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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

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This study was done to determine the effects of steeping temperature and storage period on the bioactive contents plus antioxidant and antidiabetic activities of *Pluchea* leaf infusion. The research used a randomized block design with two factors, *i.e.* steeping temperature (T) and storage period (B). The *Pluchea* leaf blades were exposed to four steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period of 0 (B1) and 5 (B2) yr – resulting in eight treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at $\alpha \leq 0.05$ showed that treatments significantly affected the bioactive contents [total phenol (TPC), total tannin (TTC), and total flavonoid (TFC)], antioxidant [DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [α-amylase (AA) and α-glucosidase (GA) inhibition] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH, and FRAP significantly increased for the storage period and the steeping temperatures. Then, TFC decreased during the storage period but significantly increased at higher steeping temperatures. The AA and GA of *Pluchea* leaf infusion increased until 70 °C of the steeping temperature but decreased until 95 °C. The DPPH and FRAP of the *Pluchea* leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA of *Pluchea* leaf infusion were not influenced by the TPC and TTC but were weakly and positively correlated with TFC. The antioxidant activity of the *Pluchea* leaf infusion was inversely proportional to the antidiabetic activity. The simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

Keywords: antioxidant, antidiabetic, bioactive compound, *Pluchea indica* Less, steeping temperature, storage period

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INTRODUCTION

Pluchea herbal tea is a product of dried *Pluchea* leaf processing introduced by world people (Srisook *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the active components in *Pluchea* leaves, as a herbal plant that has been widely used for traditional medicine and food (Chan *et al.* 2022). *Pluchea* leaves are composed of many nutrients and bioactive compounds useful to body health. The nutrient compositions in the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium, β-carotene, and vitamin C, whereas bioactive compounds are comprised, *i.e.* chlorogenic acid, caffeic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin, β-carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019; Widyawati *et al.* 2022; Chan *et al.* 2022).

The steeping process of *Pluchea* leaves can be performed with fresh or dry leaves in hot or boiling water for a few min (Suriyaphan 2014; Silva-Ramirez *et al.* 2020; Jayani *et al.* 2022). In Asia, especially in Indonesia, people usually consume the *Pluchea* infusion by steeping 2 g of powdered *Pluchea* leaves in a tea bag in 100 mL of hot or boiling water. Widyawati *et al.* (2016) claimed that steeping 2 g of *Pluchea* leaf powder at 95 °C for 5 min exhibits total phenolic and flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3, 22.0, 27.2, and 10.2 mg gallic acid equivalent (GAE)/ g sample, respectively. Werdani and Widyawati (2018) reported that drinking *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/ 100 mL) can decline blood sugar levels.

The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 min certainly determines the stability and amount of extracted bioactive compounds that influence the biological activity especially antioxidant and antidiabetic activities. Silva-Ramirez *et al.* (2020) reported that the infusion process can influence the content and composition of the bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022) stated that the infusion quality of herbal tea extract depends on a number of factors, *i.e.* storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping or brewing and storage temperatures, as well as longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, as some white and green teas are effective with hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), on rosehip tea is effective at infusion period around 6–8 min at temperatures of 84–86 °C (Ilyasoglu and Arpa 2017), on the caffeine content extracted at the brewing temperature of coffee (Zarwinda and Sartika 2018), and the high total phenol content and antioxidant activity of dark tea at 92 °C for 27 min (Wang *et al.* 2022). The study of the effect of steeping temperature on *Pluchea* infusion was carried out to afford information about the most efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds, antioxidant, and antidiabetic activities.

Storage period tea usually for several months to years *Pluchea* herbal tea also affects the levels of the bioactive compounds and biological activity (Jayani *et al.* 2022). Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or aluminum foil standing pouch or a combination of both. Many researchers reported that the storage period decreases the bioactive compounds plus antioxidant and antidiabetic activities, *i.e.* juice from *Momordica charantia* L. (Lin *et al.* 2020), dried *Piper betle* extracts (Ali *et al.* 2018), white tea (Xu *et al.* 2019), Kinnow-Amla beverages (Purewal *et al.* 2022), and whole-wheat flour (Zhang *et al.* 2021).

Therefore, this research studied the effect of steeping temperature and storage period on the bioactive compounds [total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC)], antioxidant [DPPH free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)], and antidiabetic activities [α-amylase (AA) and α-glycosidase (GA) inhibition] of the infusion from powdered *Pluchea* leaves and on the phenolic compound profile.

