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Herbal Yield, Secondary Metabolites and Antioxidant Activities of *Piper sarmentosum* Roxb. affected by Different Harvest Maturity

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The aim of this study was to assess the yields, total flavonoid content, total phenolic content, and antioxidant activities of 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and 2,2'–azion–bis-3–ethylbenzothiazo line–6-sulphonic acid (ABTS) radical scavenging activity of *Piper sarmentosum* Roxb. tested after different harvesting maturity (30, 60, 90, and 120 days after planting). A completely randomised design was determined for the field experiments at the Department of Medicinal Plant Science, Faculty of Agricultural Production, Maejo University, Thailand. Results showed that the highest weight for fresh and dried leaves and total flavonoid content were observed at harvesting maturity of 90 days after planting with values of 18.03 ± 0.04 g fresh weight (FW)/plant, 4.08 ± 0.03 g dried weight (DW)/plant, and 4.52 ± 0.16 mg quercetin equivalent (mgQE)/g dried sample, respectively. Furthermore, the harvesting maturity at 120 days also gave the highest total phenolic content (14.53 ± 0.18 mg gallic acid equivalent (mgGAE)/g dried sample) and antioxidant activities in *Piper sarmentosum* Roxb.

Keywords: Antioxidant; Flavonoid; Harvesting; Phenolic; *Piper sarmoentosum* Roxb.

I. INTRODUCTION

Thai herbs are used in various ways today; for example, medicinal (Prajuabjinda *et al.*, 2020), cosmetics (Narayanaswamy & Ismail, 2015) and functional foods (Bishnoi, 2021). Cha-plu, or *Piper sarmentosum* Roxb., is one Thai herb used as a traditional medicine to treat diabetes (Basheer & Majid, 2010; Rahman *et al.*, 2010) and as a side dish. Cha-plu is in the family of Piperaceae and is cultivated in Thailand and Southeast Asia. The plant has a tender, bright green and 50–70 cm high stem. The leaves are thin, 7–15 cm long, 5–10 cm wide, dark green (Figure 1), and have a spicy taste. The herb contains alkaloids such as amide, pyrones, and flavonoids (BGO Plant Databases, 2011). It has also been reported to have anti-cancer (Hematpoor *et al.,* 2018), anti-tuberculosis, and antioxidant properties (Hussian *et al.*, 2009).

In the past decade, herbs have been harvested from the wild, making them insufficient for use. Nowadays, medicinal plants are being cultivated to get the desired yield and can control the quality of the secondary metabolites as well. Many researchers have reported that yields and secondary metabolites of herbs cultivated are affected by various factors such as fertiliser (Mohamed *et al.*, 2016; Matłok *et al.*, 2019; Doan *et al.*, 2021; Haghighi & Nikbakht, 2021), light exposure (Dou *et al.*, 2017; Hazrati *et al.*, 2020) and amount of water received (Mahmud *et al.*, 2017; Mirzaie *et al.*, 2020) and the effects of harvesting maturity were investigated (Brasileiro *et al.*, 2015; Cezarotto *et al.*, 2017; Moradi *et al.,* 2020) but *Piper sarmentosum* Roxb. has not been studied in the effect of

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harvesting maturity before. Therefore, the aim of this study was to evaluate the effect of harvesting maturity (30, 60, 90, and 120 days) on crop yield, secondary metabolites (total flavonoid content and total phenolic content), and antioxidant activities of *Piper sarmentosum* Roxb..

