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PHYTOCHEMICAL SCREENING AND EVALUATION OF THE ENZYMATIC ANTIOXIDANT ACTIVITIES OF SELECTED *Mangifera spp*. LEAVES (ANACARDIACEAE)

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Abstract: A study was conducted on the chemical and biological properties of three different species of Mangifera i.e. *Mangifera pajang, M. indica L.*, and *M. kemanga* leaves obtained from Pitas, Sabah. The aims of this study were to determine the presence of secondary metabolites as well as the antioxidative activities especially the catalase (CAT) and guiacol peroxidase specific activities (gPOD) in the leaves part of these three species. The extraction of these samples was carried out using three different polarities of solvents**:** hexane, ethyl acetate, and methanol. The total percentage of the crude extract ~~of~~ is 7.30% for *M. pajang*, 12.87% for *M. indica* and 7.98% for *M. kemanga*. Phytochemical screening was performed with various tests for each of the crude extracts. The results showed that these three species gave positive results for alkaloids, saponins, flavonoids, phenols, carbohydrates, phytosterols, and tannins metabolites. Based on the tests, CAT specific activities were significantly higher in the leaves of *M. pajang* with 4.35 ± 1.18 units/mg protein compared to *M. indica L.* and *M. kemanga*. The guaiacol peroxidase (gPOD) specific activities showed that *M. indica L.* has the highest activity with the value of 0.0047 ± 0.0004 units/mg protein.

Keywords: Phytochemical screening, catalase (CAT), guaiacol peroxidase (gPOD), *Mangifera* spp.

Introduction

Natural product is a metabolite produced by a living organism (Altemimi *et al.*, 2017). These metabolites can be found as a large and diverse group of substances from many types of sources. Metabolites play a significant role as a defence mechanism against predators in the organism. They have limited occurrence which give particular species their characteristic features. These include saponins, flavonoids, steroids, and alkaloids (Dias *et al.,* 2012).

The genus Mangifera belongs to the Anacardiaceae family and thousands of species are distributed throughout the world. The highest diversity occurs in Malaysia, particularly in Peninsular Malaysia, Borneo and Sumatera (Bompard & Schnell, 1997). This genus is a cultivated variety and has been introduced to other warm regions of the world.

The chemical constituents of the *Mangifera spp.* are reviewed by Ross (1999) and Scartezzini & Speroni (2000). The bark is reported to have protocatechic acid, catechin, mangiferin, alanine, glycine, γ-amino-butyric acid, kinic acid, shikimic acid and the tetracyclic triterpenoids. The natural C-glucoside xanthone mangiferin has been

reported in various parts of *Mangifera spp*. leaves (Desai *et al*., 1966), fruits (El Ansari *et al*., 1971), stem bark (Bhatia *et al*., 1967; El Ansari *et al*., 1967) and roots (Nigam & Mitra, 1964). This pharmacologically active metabolite happens among different angiosperm families and ferns (Richardson, 1984). It is also widely distributed in the Anacardiaceae families, especially in the leaves and bark of the plant (Yoshimi *et al*., 2001). All these metabolites and antioxidative enzymes are necessities for plant growth and development. Any perturbation to the environment can result in oxidative damage to major biomolecules as well as induction of antioxidative enzymes. Plants defend themselves against reactive oxygen species (ROS), generated under stress condition by two ways including enzymatic and non-enzymatic processes (Azarmehr *et al*., 2013).

The medicinal purposes of *Mangifera* spp. leaf have been widely studied. For example, it has been reported that extract of *M. indica* leaf inhibited lipid peroxidation (Badmus *et al.,* 2011), exerted antifungal activity (Kanwal *et al*., 2010), and antiulcerogenic action (Severi *et al*., 2009). However, the enzymatic activities of CAT and gPOD of *Mangifera* spp. leaves have not been reported so far. This present work, therefore, undertaken to examine the CAT and gPOD activities and to screen the phytochemicals for the *M. pajang, M. indica L*., and *M. kemanga* leaves. CAT function is mainly to detoxify hydrogen peroxide (H_2O_2) to oxygen and water whereby gPOD analysis can protect cells against the destructive influence of H_2O_2 by catalyzing its decomposition through oxidation of phenolic and other substrates (Hafsi *et al*., 2011).

