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Hydrocyanic Acid, Protein Concentration, and Phytochemical Compounds of Pulut and White Varieties in Young and Matured Cassava (*Manihot Esculenta*, Crantz)

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ABSTRACT

In Malaysia, there is a demand and a promising market for cassava (*Manihot esculanta*, Crantz) leaves as a supplementary animal feed because of their nutritional value and availability throughout the year. However, cyanide accumulation has been a problem due to its toxicity in animal feed; therefore, finding the best variety with low cyanide, high protein, and phytochemical content can address this issue. The hydrocyanic (HCN) contents were analyzed for two local varieties, White and Pulut cassava, distinguished from leaf shapes and the color of the leaf petioles. Young leaves were identified from the plant's top leaves, while matured leaves were defined from the plant's bottom leaves. Two-way ANOVA was conducted to determine the interaction between the maturity and variety of cassava leaves for the cyanide and protein concentrations with Tukey's multiple ranges to observe the significant difference at $p < 0.05$. The findings indicated significant differences in the HCN content of cassava leaves between different maturities, while other varieties significantly affected protein concentration. The maturity and variety of cassava leaves showed significant interactions with the HCN content. The young Pulut variety had the highest protein concentration and low HCN content. Thus, it is the best option as an animal feed by reducing its HCN content and maintaining its total phenolic (TPC) and total flavonoid content (TFC). The results imply that variety, as well as maturity, have significant effects on the protein and cyanide concentration of cassava leaves.

Keywords: Cassava leaves, cyanide concentration, phytochemical contents, protein concentration

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INTRODUCTION

Manihot esculenta, Crantz (cassava), also known as yucca, manioc, tapioca and commonly known as "ubi kayu" in Malaysia, belongs to a woody shrub in the Euphorbiaceae family (Jamil & Bujang, 2016; Latif & Müller, 2015). Cassava roots have a high carbohydrate. Therefore, it is utilized to produce chips and starch. The aerial components (leaves and stems) are usually thrown as waste or used as animal feed after the roots have been harvested (Chaiareekitwat et al., 2022; Idris et al., 2021). Since cassava plants can withstand drought and grow in acidic, low-fertility soil, their leaves are widely available throughout the year (Ubwa et al., 2015). Cassava cultivation is widespread in many tropical countries, with an estimated annual production of 291.9 million tonnes (Lansche et al., 2020). In Asia, including Malaysia, Indonesia, and sub-Saharan Africa, tender leaves are edible as a traditional vegetable commonly served with rice (Latif & Müller, 2015).

It is crucial to evaluate the protein content of cassava leaves because it has been known that green vegetables are the lowest-priced source of protein. Since cassava leaf has three times more crude protein than various cassava plants' components, it can be used as a ruminant protein source (Idris et al., 2021). Another study reported that the crude protein content of cassava leaf was 17.7%–38.1% D.M. (Awoyinka et al., 1995). Crude protein levels in Malaysian cassava cultivars ranged from 21.51 to 30.31% D.M. (Jamil & Bujang, 2016). Since the root of the cassava contains low protein, the leaves should receive better attention as human food and animal feed.

Cyanogen glucosides are present throughout cassava, and the leaf is six to 20 times higher than in the root (Ekpo & Baridia, 2020; Jamil & Bujang, 2016; Eleazu & Eleazu, 2012). It determines the bitterness of the root and other parts of the plant, and it is vital as a defense mechanism against insect herbivores (da Silva Santos et al., 2020; Roslim et al., 2016). The variety, age, soil, geographic location, and environmental factors affect how much cyanogen glucosides are synthesized (Nwokoro et al., 2010; Ubwa et al., 2015). Cassava is divided into two types, sweet and bitter, based on the concentration of cyanogen glucosides in the root (CIAT, 1983). The bitter type contains 50 mg HCN/kg and above (wet basis), while the sweet type has less than this amount (Ojiambo et al., 2017; Pendak, 2011). Sweet cassava matures 6–12 months after planting (MAP) and loses quality if not harvested on time, while bitter cassava matures 10–14 MAP, becoming fibrous if not harvested on time (Pendak, 2011). Previous research has shown that HCN is synthesized at the cassava plant shoot apex and transported to the root (Chaiareekitwat et al., 2022; Latif & Müller, 2015). Therefore, the concentration of cyanogen glucosides in cassava leaves is highest at the top and decreases as it descends. Cyanogen glucosides are hydrolyzed by endogenous enzymes when the plant tissue is damaged during harvesting, by herbivore chewing, food preparation, or within the digestive system to sugar and alpha-hydroxynirile (Ubwa et al., 2015). Alpha-hydroxynirile has undergone an intramolecular