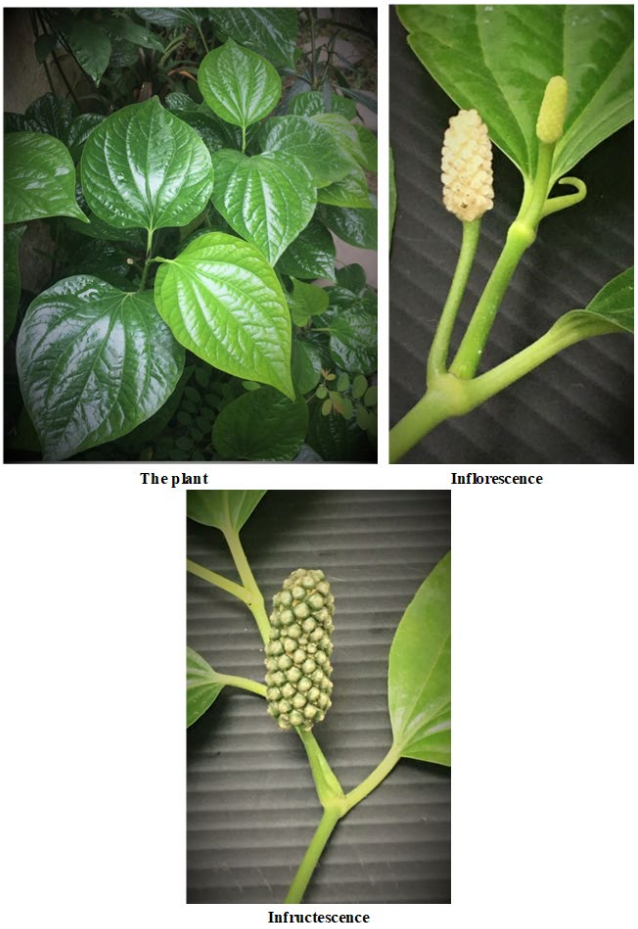


Figure 1. *Piper sarmentosum* Roxb.

II. MATERIALS AND METHODS

A. Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH) and Sodium carbonate anhydrous were obtained from Fluka (Germany), Folin-Ciocalteu phenol reagent and Aluminum chloride were purchased from the Merck Co., Ltd. (Germany). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), 2,2'–azion–bis-3–ethylbenzothiazo line–6-sulphonic acid (ABTS) and Quercetin were obtained from Sigma Aldrich (Sigma–Aldrich GmbH, Germany). All other chemicals were analytical reagent grade.

B. Plant Material and Experimental Design

Piper sarmentosum Roxb. was obtained from Chiang Mai Royal Agricultural Research Centre, Chiang Mai, Thailand. The sample was identified by the Queen Sirikit Botanical Garden, Ministry of Natural Resources and Environment, Thailand. A voucher specimen was deposited at the Queen Sirikit Botanical Garden Herbarium (QBG no. 127401). The shoot tip was cut 10-15 cm long, planted in the pots containing a 1:1 mixture of soil and husk, and cultivated in a greenhouse under the natural light condition for 30 days.

The experiment was conducted between September 2017 to January 2018 at the demonstration farm of Maejo University, Chiang Mai (383 m AMSL, 18°55'05"N, and 90°03'06'E). The experiment was performed in a complete randomised design (CRD) with three replications. In all, a total of 48 plots were used to collect data. The herb materials were transplanted in 15-inch pots containing soil: husk (1:1). After planted 7 days the chicken manure was applied with 125 g /pot. The crop was watered on alternate days, and all plants were harvested at 30 60 90 and 120 days after planted. The freshly harvested leaves were immediately weighed for the fresh weight determination and were dried at 45 °C for 72 hours in a drying oven for dry weight determination and then ground to the fine powders.

C. Total Flavonoid Content (TFC)

The determination of total flavonoid content was estimated with minor modifications from Pumtes *et al.* (2016). Briefly, three grams of dry powder samples were extracted by the sonication method, a 10 mL of ethanol was added to the sample and sonicated for 15 minutes. The extract solution was then filtered and transferred to a 25 mL volumetric flask. The residue was re-extracted under equivalent conditions two times. The extract solutions were combined, and the volume was completed to 25 mL of ethanol. A 0.5 mL of sample solution was diluted in 2 mL of ethanol, resulting in the test sample solution.

TFC was measured by using the Aluminium chloride assay (Patil *et al.*, 2012). The test samples (0.5 mL) were mixed with water (2.9 mL) and ethanol (1.5 mL), then mixed with 10 % aluminum chloride solution (0.1 mL) and 0.1 mL of Na-k tartarate. The mixture solution was subjected to

measurement at 410 nm by using a spectrophotometer (Genesys 10S, Thermo Scientific, USA). Quercetin was used as a standard to prepare calibration curve solutions with concentrations of 10-100 µg/mL. TFC was expressed as mg quercetin equivalent per gram dried sample (mgQE)/g DW).