Materials and Methods

Chemical Reagents

The chemical solvents that have been used for sample extraction are hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar). The chemical reagents used for phytochemical screening are Wagner's reagent, Fehling's reagent, ferric chloride (, chloroform (), sodium hydroxide solution (NaOH), ethanol (), diethyl ether ((O), ammonia (), and copper sulphate ().

The chemical reagents that were used for antioxidant assays are linoleic acid (), Tween 20 (potassium phosphate buffer (), Coomasie Brilliant Blue G-250 (), Bradford reagent, guaiacol (), hydrogen peroxide (), methanol (), Bovine Serum Albumin (BSA), and ortho-phosphoric acid ().

Plant Materials

The fresh leaves *of M. pajang, M. indica* L., and *M. kemanga* were collected from Pitas, Sabah. Five replicates for each species were used in this study.

Sample Preparation

The fresh leaves were cleaned under running tap water, and shade dried at room temperature for five days before pulverized into fine power. The plant powder was then stored in sealed containers at 4^{o}C until further analysis.

Phytochemical Screening

The phytochemical screening analysis was carried out to determine the presence of secondary metabolite compounds in the crude extract of the selected plants. In this study, nine tests were conducted including alkaloids, flavonoids, saponins, tannins, phlobatannins, carbohydrates, proteins, phenols and phytosterols test.

Detection of alkaloids (Wagner's Test)

About 0.1 g of the crude extract was treated with a few drops of Wagner's reagent (iodine in potassium iodide). The appearance of reddish brown precipitate indicated the presence of alkaloids.

Detection of flavonoids (Alkaline Reagent Test)

About 0.1 g of the crude extract was treated with a few drops of sodium hydroxide solution. The formation of intense yellow colour, which became colourless on addition of diluted hydrochloric acid, indicated the presence of flavonoids.

Detection of saponins (Foam Test)

About 0.1 g of the crude extract was shaken in a test tube with 2 mL of distilled water. The formation of foam that persisted for 10 minutes indicated the presence of saponins.

Detection of tannins (Tannins Test)

About 0.1 g of the crude extract was treated with chloroform and the extract was filtered. The filtrates then were treated with a few drops of concentrated sulphuric acid, shaken and were allowed to stand. Appearance of golden yellow colour indicated the presence of tannins.

Detection of phlobatannins (Phlobatannins' Test)

About 0.1 g of the crude extract was boiled with 2.0 M hydrochloric acid solution. The formation of red precipitate indicates the presence of phlobatannins.

Detection of carbohydrates (Fehling's Test)

About 0.1 g of the crude extract was dissolved individually in 5 mL of deionized water and was filtered. The filtrates were then hydrolysed with diluted hydrochloric acid, neutralized with sodium hydroxide solution and heated with Fehling's A and B solutions. The appearance of red precipitate indicated the presence of carbohydrates.

Detection of proteins (Biuret Test)

The formation of purple colour when the crude extract (about 0.1 g) was treated with an equal volume of 1% strong base (sodium hydroxide) followed by a few drops of aqueous copper (II) sulphate indicated the presence of protein.

Detection of phenols (Ferric Chloride Test)

About 0.1 g of the crude extract was treated with 2-3 drops of ferric chloride solution. The formation of bluish black colour indicated the presence of phenols.

Detection of phytosterols (Libermann Burchard's Test)

About 0.1 g of the crude extract was treated with chloroform and filtered. The filtrates were then treated with a few drops of acetic anhydride. After that, 2.0 M of sulphuric acid was added. The formation of brown ring at the junction showed the presence of phytosterols.