reaction to release the toxic HCN gas (Müller-Schwarze, 2009; Zitnak, 1973). HCN inhibits cytochrome oxidase, eventually leading to death, as shown in Figure 1. The tissues readily absorb the HCN through the bloodstream because of their tiny size and low charge density (da Silva Santos et al., 2020).

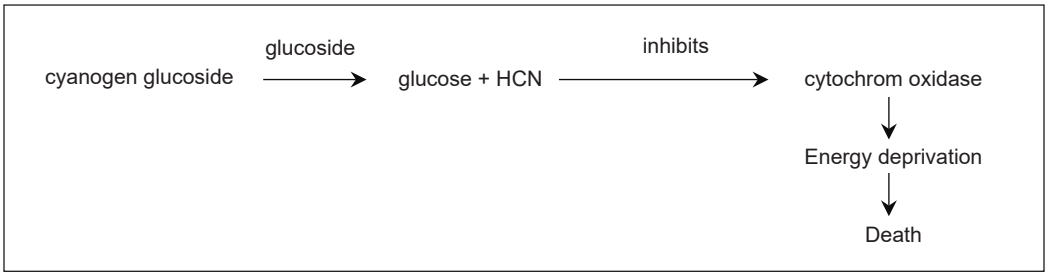

Figure 1. Mechanism of action of cyanogens according to Makkar et al. (2007)

WHO recommends that the maximum lethal dose of HCN in oral foods is 10 mg HCN/kg of body weight (Ojiambo et al., 2017; Ubwa et al., 2015; Umuhozariho et al., 2014). However, ruminants are more vulnerable to HCN poisoning than non-ruminants (Ajayi & Joseph, 2019). It has been demonstrated that an animal's lethal dose of HCN is between 2 and 2.5 mg/kg. Moreover, if the HCN concentration is over 1000 ppm, it may cause death among ruminants (Gensa, 2019).

In the last few decades, there has been much research on polyphenols, phenolics, and flavonoids as alternative bioactive compound-based functional food supplements. Phytochemicals benefit health by acting as preventatives and lowering the chance of non-communicable diseases (NCDs), but they are unnecessary for survival. In animal husbandry, polyphenol supplements are needed in feeds to promote immunity against foreign pathogens by activating signaling pathways (Dragoș et al., 2022).

Furthermore, polyphenols hold benefits such that they can induce epigenetic changes in cells, regulate intestinal musical immune responses, allergic diseases, and antitumor immunity. In the previous literature, cassava has been reported to have secondary metabolites that may add value to animal feed. Thus, this paper aims to determine the level of HCN concentration, protein concentration, and phytochemicals in two cassava varieties at different maturity stages.

MATERIALS AND METHODS

Cassava Leaf Samples

One kg of cassava leaves from two varieties, i.e., White and Pulut, as shown in Table 1, was collected from a local planter in Banting, Malaysia. The samples were collected on the same day to obtain samples with the same ages at six MAP. To describe young and mature leaves,

respectively, leaves were randomly selected from the defined heights from the plants' top (tender shoot apex) and bottom (woody stem). The leaves were harvested, bagged in plastic, and preserved in a polystyrene box with ice during transportation because the protein and cyanide content is thermolabile. Therefore, the samples must be kept between 0 and 4°C. The samples were cleaned, and 12 g of each was randomly taken over the homogenization process. Then, the sample was ground using a kitchen blender. It was packaged in zip-lock bags and kept at -80°C for further analysis (Chaiareekitwat et al., 2022).