MATERIALS AND METHODS

Raw Materials and Preparation

The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya, East Java, Indonesia. The *Pluchea* plants were included in the Asteraceae family with specifications according to the GBIF taxon ID number database:3132728 (Ferraris 2023). *Pluchea* leaves at 1–6 levels of each branch from the shoot were collected, sorted, washed, and dried to get a moisture content of around 11.16 ± 0.09% dry basis (Widyawati *et al.* 2022). The dried *Pluchea* leaves were pulverized to a 45-mesh size powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder was packed into a paper filter infusion bag. Packed samples were stored for 0 (unstored) and 5 (stored) yr in a standing pouch before analysis.

In the research, one tea bag of *Pluchea* herbal tea that was stored for 0 (B1) and 5 (B2) yr was steeped with 100-mL hot water at various temperatures – including 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C for 5 min – with infusion method obtaining eight treatment combinations – namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, and T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

Reagents

The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, p-nitrophenyl-α-glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin-Ciocalteu's phenol, sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade except for distillated water which was purchased from PT Aqua Industry Surabaya.

Analysis of the Bioactive Compounds

Total phenolic content (TPC) analysis. The TPC of treated *Pluchea* infusion was carried out using the technique by Gao *et al.* (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flash and incubated for 5 min. Then, 2 mL Na_2CO_3 7.5% was added and filled up to 10 mL volume with distilled water. The blue color intensity of the solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ = 760 nm, with gallic acid as the reference standard. The TPC was calculated using the following formula: y = 0.00009x + 0.008, with R^2 = 0.9941. The results were expressed as mg GAE/g samples.

Total flavonoid content (TFC) assay. The TFC of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30-µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5% in 10-mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10% for 5 min. Then, 2-mL NaOH 1 M and distilled water were added to a 10-mL volume. Then, the red solution was produced after NaOH solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 510 nm, with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/ g samples using the following formula: y = 0.00008x – 0.0023, with R^2= 0.9980.

Total tannin content (TTC) analysis. The TTC of the samples was analyzed using the Folin-Ciocalteu method (Chandran and Indira 2016). Approximately 10-µL *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2-mL Na_2CO_3 7.5% and filled up to 10-mL volume with distilled water. The blue dark color solution was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ = 760 nm, with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/ g samples using the following formula: y = 0.00009x + 0.0021, with R^2 = 0.9993

Analysis of the Antioxidant Potential

DPPH free radical scavenging activity assay. The DPPH free radical scavenging activity (DPPH) was measured by the spectrophotometric method (Widyawati *et al.* 2017) to determine the ability of the phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atoms to the nitrogen atom in DPPH, resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was poured into the reaction tube, into which 3-mL DPPH solution (4 mg/ 100 mL) was added. After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at . = 517 nm. The reference standard compound was gallic acid, and the results of the analysis were expressed as mg GAE/g samples calculated using the following formula: y = 0.146x + 1.7896, with R^2 = 0.9975.

Ferric-reducing power (FRAP) analysis. FRAP was determined following the method used by Widyawati *et al.* (2014) method. Approximately 10 µL of samples were added to 2.5 mL phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube. Then, the mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5-mL supernatant, 2.5 mL distilled water and 0.5 mL ferric chloride 0.1% w/v were added, and the mixture was incubated for 10 min. The potency of the samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 700 nm. The intensity of the blue color indicated a higher reducing capacity. The reducing power, expressed as mg GAE/g samples, was calculated using the following formula: y = 0.0002x + 0.0256, with R^2 = 0.9906.