D. Total Phenolic Content (TPC)

The total phenolic content in the *Piper sarmentosum* Roxb. leaves extract was determined with minor modifications from Pumtes *et al.* (2012). Briefly, three grams of ground sample were extracted in 30 mL methanol and heated at 40°C for 3 hours in the water bath. The extracted aqueous phase was subsequently filtered by using Whatman No.1, then evaporated and the crude extracts were weighed and dissolved the crude extract in 5 mL of methanol. The sample solution was stored at 4°C to estimate TPC, DPPH and ABTS scavenging assay.

TPC was measured by using the Folin-Ciocalteu assay (Namjooyan *et al.,* 2010). An aliquot of 0.1 mL extracts was mixed with 2 mL of 2% w/v sodium carbonate followed by adding Folin Ciocalteu's phenol reagent (0.1 mL). The mixture solution was completely mixed and stood for 30 minutes at room temperature. After that, was measured the absorbance by using a spectrophotometer (Genesys 10S, Thermo Scientific, USA) at 750 nm. Gallic acid was used as the standard curve (10-100 µg/mL), and the total phenolic content was expressed as mg gallic acid equivalent per gram dried sample (mgGAE/ g DW).

E. Antioxidant Activities Analysis

1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging activity was determined based on the following method (Singh *et al.*, 2002). The assay of DPPH radical scavenging was performed in a spectrophotometer (Genesys 10S, Thermo Scientific, USA) with 3.0 mL of DPPH solution (0.5 mM in ethanol) and 0.1 mL of sample solution (obtained from the above TPC extract). The mixture solution was allowed to stand for 30 minutes at room temperature and was measured at 517 nm, and the DPPH radical scavenging activity (%) was calculated as below:

$$\text{DPPH radical scavenging activity (\%)} = (A_{Control}-A_{Sample})/(A_{Control})x100$$

where A_{Sample} is the absorbance of the sample, and $A_{Control}$ is absorbance measured in the absence of the sample. The IC_{50} value is defined as the concentration of the sample leading to a 50% reduction of the initial DPPH concentration. It was obtained from the linear regression of plots of the percentage of the radical scavenging activity against the concentration of the test extracts (mg/mL) obtained from three replicate assays. The antioxidant activity was also expressed as mg Trolox equivalent (TEAC)/g dried basis using Trolox as a standard curve (10-30 µg/mL) and mg Ascorbic equivalent (AEAC)/g dried basis using Ascorbic as a standard curve (10-30 µg/mL).

2. 2,2'–azion–bis-3–ethylbenzothiazo line–6-sulphonic acid (ABTS) radical scavenging assay

The ABTS radical scavenging assay was measured based on the method (Thaipong *et al.*, 2006). Briefly, ABTS mixture solution was first prepared by mixing an equal amount of 7.4 mM ABTS and 2.6 mM of potassium persulfate solutions ($K_2S_2O_8$) for 24-h in the dark at ambient temperature. Subsequently, ABTS working solution was prepared by dilution of the 1.0 mL of ABTS mixture solution in 60 mL of methanol before use. The reaction proceed was started by the addition of 3.0 mL of the ABTS working solution into the 0.1 mL of sample solution (obtained from the above TPC extract), then allowed in the dark at room temperature for 6 minutes. The absorbance of the mixture solution was measured at 734 nm. The ABTS radical scavenging activity and IC_{50} were calculated the same as DPPH radical scavenging assay. The antioxidant activity was also expressed as mg Trolox equivalent (TEAC)/ g dried basis and mg Ascorbic equivalent (AEAC)/g dried basis the same as DPPH radical scavenging assay.