Table 1
Variety of cassava leaf samples

Variety	Shapes	Description
White		• The color of the leaf is green • The color of the petiole is green • The shape of the lobes is lanceolate
Pulut		• The color of the leaf is green • The color of the petiole is reddish-green • The shape of lobes is linear

HCN Analysis Based on Reduction of Picrate

The HCN analysis based on the reduction of picrate is the ability to reduce sodium picrate to a red compound, as described by Makkar et al. (2007) (Figure 2). A flat-bottomed screw-capped glass bottle was filled with a 20 mg sample and 1 mL of the 0.2 M phosphate buffer (pH 8). A picrate paper attached to a plastic strip was glued on the screw cap of the bottle above the solution and immediately closed. The bottle was shaken slowly to mix the sample with the buffer. The bottle was incubated at room temperature (30 ± 2°C) for 16 hr. Later, the picrate paper was taken out and submerged for 30 minutes in 5 mL of distilled water. The colorless filter paper was removed, and the picrate solution was boiled for 5 minutes in a water bath. The solution was centrifuged at 4000 rpm for 5 min. The absorbance of the colored solution was measured against the reagent blank (0 ml of sample, 1 ml of buffer, and 5 ml of picrate solution) at 510 nm. HCN content was expressed in ppm using the calibration curve according to the potassium cyanide (KCN) standard.

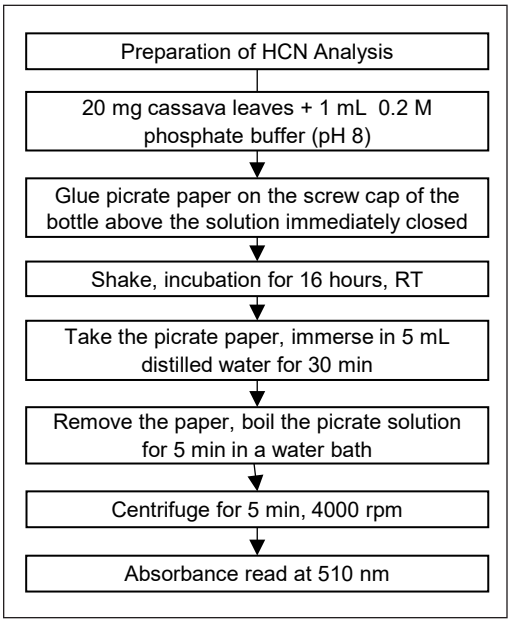

Figure 2. Flowchart representing steps of HCN determination (Makkar et al., 2007)

Protein Analysis Based on Bradford Method

The Bradford method, which analyses the protein-dye complex of a sample, is based on the absorbance shift seen in an acidic solution of the dye Coomassie® Brilliant Blue G-250. The complexes remain dispersed in the solution for about an hour after the dye has a steady affinity for protein and starts to aggregate after one hour (Bonjoch & Tamayo, 2001). The protein concentration was measured using a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510) at 595 nm. The protein concentration in each cassava leaf was determined by using the BSA (bovine serum albumin) calibration curve as a standard.

The samples were powdered using liquid nitrogen. In 1 mL of extraction buffer containing 0.05 M tris base, 0.1% ascorbic acid, 0.1% cysteine hydrochloride, 1% polyethylene glycol, 0.15% citric acid (monohydrate), and 0.008% 2-mercaptoethanol in distilled water. 0.05 g of the antioxidant polyvinyl polypyrollidone (PVPP) is added to each sample during homogenization. Homogenates are transferred to 2 ml Eppendorf tubes and centrifuged for 20 minutes at 12000 rpm at 4°C. The supernatant was collected for protein determination using the Bradford assay described in Figure 3.