Analysis of the Antidiabetic Properties

α-amylase enzyme inhibition (AA) capacity assay. *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500 µL of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250 µL of the mixture, an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) was added and then dissolved in 50 mL of 0.2 M sodium acetate pH 5. The mixture was shaken, into which 2-mL sodium hydroxide 1M was added. Before the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 540 nm. The inhibition percentage of α-amylase was assessed using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 100% – where ACb is the absorbance of 100% enzyme activity (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without the enzyme), As is the absorbance of the test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

α-glucosidase enzyme inhibition (GA) capacity assay. The analysis of the α-glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150-µL samples containing 100-µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50-µL α-glycosidase 2 mM (0.0833 unit/mL); then, the mixture was incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000-µL sodium carbonate 0.2 M. The amount of these enzymes that did not react with bioactive compounds of *Pluchea* infusion hydrolyzed pNPG as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea* infusion was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ = 405 nm. The inhibition percentage of α-glycosidase was calculated using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 100% – where ACb is the absorbance of 100% enzyme activity (solvent with enzyme), ACa is the absorbance of 0% enzyme activity (solvent without enzyme), As is the absorbance of test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

Analysis of Phenolics

The phenolic compounds of the samples were analyzed using high-performance liquid chromatography (HPLC) based on the method of Kongkiatpaiboona *et al.* (2018) with modifications. Each *Pluchea* infusion was sonicated for 15 min (Branson 1510); then, the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of the sample was injected in an HPLC (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm x 4.6 mm). The mobile phase used consisted of a solution of [A] 0.5% acetic acid in water and [B] absolute methanol. Analysis was carried out using a gradient system in the following order: initial conditions of 10% B in A to 50% B in A were maintained for 40 min; then, 100% B was maintained for 20 min. Next, the column was re-equilibrated with 10% B in A and maintained for 10 min before analysis of the next sample. The sample flow rate was set at 1.0 mL/min with a controlled temperature of 40 °C. Detection was used at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water and prepared similarly to the samples before being injected in HPLC.

Experiment design and statistical analysis. The research design used a randomized block design with two factors, *i.e.* the steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and the storage period of 0 year /unstored (B1), and 5 year /stored (B2) resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated for six periods. The data analysis of samples was repeated for six periods. The data were analyzed using a paired t-test at $\alpha \leq 0.05$, treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSIONS

Bioactive Compounds

Phenolic compounds. Bioactive compounds are active compounds in plants that are essential to protect body health (Nguyen and Chuyen 2020). These compounds usually have many biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer, antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan 2014; Acar *et al.* 2022). Phenolic compounds have potential redox properties that can scavenge free radicals that can cause a number of chronic diseases (Noreen *et al.* 2017; Aryal *et al.* 2019; Acar *et al.* 2022).

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage period based on paired t-test at $\alpha \leq 0.05$ (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unstored. Further, the highest TPC was observed in samples infused at 95 °C and stored for 5 yr (at 71.38 ± 4.14 mg GAE/g sample), whereas the lowest was measured in the unstored samples and infused at 60 °C (at 4.39 ± 0.49 mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 °C showed a significant increase in their phenolic content. This implies that the steeping temperature and the storage periods significantly resulted in the high amounts of phenolic compounds in the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage periods. This could have been due to the fact that the steeping temperature and storage period could cause the process of degradation, oxidation, and leaching or release of phenolic compounds. Phenolic compounds are water-soluble and, thus, soaking in hot water for a certain period, as steeping causes the migration process of more phenolic compounds to the water because of longer exposure of phenolic compounds to water (Castiglioni *et al.* 2015; Kilic *et al.* 2017; Acar *et al.* 2022). Su *et al.* (2019) reported that temperature treatment can stimulate the release of phenolic compounds and increase the antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and different long storage (fresh and 72 h).

Temperature treatment degrades (or hydrolyzes) the hydrogen bond between phenolic compounds and proteins, resulting in an increase of phenolic compounds when exposed to higher temperatures (Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are not completely stable but are easily degraded during storage after harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded with increasing temperature. Fibrianto *et al.* (2021) also stated that the brewing temperature has an effect on the extracted antioxidant compounds such as alkaloids, catechins, and tannins. Thus, there is an assumption that temperature and storage caused the degradation, oxidation, and hydrolysis of the phenolic compounds period, resulting in the increased amount of the phenolic compounds at higher steeping temperatures and longer storage periods.

Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids is shown in Table 1. The treatment effects using the t-test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol content were insignificantly different at various steeping temperatures and storage periods. The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the unstored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 °C, whereas (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 °C. The myricetin content was significantly different at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed significant difference at 60, 80, and 95 °C, whereas 4,5-di-O-caffeoylquinic acid content was only significantly different at 60 °C.