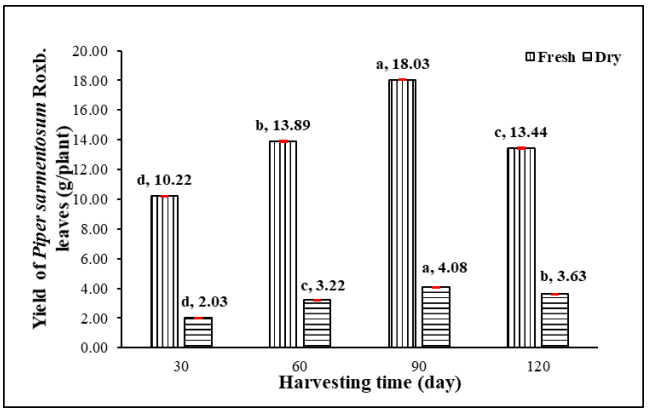
F. Statistical Analysis

All experiments were conducted in three replicates. The results were expressed in mean ± standard deviation (SD). Analysis of variance using one-way ANOVA at 0.05 significance level and Duncan's New Multiple Range Test was used. Pearson's correlation coefficient (r) was used to determine the relationship between variables.

III. RESULT AND DISCUSSION

A. Effect of Harvesting Maturity on The Yield of Piper sarmentosum Roxb. Leaves

The highest yield of *Piper sarmentosum* Roxb. was obtained at a harvesting maturity of 90 days and the difference between harvesting maturity was significant ($p<0.05$). The fresh and dry weights of the harvested leaves were 18.03 ± 0.04 g FW/plant and 4.08 ± 0.03 g DW/plant (Figure 2), respectively. The lowest yields per plant (10.22 ± 0.02 g FW/plant and 2.03± 0.02 g DW/plant) were found at the harvesting maturity of 30 days. From these results, the yield of leaves increased until 90 days after that, the yield decreased because the leaves had older and were defoliated. This result was similar to the results of Fetiandreny (2013), who reported that the yield *Piper sarmentosum* Roxb. leaves increased for harvesting maturity of up to 98 days, then the yield of leaves decreased at 112 days.



Means with different letters indicate significantly different between harvesting maturity ($p<0.05$, Duncan test).

Figure 2. Yields of *Piper sarmentosum* Roxb. at difference harvesting maturity. Means with different letters indicate significantly.

B. The Effect of Different Harvesting maturity on The Total Flavonoid Content (TFC) and Total Phenolic Content (TPC)of Piper sarmentosum Roxb. Leaves

Flavonoids are a group of secondary metabolites that can be found naturally in plants. Studies have indicated that increased intake of dietary flavonoids is related to a decreased risk of cardiovascular diseases (Arts & Hollman, 2005). The cardiovascular preventative effects of flavonoids may be mediated by multiple mechanisms such as anti-hypertensive, antioxidant, hypoglycemic, antiplatelet, and hypoglycemic effects (Ushida *et al.*, 2008). *Piper sarmentosum* Roxb. extracts have been reported to possess multiple cardiovascular protective effects. The researcher reported rutin and vitexin as the main flavonoids in this herb (Ugusman *et al.*, 2012).

Table 1. Total flavonoid content and total phenolic content at difference harvesting maturity of *Piper sarmentosum* Roxb.

Harvesting maturity(day)	Total flavonoid content (mgQE/g DW)	Total phenolic content (mgGAE/g DW)
30	2.02±0.10^{d}	10.22±0.49^{c}
60	3.02±0.09^{c}	10.57±0.24^{c}
90	4.52±0.16^{a}	11.98±0.29^{b}
120	3.64±0.08^{b}	14.53±0.18^{a}

Note: Different letters within a column indicate significant differences at $p<0.05$.

The results of TFC in this study ranged from 2–5 mgQE/g DW (Table 1). The results of this study showed significant differences ($p<0.05$) for TFC due to the effect of different harvesting maturity. The highest TFC was recorded at a harvesting maturity of 90 days after planting, 4.52 ± 0.16 mg QE/g DW. The lowest TFC was obtained at a harvesting maturity of 30 days, 2.02 ± 0.10 mg QE/g DW.