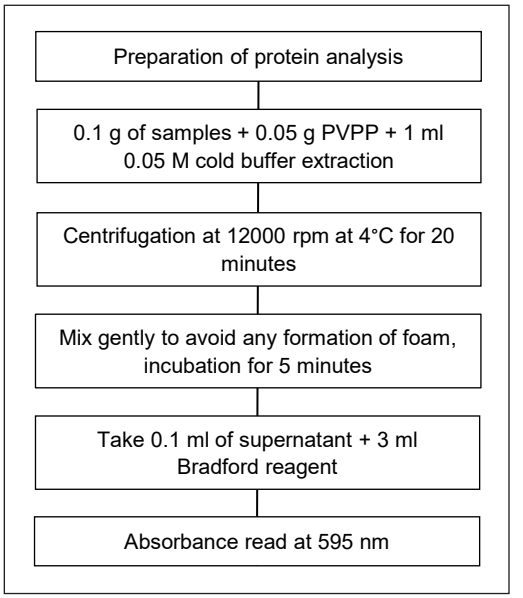

Figure 3. Flowchart representing steps in protein concentration

Phytochemical Analysis

Total phenolic and flavonoid content extraction was analyzed using procedures described by Hosni et al. (2023) with minor modifications. Then, 0.5 g each of cassava roots and leaves was ground using mortar and pestle. Each sample of cassava leaves was extracted in 10 ml of distilled water in sealed bottles using an ultrasonic bath at 60°C for 2 hours. Then, all extracts were separated from the residues by filtering through Whatman No.1 filter paper and stored at -20°C for further quantification.

The phytochemical analysis of TPC and TFC in cassava leaves was conducted using Folin-Ciocalteu reagent and aluminum chloride assay (Samat et al., 2020), respectively, as shown in Figure 4. The TPC was analyzed with slight modifications. Firstly, 200 µL of samples were added to 5 mL of Folin-Ciocalteu reagent, allowing the mixture to react for 10 min at 25°C. Later, 4 mL of sodium carbonate was added to the solutions kept in the dark for 20 minutes at room temperature. The absorbance was quantified using a Multiskan

GO microplate spectrophotometer (Thermo Scientific 1510) at 750 nm. The TPC value was stated as mg gallic acid equivalents per 100 g of fresh weight.

To determine the TFC, 1 mL of each extract was mixed with 4 mL of distilled water. Next, 0.3 mL of a sodium nitrite ($NaNO_2$) solution (1:5, w/v) was added to the flasks and left at room temperature for 6 min. Afterward, 0.3 mL of a 1:10 solution of aluminum chloride ($AlCl_3$) was added, and the mixture was left at room temperature for 6 min. To the resulting solution, 2 mL of a 1 M sodium hydroxide solution was added, and the mixture was incubated for 10 minutes at 25°C. The absorbance of the mixture was measured at 510 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510). The TFC was calculated using the calibration curve with the quercetin standard and expressed as mg QE/100 g fresh weight.