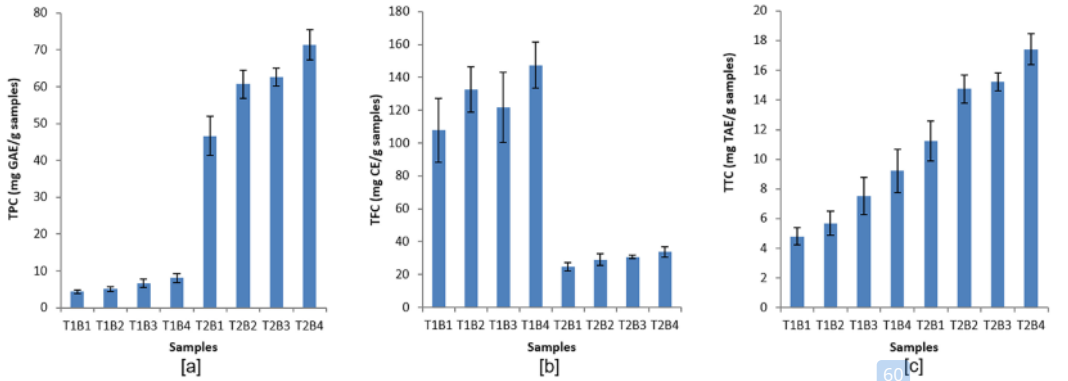

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperatures and storage periods.

Phenolic compounds	Steeping temperature (°C)	Mean ± SD (unstored)	Mean ± SD (stored)	Mean difference ±SD	Sig. (two-tailed)
Gallic acid (µg/g samples)	60	0.2132 ± 0.0027	0.2364 ± 0.0015	0.0375 ± 0.0175	0.2030
	70	0.2157 ± 0.0013	0.2324 ± 0.0214	0.0167 ± 0.0227	0.4870
	80	0.2234 ± 0.0122	0.2347 ± 0.0078	0.0386 ± 0.0264	0.2870
	95	0.2316 ± 0.0104	0.2402 ± 0.0169	0.0086 ± 0.1990	0.8500
(+)-Catechin (µg/g samples)	60	0.3425 ± 0.0110	0.5085 ± 0.0111	–0.1576 ± 0.0885	0.241
	70	0.3260 ± 0.0265	0.5448 ± 0.0006	–0.2188 ± 0.0259	0.053
	80	0.3240 ± 0.0222	0.5023 ± 0.0773	–0.1451 ± 0.0248	0.077
	95	0.4039 ± 0.0320	0.5995 ± 0.0372	–0.2049 ± 0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756 ± 0.1234	1.4762 ± 0.0271	–1.2887 ± 0.3222	0.111
	70	0.2587 ± 0.0160	1.4245 ± 0.2526	–1.1657 ± 0.2695	0.103
	80	0.4175 ± 0.0104	1.4570 ± 0.0925	–1.0391 ± 0.0841	0.036*
	95	0.8786 ± 0.0434	2.6138 ± 0.0695	–1.1735 ± 0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220 ± 0.0268	0.6220 ± 0.0706	–0.5999 ± 0.9733	0.544
	70	0.1530 ± 0.0511	1.0708 ± 0.0289	–0.9177 ± 0.0222	0.011*
	80	0.3666 ± 0.0103	0.8629 ± 0.0815	–0.1082 ± 0.4462	0.790
	95	0.6559 ± 0.0570	2.0230 ± 0.0573	–1.4123 ± 0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394 ± 0.0202	0.3675 ± 0.0183	–0.3207 ± 0.1122	0.154
	70	0.0514 ± 0.0037	0.3726 ± 0.0944	0.3213 ± 0.0907	0.125
	80	0.3699 ± 0.0924	0.7966 ± 0.0366	–0.4267 ± 0.2727	0.271
	95	0.5913 ± 0.0239	0.9478 ± 0.0287	–0.3565 ± 0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103 ± 0.0628	2.4863 ± 0.0270	–1.8760 ± 0.2074	0.050*
	70	0.6271 ± 0.0099	2.3403 ± 0.0325	–1.7131 ± 0.3152	0.082
	80	0.7967 ± 0.03060	2.6278 ± 0.0211	–1.8311 ± 0.0095	0.002*
	95	1.5386 ± 0.0668	4.0211 ± 0.0851	–2.4825 ± 0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635 ± 0.0628	0.9449 ± 0.0501	–0.2814 ± 0.4458	0.536
	70	0.6162 ± 0.0099	0.9485 ± 0.0794	–0.3323 ± 0.0301	0.041*
	80	0.6601 ± 0.0306	0.9099 ± 0.0387	–0.2498 ± 0.3127	0.461
	95	0.6642 ± 0.0668	1.3156 ± 0.0166	–0.6514 ± 0.2666	0.179
4,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.4906 ± 0.0060	1.1842 ± 0.0120	–0.6886 ± 0.2723	0.018*
	70	0.4807 ± 0.0034	1.0089 ± 0.0736	–0.5281 ± 0.0702	0.060
	80	0.5299 ± 0.0053	1.2382 ± 0.1435	–0.7082 ± 0.1489	0.094
	95	1.0018 ± 0.0526	1.3797 ± 0.2170	–0.3086 ± 0.3086	0.333

Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation ($n = 6$). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

Results further showed that gallic acids and kaempferol were relatively stable, as reflected by the insignificant changes when exposed to the different steeping temperatures and storage periods. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a drastic increase at higher steeping temperatures and longer storage periods, implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-catechin, and 3,4-di-

O-caffeoylquinic acid were easier to dissolve or degrade to form simple phenolic acids at higher temperatures and storage period (Su *et al.* 2019; Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023; Zhang *et al.* (2021). Degradable polyphenol compounds have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's phenol reagent, resulting in a complex blue solution that can detected as TPC.

Flavonoid content (TFC). Flavonoids are the major phenolic compounds that have potential chemical and biological activities such as radical scavenging and antimicrobial activities (Ayele *et al.* 2022; Chandra *et al.* 2014) that can protect the human body from the oxidative stress caused by many degenerative diseases – especially cancer, cardiovascular problems, and aging (Mathur and Vijayvergia 2017). The TFC of steeped *Pluchea* infusion decreased with a longer storage period. Unstored samples exhibited higher flavonoid content than the stored samples. The statistical analysis using a paired t-test at $\alpha \leq 0.05$ showed that the TFC of *Pluchea* infusion was significantly different between the steeped unstored and steeped stored samples (Figure 1b). The highest TFC was exhibited by the unstored samples steeped at 95 °C at about 147.42 ± 14.03 mg CE/g sample. The TFC was significantly lower in the stored samples than those of the unstored samples, implying that the increase in the flavonoid content of the infusion was affected primarily by the steeping temperature.

Tannin content (TTC). Tannins are bioactive compounds that provide properties, such as astringent, anti-diarrheal, antibacterial, and antioxidant (Malangngi *et al.* 2012). Generally, results indicated that the TTC of *Pluchea* infusion significantly increased with increasing steeping temperature and storage period (Figure 1c). Among the unstored steeped samples, the tannin content was significantly lowest in the samples infused at 60 °C at about 4.81 ± 0.58 to 17.42 ± 1.04 mg TAE/g samples, which was significantly different lower from that of the lowest tannin content of the stored samples. Among the stored and steeped samples, the highest tannin content was observed at samples steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples and was significantly different from that of the highest tannin content of the unstored steeped samples at 95 °C about 9.22 ± 1.48 mg TAE/g samples. Indicating that the tannin content was primarily affected by a longer storage period than high steeping temperature. The condensation of catechins to tannins is a dominant process occurring in tea leaves that is accelerated during the maceration of raw tea leaves (Kowalska *et al.* 2021) and could have contributed to the observed increase in the tannin content in the treated samples.

Nonetheless, high temperatures and long storage periods can cause the degradation of tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar thermostable complex compounds that are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples period.

Antioxidant activity. Antioxidant activity is the capability of compounds to inhibit the oxidation of macromolecules from biological targets that are involved in oxidative chain reactions (Ali *et al.* 2005; Oh *et al.* 2013). The antioxidant activity assay was done in this research using DPPH and FRAP methods. The phenolic compounds are active antioxidants with antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atoms, which is negatively correlated with the O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the weak hydrogen bonds in the OH group of the phenolic compound, so that it is easier to donate hydrogen atoms (Kruk *et al.* 2022). The mechanism of phenolic compounds as antioxidants depends on their ability to donate hydrogen atoms and transfer electrons, as well as reducing agents and singlet oxygen quenchers (Ali *et al.* 2005; Huang *et al.* 2005).