Enzymatic Antioxidant Assays

CAT Assay

CAT specific activity was analysed according to the method suggested by Claiborne (1985). About 0.05 g of each crude extract was mixed with 1.0 mL methanol and 1.0 mL of 50 mM phosphate buffer pH 7.4. The mixture was centrifuged at 10,000 rpm at 4˚C for 10 minutes. The reaction mixture consisted of 3.0 mL of reaction buffer (19 mM hydrogen peroxide in 50 mM phosphate buffer, pH 7.0) and 100 µL of enzyme extract. The rate of changes in the absorbance of the reaction mixture solution was monitored at 240 nm for 3 minutes using spectrophotometer (Shimadzu 1800). CAT specific activity was expressed in µmoles of hydrogen peroxide consumed per minute per mg protein.

Guaiacol peroxidase (gPOD) Assay

Guaiacol peroxidase (gPOD) specific activity was determined based on method by Agrawal and Patwardhan (1993). About 0.05 g of each crude extract was mixed with 1.0 mL methanol and 1.0 mL of 100 mM phosphate buffer pH 7.0. The mixture then was centrifuged at 10,000 rpm at 4˚C for 5 minutes. The reaction mixture contained 1.0 mL of 50 mM phosphate buffer pH 7.5,

1.0 mL of 20 mM guaiacol, and 1.0 mL of 30 mM hydrogen peroxide was added to 200 µL enzyme extract (supernatant). After that, the rate of changes in absorbance of the reaction mixture was monitored at 470 nm for 3 minutes.

Determination of protein

Determination of protein content was done according to the method by Bradford (1976). About 25 mg of Coomasie Brilliant Blue G-250 was dissolved in 12.5 mL of 95% methanol. Then, 25 mL of concentrated phosphoric acid was added and the mixture was diluted to 250 mL. The solution was filtered through filter paper and stored at room temperature in light-proof bottle. 100 µL of enzyme extract was added to 3.0 mL of Bradford's reagent and the absorbance was taken at 595 nm after 10 minutes. Next, the protein concentration was determined according to the standard curve prepared with various concentrations of BSA (Bovine Serum Albumin) at 0 mg/L to 100 mg/L BSA.

Statistical Analysis

Data was analysed using Statistical Package for Social Sciences (SPSS) software version 22.0. All data recorded were reported as means ± standard error. The data of the specific enzymatic activities was analysed using Welsch F method with One-Way ANOVA to evaluate the differences in activity of enzymatic antioxidants in different species of Mangifera (*M. pajang, M. indica L.,* and *M. kemanga*).

Results and Discussion

Phytochemical Study

The phytochemical screening of *Mangefira* spp., showed the presence of tannins, flavonoids, phytosterols and carbohydrates. Alkaloids, saponins and phlobatannins were only detected in methanolic extracts. Table 1 below shows the complete results for the phytochemical screening analysis.

Table 1: Phytochemical Screening Analysis of 3 selected *Mangifera* spp.

Phytochemical	Hexane			Ethyl acetate			Methanol		
Test	MP	MIL	MK	MP	MIL	MK	MP	MIL	MK
Alkaloids	-	-	-	-	-	-	+	+	+
Saponins	-	-	-	-	-	-	+	+	+
Flavanoids	-	-	-	+	+	+	+	+	+
Phlobatannins	-	-	-	-	-	-	+	+	-
Phenols	-	-	-	-	+	-	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+
Proteins	-	-	-	-	-	-	-	-	-
Phytosterols	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+

MP = *Mangifera pajang,* MIL = *Mangifera indica L.* MK = *Mangifera kemanga*

Enzymatic Antioxidant Assays

Catalase (CAT) specific activities

In this study, CAT specific activities in the leaf extracts *of M. pajang, M. indica L.*, and *M. kemanga* were in the range of 0.80 ± 0.14 to 4.35 ± 1.18 units/mg proteins. CAT specific activity was significantly higher ($P<0.05$) in *M. pajang,* followed by

M. indica L., and the least was found in *M. kemanga* (Figure 1). The increase of specific activity in *M. pajang* compared to *M. indica L.* and *M. kemanga* might be due to the UV irradiation and changes in the temperature that occurred. It shows that the removal of H_2O_2 produced in peroxisomes was higher in *M. pajang* compared to *M. indica L.* and *M. kemanga*.