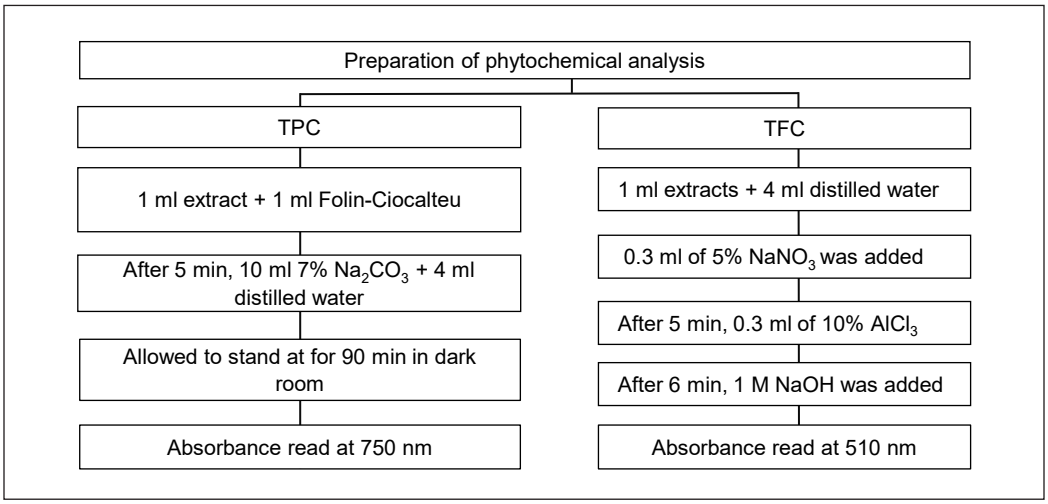

Figure 4. Flowchart representing steps in TPC and TPC determination

Statistical Analysis

Two independent variables influenced by dependent variables were analyzed using Minitab software (Version 19, State College, Pennsylvania, USA). A two-way analysis of variance (ANOVA) at 95% ($p < 0.05$) confidence interval was carried out to test differences between the means of more than two groups. Tukey's multiple comparison tests identified the significant difference between the means and the final value as the average±standard deviation. The interaction between the one independent variable and the effect on the other independent variable was examined.

RESULTS AND DISCUSSION

Table 2 demonstrates the amount of HCN, protein concentration, and phytochemical contents in cassava leaf samples according to the KCN, BSA, gallic acid, and catechin

standard curves, respectively. The cassava leaves of different maturity and variety had different HCN and protein values. The findings indicated that the HCN varied significantly ($p < 0.05$) among different maturities from the White variety. The HCN value ranged from 5.72 to 8.67 mg/100 g. This finding is consistent with that of Jamil and Bujang (2016), who found that the cyanide content in cassava leaves in Malaysia was 8.867–14.72 mg/100 g. Cassava leaves from the White variety demonstrated the highest HCN, followed by the young Pulut, mature Pulut, and young White. The significant differences could be due to the different leaf positions representing leaf maturity. The youngest leaves are collected from the shoot apex (top position), and the matured leaves are located at the bottom, resulting in different amounts of HCN content. According to Ravindran (1992), the cyanide concentration in cassava leaves was highest at the top position and declined towards the lower position. Generally, young cassava leaves contain the highest level of HCN and will decrease to 50–70% in mature leaves (Nambisan & Sundaresan, 1994).

Furthermore, the results showed that HCN content in all cassava leaves exceeded the lethal dose intake of 1 mg HCN equivalent/100 g of the sample (Jamil & Bujang, 2016; Latif & Müller, 2015). Also, ruminants (based only on a studies on goats) may tolerate an intake of 0.25 ppm of HCN (Gensa, 2019). Therefore, the leaves should be detoxified before consumption because they are highly poisonous. The presence of HCN in all study leaves confirmed earlier reports that all cassava cultivars contain a cyanogenic glucoside in wide disparities according to varieties (CIAT, 1983). Hydrolysis of cyanogen glycosides at a temperature above 25.6°C will release cyanide gas, which may cause dizziness, headache, fatigue, and sometimes death (Latif & Müller, 2015).

The final value was expressed as mean ± standard deviation; n=3. Means with the same letters (a, b) in the same column are not significantly different at $p < 0.05$ according to the Tukey multiple range test.

Furthermore, the results showed that HCN content in all cassava leaves exceeded the lethal dose intake of 1 mg HCN equivalent/100 g of the sample (Jamil & Bujang, 2016; Latif & Müller, 2015). Additionally, ruminants (based only on studies on goats) may tolerate an intake of 0.25 ppm of HCN daily (Gensa, 2019). Therefore, the leaves should be detoxified before consumption because they are highly poisonous. The presence of HCN in all study

Table 2
Cyanide, protein, phytochemicals concentrations of different varieties, and maturity of cassava leaves

Samples	Maturity	HCN (mg/100 g)	Protein (mg/100 g)	Total Phenolic compound (mg GAE/100 g)	Total Flavonoid compound (mg CE/100 g)
White	young	8.67±0.25^{a}	1.91±0.69^{b}	242.9±32.7^{a}	64.12±0.65^{b}
	mature	5.72±0.47^{b}	129.46±0.2^{a}	305.2±63.2^{a}	76.81±3.53^{a}
Pulut	young	7.27±0.47^{a,b}	233.17±0.46^{a}	302.4±61.6^{a}	59.73±2.73^{b}
	mature	7.41±0.41$^{a, b}$	232.7±91.1^{a}	291.7±51.8^{a}	88.01±7.73^{a}

leaves confirmed earlier reports that all cassava cultivars contain a cyanogenic glucoside in wide disparities according to varieties (CIAT, 1983).