Various factors such as species, variety, cultivation, weather, region conditions and harvesting maturity could influenced the phytochemical composition of plants (Jan *et al.*, 2021). Therefore, increasing the harvesting maturity may increase the TFC. Flavonoids are synthesised through the phenylpropanoid pathway, transforming phenylalanine into 4-coumaroyl-CoA, which enters the flavonoid biosynthesis pathway (Ferreyra *et al.*, 2012). Increasing the period of plant growth, the leaves increase photosynthesis and glucose cumulative, which are the major molecules of the phenylalanine biosynthesis pathway (Figure 3) (Barros & Dixon, 2020). This increase may be another reason to explain why the highest TFC was found in *Piper sarmentosum* Roxb. at a harvesting maturity of 90 days. According to previous reports have found that increased harvesting maturity increased flavonoids. Cezarotto *et al.* (2017) studied the harvested leaves of rabbiteye blueberry (*Vaccinium ashei*) at

different times (December 2013 and March 2014) and the results found that increased harvest time resulted in the highest TFC.

Phenolic compounds are important secondary metabolites as the flavonoids because of their free radical scavenging ability, which is due to their hydroxyl groups. Therefore, the TPC and TFC of plants may contribute directly to their antioxidant capabilities. In the present study, the TPC of the leaves was determined by the Folin–Ciocalteau method. The TPC results presented in Table 1 showed significant differences (p<0.05) among at the different harvesting maturity. The results also show that a harvesting maturity of 120 days had the highest mean TPC, 14.53 ± 0.18 mg GAE/g DW, while harvesting maturity of 30 and 60 days had the least mean TPC, 10.22 ± 0.49 mg GAE/g DW and 10.57 ± 0.24 mg GAE/g DW, respectively. As the harvesting maturity increased, the TPC increased. The TPC results in this current study were similar to those reported by Routray and Orsat (2014) who observed that the TPC of blueberry leaves increased when harvesting maturity increased due to environmental stresses. In the same way, the study by Ozcan *et al.* (2019) reported that the composition of the bioactive compounds in vegetables and herbs, including phenolics, varied at different harvesting maturity.