DPPH free radical scavenging activity (DPPH). DPPH is a free radical that is often used to evaluate antioxidant activity because this method is simple and is suitable for measuring the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to a yellow color (Munteanu and Apetrei 2021; Baliyan *et al.* 2022). Figure 2a shows that the free radical scavenging properties of the stored and steeped samples were significantly higher than the unstored steeped samples. It can also be observed that the free radical scavenging property was significantly different among the stored and steeped samples but insignificant among the unstored and steeped sample period. *Pluchea* infusion stored at room temperature for 5 yr resulted in high free radical scavenging activity by more than 10%. Steeping at higher temperatures significantly increased the DPPH free radical scavenging activity in stored *Pluchea* infusion by around 15–25%. This implies that the higher free radical scavenging property was primarily affected by the storage period than the steeping temperature. During the storage process, it is possible to form complex phenolic compounds that provide a high ability to scavenge free radicals (Thanajiruschaya *et al.* 2010).

The scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels (Table 2). The antioxidant activity was strongly and negatively correlated with flavonoid content. The storage period could be reduced flavonoid content. The study also demonstrated that longer storage period and higher infusion temperatures produced many simple phenolic compounds with free

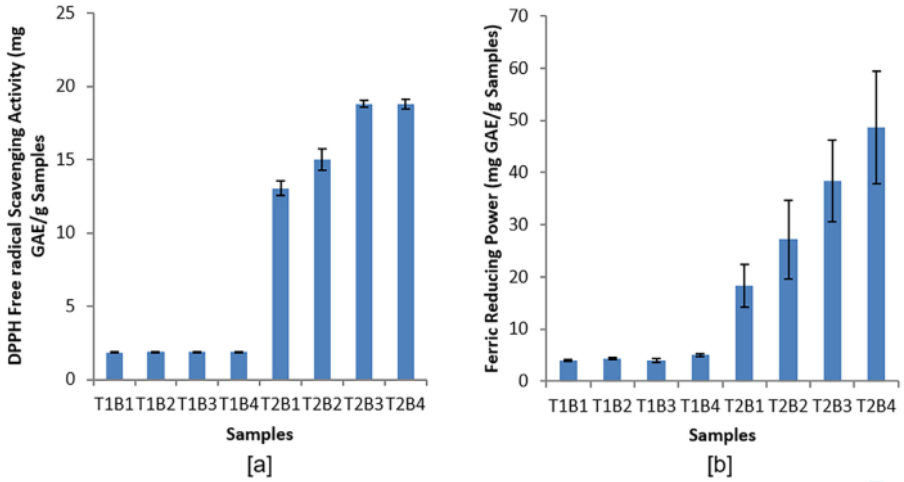

Figure 2. Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 6). Samples : T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC, and TAC), antioxidant activity (DPPH and FRAP), and antidiabetic activity (AA and GA).

	TPC	TFC	TTC	DPPH	FRAP	α-glucosidase	α-amylase
TPC	1						
TFC	–0.93589	1					
TTC	0.960028	–0.81321	1				
DPPH	0.992776	–0.93992	0.942273	1			
FRAP	0.953366	–0.82636	0.947778	0.956242	1		
α-glucosidase	–0.55512	0.349873	–0.71534	–0.5272	–0.55947	1	
α-amylase	–0.70842	0.429393	–0.8569	–0.69579	–0.80548	0.725161631	1

Significant at the 0.05 level (two-tailed)

hydroxyl groups capable to donate hydrogen atoms to DPPH free radicals. Many phenolic acids such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel 2019) (Table 1). Kruk *et al.* (2022) informed that the capability of phenolic compounds to donate hydrogen atom depends on the chemical structure, number, and position of hydroxyl groups attached to a benzene ring, a double bond between C2 and C3 rings, and a carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to donate hydrogen atoms is determined by O-H bond dissociation energy.

The free radical scavenging property observed in the study was not consistent with the results of the study by Moraes–de Souza *et al.* (2008). The research shows that the TPC of herbal infusion is lowly correlated with free radical scavenging activity. However, Dobrinas *et al.* (2021) informed that TPC is positively and significantly correlated with the free radical scavenging property of tea infusion.

Ferric reducing antioxidant power (FRAP). FRAP is an analysis of the antioxidant power of the phytochemical compounds that is based on the ability of antioxidant compounds to reduce iron ions of potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). Potassium ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati *et al.* 2017; Raharjo and Haryoto 2019).