The safe level of HCN from cassava leaves varies for animals depending on the species and the animal's age. The Food and Agriculture Organization (FAO) recommends a maximum safe level of 50 mg HCN per kilogram of dry matter in the diet of ruminants and 10–20 mg HCN per kilogram of dry weight in the diet of monogastric animals (Gensa, 2019). High levels of HCN in the ruminant diet can adversely affect their health and performance.

Regarding polyphenols, not many differences were recorded for both varieties and maturities. Although no significant variances were found between flavonoid varieties, flavonoids were higher in mature leaves than in young cassava leaves. The current results agreed with the previous study conducted by Nur et al. (2013) in cultivating cassava leaves in Malaysia, where the value of TPC is five times higher than TFC—according to Fritz et al. (2001) and Ghasemzadeh et al. (2014) stated that TPC and TFC increase their production following the maturity of plant's capability to devotes the resources. It explained why flavonoids as secondary metabolites and primary metabolites were accumulated in mature leaves rather than young leaves.

The results also revealed that the protein concentration demonstrated a significant difference between young White cassava leaves and others, with values ranging from 129.46 to 233.17 mg/100 g. The highest protein content was exhibited by the young Pulut (233.17 mg/100g), and the lowest was from the young White (1.91 mg/100g) cassava leaves. The protein concentration was significantly lower ($p < 0.05$) for young White cassava leaves compared to the other samples. On a fresh basis, protein content was reported to be 4.0-9.6%, while dry basis content had higher crude protein at 20.6–36.4% (Latif & Müller, 2015). Therefore, to use cassava leaves as a protein source, the Pulut variety is more suitable than the White variety. Two-way ANOVA was conducted to investigate two main effects (variety and maturity) on cassava leaves' HCN and protein content (Tables 3 and 4). The two-way interaction showed that the leaf maturity and interaction (variety × maturity) statistically significantly affected the HCN content.

Table 3
Two-way ANOVA test of the effect of variety and maturity of cassava leaf on HCN concentration

Source of variation	df	Sum of squares	Mean square	*F*-value	*p*-value
Variety	1	0.04	0.04	0.10	0.77
Maturity	1	3.93	3.93	8.96	0.04
Variety × Maturity	1	4.75	4.74	10.81	0.03
Error	4	1.76	0.44		

Note. R squared = 83.24% (adjusted *R* squared = 70.67%)
df – degree of freedom; dependent variable –HCN concentration

Table 4
The two-way ANOVA test of the effect of variety and maturity of cassava leaf on protein concentration

Source	df	Sum of squares	Mean square	*F*-value	*p*-value
Variety	1	55941	55941	26.97	0.01
Maturity	1	8073	8073	3.89	0.12
Variety × Maturity	1	8197	8197	3.95	0.12
Error	4	8296	2074		

Note. R squared = 89.70% (adjusted *R* squared = 81.97%)
df – degree of freedom; dependent variable –protein concentration

CONCLUSION

This study has shown that cassava leaves are rich in protein. However, it also contained HCN, which is harmful to humans and animals and was above the safe level recommended by WHO/FAO. There is a variation of protein and HCN content found in different varieties and maturity of the cassava leaves. The highest protein content was found in young Pulut leaves; therefore, it was selected as a potential ingredient in ruminant feed. The influence of maturity significantly affects the HCN content of cassava leaves. These results could be preliminary data on nutrient sources related to cassava leaves that can be used as a reference for future research.

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