al*., 2018).

3.2 Effect of different temperature extraction on antioxidant enzymes activities

3.2.1 Catalase activity

Table 3 shows that catalase activity at 50°C, which is 0.36 U/mg was considerably the highest ($p<0.05$). Other samples except T50 showed no significant difference between themselves ($p>0.05$). As contended by Alici and Arabaci (2016), the optimum temperature value for antioxidant enzymes in plant samples ranges from 20°C to 40°C. Another study by Zargoosh *et al*. (2019) reported that optimum temperature value for antioxidant

Table 3. Antioxidant enzymes activities in different temperature extraction

Extraction	CAT (U/mg)	POD (U/mg)	PPO (U/mg)	SOD (U/mg)
T20	0.19±0.02^{d}	1.26±0.25^{d}	0.22±0.01^{e}	0.21±0.01^{d}
T30	0.21±0.01^{c}	1.72±0.05^{a}	0.63±0.08^{a}	0.42±0.02^{a}
T40	0.21±0.02^{c}	1.58±0.37^{b}	0.46±0.02^{b}	0.32±0.01^{b}
T50	0.35±0.04^{a}	1.42±0.10^{c}	0.38±0.01^{c}	0.27±0.01^{c}
T60	0.25±0.01^{b}	1.37±0.21^{cd}	0.37±0.04^{c}	0.22±0.004^{d}
T70	0.23±0.03^{bc}	0.89±0.17^{e}	0.24±0.02^{d}	0.17±0.004^{de}
T80	0.22±0.00^{bc}	0.76±0.16^{f}	0.21±0.05^{d}	0.11±0.004^{f}

Values are presented as mean±SD, n = 3. Values with different superscript within the same column are significantly different ($p<0.05$).

enzymes in plant samples varies depending on types and parts of plant uses, usually in the range of 25°C to 50°C. It was found that the optimum temperature value for catalase enzyme in *M. citrifolia* L. leaves extract was at 50°C and this is in support of a previous study by Wattanathorn *et al*. (2018). There is a higher amount of enzymatic antioxidants like SOD and catalase in this study, which could be attributable to several compounds such as alkaloids, flavonoids, and certain glycosides.

3.2.2 Peroxidase activity

All samples undergo an increment of absorbance at 420 nm every 10 s for 1 min, caused by oxidation of 4-methylcatechol by H_2O_2. The data shows that T70 and T80 (high temperature) have lower absorbance readings. On the other hand, T20, T30, T40, T50, and T60 have high absorbance values (Table 3). During this observation, T20 has the highest absorbance reading values and T70 expressed the lowest absorbance values. 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. Doğan *et al*. (2011) reported that the rate of enzymatic browning in fruits and vegetables is governed by the active polyphenol oxidase and peroxidase content of the tissues, the phenolic content of the tissues, pH, temperature and oxygen availability with the tissue. The data in Table 2 also indicated the effect of different temperature treatments on peroxidase activity. The highest peroxidase activity was found in a T30 sample (1.72 U/mg). A previous study by Mizobutsi *et al*. (2010) revealed that from 10°C to 20°C, the peroxidase activity in litchi pericarp increased slowly, at 30°C the rate of activity increase was higher, and at 70° C, the activity reached its peak. As studied by Alici and Arabaci (2016), antioxidant enzymes in plant samples usually have optimum temperatures in the range of 20°C to 40°C.

3.2.3 Polyphenol oxidase activity

The result (Table 3) revealed that polyphenol oxidase (PPO) activity in the T30 sample (0.64 U/mg) was found to be significantly ($p<0.05$) higher compared to other samples. Alici and Arabaci (2016) stated that the optimum temperature for PPO activity ranges between 20°C to 40°C. Meanwhile, Sikora *et al*. (2019) claimed that the optimum temperature of polyphenol oxidase enzyme ranges from 15°C to 40°C. However, as mentioned by Taranto *et al*. (2017), there are often discrepancies in the published values for PPO from the same source and differences in pH optimum depending on cultivars and maturity. Sellés-Marchart *et al*. (2006) reported that differences in optimum pH and temperature for polyphenol oxidase activity depended on the plant sources, extraction methods, purities of the enzymes, buffers and substrates.

3.2.4 Superoxide dismutase activity

Table 3 also indicates that the T30 sample (0.43 U/ mg) exhibited significantly the highest ($p<0.05$) superoxide dismutase (SOD) activity while the T80 sample (0.11 U/mg) was found to be the lowest. Several studies on antioxidant enzymes activities have depicted that the range of optimum temperature varies depending on the types and parts of the plant used in the analysis, usually in the range of 25°C to 50°C (Haida and Hakiman, 2019), 20°C to 40°C (Alici and Arabaci, 2016) and 15°C to 40°C (Nicholas *et al.,* 2003). It was revealed that the optimum temperature value for superoxide dismutase enzyme in Mengkudu leaves extract was 30°C which is in agreement with the study by Alici and Arabaci (2016) and Nicholas *et al.* (2003).

4. Conclusion

The optimum pH for the antioxidants enzymes activities of *M. citrifolia* leaves extract lies between pH 6 (peroxidase and catalase) and pH 7 (superoxide dismutase). All pH treatment given does not significantly affect polyphenol oxidase activities. Meanwhile optimum temperature in the range of 30°C (polyphenol oxidase and superoxide dismutase) to 50°C (superoxide dismutase enzyme and catalase). For peroxidase, it was found that all temperature treatments given did not

significantly affect the peroxidase activity. This result showed that *M. citrifolia* leaves exhibited somewhat a good antioxidant potential. The plant is a promising source of natural antioxidants and might be used in the treatment of diseases associated with oxidative stress.

Conflict of Interest

The authors declare that there is no conflict of interest in conducting this study.

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