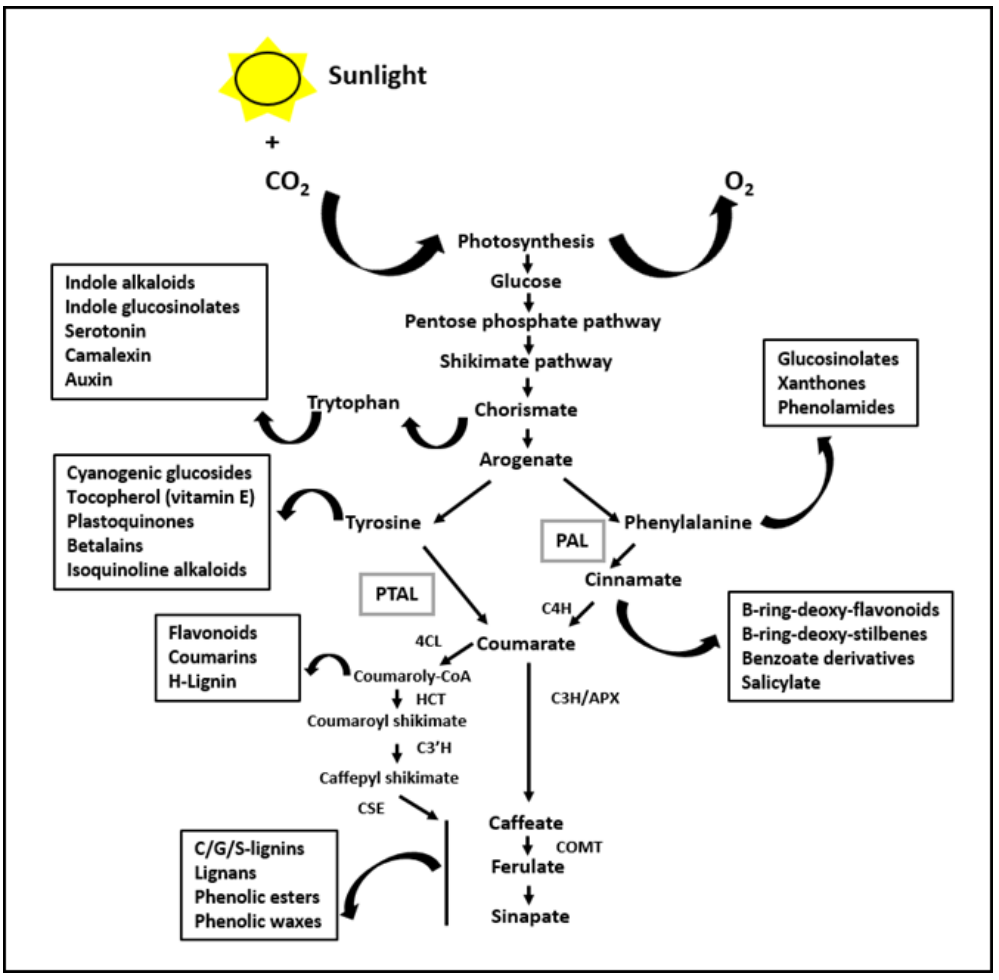


Figure 3. Flavonoid synthetic pathway (Barros & Dixon, 2020).

(PAL: L-phenylalanine ammonia-lyase; PTAL: L-phenylalanine/L-tyrosineammonia-lyase; C4H: trans-cinnamate 4-hydroxylase; CH3/APX: coumarate 3-hydroxylase/cytosolic ascorbate peroxidase; COMT: caffeic acid 3-O-methyltransferase, 4CL: 4-coumarate: CoA ligase; HAT: hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase; C3'H: p-coumaroyl quinate/shikimate 3'-hydroxylase; CSE, caffeoyl shikimate esterase)

C. Antioxidant Activities at Different Harvesting Maturity

1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging

The antioxidant properties of the extracts of the *Piper sarmentosum* Roxb. leaves harvested at different times due to DPPH (2,2-diphenyl-2-picrylhydrazyl) free radical scavenging are shown in Table 2. The radical scavenging ranged from 17 to 14 %, 32 to 43 mgTEAC/g DW, 19 to 26 mgAEAC/g DW and the IC_{50} between 0.32 to 0.42 mg/mL, respectively. Different harvesting maturity had a significant impact (p<0.05) on DPPH radical scavenging activity in *Piper sarmentosum* Roxb. The harvesting maturity at 120 days showed higher average antioxidant properties than other harvesting maturity, with values of radical scavenging 23.78 ± 1.92 %, 42.60 ± 3.17 mgTEAC/ g DW, 25.91 ± 2.04 mgAEAC/ g DW and IC_{50} with 0.32 ± 0.024 mg/mL, respectively. These results demonstrated that variation in the antioxidant activity depended on the harvesting maturity. As previously discussed, changes in environmental conditions during the different seasons of increased harvesting maturity explained TPC variations (Howard *et al*., 2002) and also DPPH activity.

2. 2,2'–azino–bis-3–ethylbenzothiazoline–6-sulphonic acid (ABTS) radical scavenging

Various methods are known to estimate the total antioxidant capacity of herbs, including the ABTS assay, which we have applied in this study. It can be seen from the data in Table 2 that the ABTS radical scavenging antioxidant potential of *Piper sarmentosum* Roxb. increased with increased harvesting maturity, and significant differences (p<0.05) were observed between different harvesting maturity, similarly with the results of DPPH radical scavenging.

The harvesting maturity of 120 days had the highest ABTS radical scavenging values than other harvesting maturities. The highest antioxidant activity of *Piper sarmentosum* Roxb. was 28.07 ± 2.24 %, 36.83 ± 0.58 mgTEAC/ g DW, 17.13 ± 0.01 mgAEAC/ g DW and IC50 with 2.67 ± 0.04 mg/mL, respectively. According to the ABTS radical scavenging results, the ABTS radical scavenging in *Piper sarmentosum* Roxb increased similarly with TPC and DPPH radical scavenging with increased harvesting maturity.