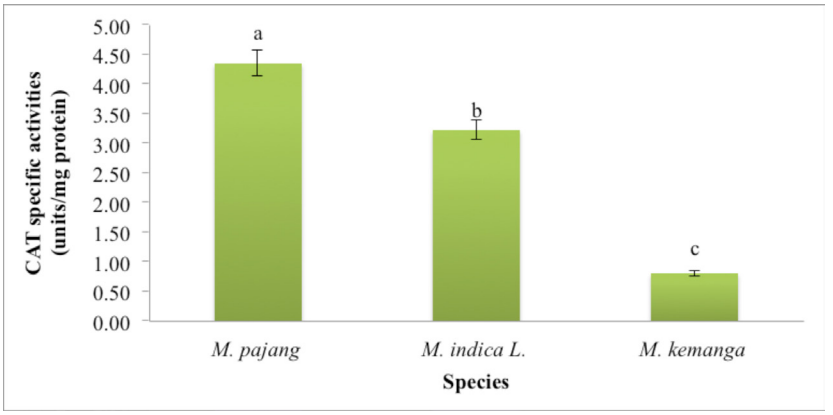

Figure 1: Catalase specific activity in leaves of *M. pajang, M. indica L.,* and *M. kemanga*. Vertical bars represent mean ± SEM (n=5). Means with different letters are significantly different at $p < 0.05$.

Guaiacol peroxidase (gPOD) specific activities

Figure 2 shows gPOD specific activities in the leaf extracts of *M. pajang, M. indica L.,* and *M. kemanga*. In this study, the gPOD specific activities were in the range of 0.0028 ± 0.0007 to 0.0047 ± 0.0004 units/mg proteins. *M. indica* exhibited the highest gPOD specific activities whereas no significant difference ($P>0.05$) was observed between leaves of *M. pajang* and *M. kemanga*. The gPOD specific activity occurred in normal metabolism and during stress condition to eliminate excess amount of H_2O_2. In this study, it shows that the elimination process of excess H_2O_2 was higher in the leaves of *M. indica L.* compared to the other species. This is due to the difference in the presence of secondary metabolites that affected the biosynthesis process of lignin.

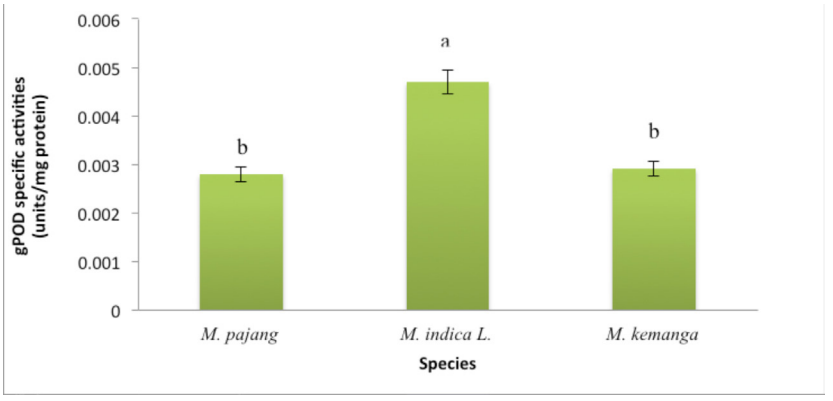

Figure 2: Guaiacol peroxidase (gPOD) specific activities in leaves of *M. pajang, M indica L.,* and *M. kemanga.* Vertical bars represent mean ± SEM (n=5). Means with different letters are significantly different at $p < 0.05$.

Conclusion

The phytochemical analysis showed that the *Mangifera* spp. contains a mixture of phytochemicals including alkaloids, saponins, flavonoids, phenols, carbohydrates, phytosterols, and tannins that are very important for plant growth and development. The CAT activities were higher in *M. panjang*, whereas *M. indica* exhibited higher activities in gPOD specific activities. This indicated the significant responses of these plants towards oxidative stress.

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