D. The correlation between secondary metabolites and antioxidant properties at different harvesting maturity

The correlation between secondary metabolites and antioxidant properties was analysed, and the outcomes are shown in Table 3. The correlation coefficient values showed a significant (p<0.05) and strong positive correlation between the TPC, DPPH and ABTS activities, while weak correlation between the TFC, DPPH and ABTS activities. The Pearson correlation coefficients or r-values of TPC with DPPH and ABTS inhibition activities were 0.97 and 0.81, meanwhile, r-values of TPC with DPPH (mgTEAC, mgAEAC) and ABTS (mgTEAC, mgAEAC) activities were 0.91, 0.91, 0.86 and 0.88 respectively, which revealed that the secondary metabolite (TPC) in *Piper sarmentosum* Roxb. leaves mainly contribute to the antioxidant capacity. Similar results were observed by Hussain *et al.* (2009), they found a *Piper sarmentosum* Roxb. showed higher antioxidant activity as well as a higher amount of polyphenols.

Table 2. Effect of different harvesting maturity on antioxidant activities of *Piper sarmentosum* Roxb.

Harvesting maturity (day)	**DPPH radical scavenging**				**ABTS radical scavenging**			
	Inhibition (%)	**IC_{50} (mg/mL)**	**mgTEAC/ g DW**	**mgAEAC/ g DW**	**Inhibition (%)**	**IC_{50} (mg/mL)**	**mgTEAC/ g DW**	**mgAEAC/ g DW**
30	17.44±0.12^{b}	0.42±0.003^{b}	32.12±0.20^{b}	19.14±1.30^{b}	21.49±0.41^{bc}	3.50±0.06^{c}	28.04±0.47^{c}	5.27±0.10^{c}
60	17.44±0.12^{b}	0.42±0.002^{b}	33.12±0.20^{b}	19.14±1.30^{b}	17.68±0.43c	4.15±0.09^{d}	23.65±0.50^{d}	4.34±0.10^{d}
90	18.33±0.61^{b}	0.40±0.012^{b}	33.60±1.01^{b}	20.09±0.09^{b}	23.06±3.75^{bc}	2.98±0.16^{b}	33.02±1.78^{b}	15.33±0.04^{b}
120	23.78±1.92^{a}	0.32±0.024^{a}	42.60±3.17^{a}	25.91±2.04^{a}	28.07±2.24^{a}	2.67±0.04^{a}	36.83±0.58^{a}	17.13±0.01^{a}

Note: Different letters within a column indicate significant differences at p<0.05.

Table 3. Correlation matrix of the studied parameters (Pearson correlation coefficients).

	TPC	TFC	IC_{50} (DPPH	IC_{50} (ABTS)	% Inhibition (DPPH)	% Inhibition (ABTS)	mgTEAC (DPPH)	mgAEAC (DPPH)	mgTEAC (ABTS)	mgAEAC (ABTS)
TPC	1									
TFC	0.57	1								
IC_{50}(DPPH)	-0.98	-0.33	1							
IC_{50}(ABTS)	-0.80	-0.53	0.73	1						
% Inhibition (DPPH)	0.97	0.31	-0.99	-0.71	1					
% Inhibition (ABTS)	0.81	0.35	-0.70	-0.84	0.69	1				
mgTEAC (DPPH)	0.91	0.31	-0.99	-0.71	1	0.69	1			
mgAEAC (DPPH)	0.91	0.31	-0.99	-0.71	1	0.69	1	1		
mgTEAC (ABTS)	0.86	0.56	-0.79	-0.99	0.77	0.85	0.77	0.77	1	
mgAEAC (ABTS)	0.88	0.78	-0.74	-0.92	0.73	0.78	0.73	0.73	0.94	1

IV. CONCLUSION

The harvesting maturity of *Piper sarmentosum* Roxb. significantly affected the amount of TFC, TPC, and their antioxidant activities, as measured by the DPPH and ABTS radical scavenging methods. Although a harvesting maturity of 120 days resulted in the highest TPC and antioxidant activities, the growing period was increased to 120 days, and the cost of management increased, but the weight of the produce decreased. When considering the increased costs, it may not be worth harvesting during that period. Among the four harvesting maturities studied (30, 60, 90, and 120 days), a harvesting maturity of 90 days is recommended as the optimal for *Piper sarmentosum* Roxb.

V. ACKNOWLEDGEMENT

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