The results showed that the FRAP increased at higher steeping temperatures and longer storage periods. The lowest FRAP was observed in the unstored samples, which were steeped at 60 °C at 3.95 ± 0.17 mg GAE/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 yr at 95 °C at 48.63 ± 10.83 mg GAE/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 yr was also significantly higher than the unstored samples at $\alpha \leq 0.05$.

This is in contrast with the study on the antioxidant activity of DPPH and FRAP of matcha. The longer storage period reduces the levels of catechin content due to the catechins such as epigallocatechin gallate, epicatechin gallate, epigallocatechin, and epicatechin, which are bioactive compounds that have high antioxidant activity (Kim *et al.* 2020). The ferric-reducing capability of *Pluchea* could have been due to the presence of simple phenolic acid that can transfer electrons from their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and positively significantly correlated with the DPPH, TPC, and TTC but inversely to TFC.

Antidiabetic Activity

α-amylase enzyme inhibition activity (AA). Antidiabetic activity is a measure of the potency of phenolic compounds to regulate the uptake of glucose by the cells from the blood through the mediation of two digestive enzymes, *i.e.* α-amylase and α-glucosidase, which are involved in the control of dietary carbohydrate digestion and release in the postprandial blood glucose in human body (Fu *et al.* 2017). The phenolic compounds have the capability to bind with the protein component of α-amylase and α-glucosidase enzymes (Martinez-Solis *et al.* 2022), resulting in the reduced activity of the enzymes. The results showed that lower steeping *Pluchea* leaf infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good AA of more than 50% and even almost 100% in unstored *Pluchea* infusion steeped at 60, 70, and 80 °C, with the highest at 60 °C and in stored *Pluchea* leaf infusion, which was steeped at 60 °C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5 min had lower enzyme inhibition activity of less than 50%, with the lowest at 95 °C around 13%. Widyawati *et al.* (2017) found that the ability to inhibit the α-amylase enzyme in unstored *Pluchea* infusion steeped at 95 °C for 5 min was also low at 28.79%. Increasing the steeping temperature and storage period reduced the ability of the phytochemicals in the *Pluchea* infusion to inhibit the α-amylase enzyme activity period. Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significantly correlated with TPC, TTC, DPPH, and FRAP, but it was weakly and positively significantly correlated with TFC.

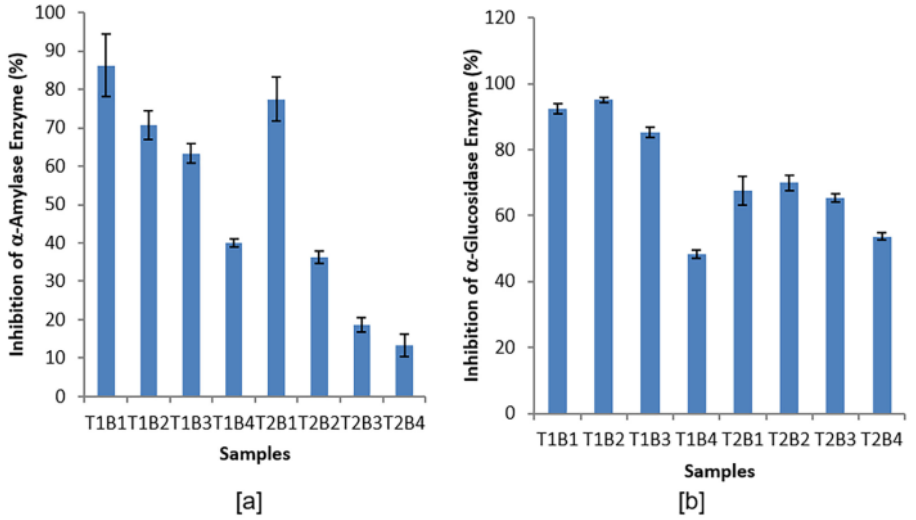

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a] α-amylase; [b] α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

This inhibitory activity was thought to be contributed by other bioactive compounds besides phenolics, which are sensitive to steeping temperature and storage period. Li *et al.* (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the α-amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitors. Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the α-amylase enzyme inhibitor in this extract may be proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory activity in *Pluchea* infusion also was determined by their protein and polyphenolic content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to α-amylase enzyme depending on their structures. There are C=C double bonds conjugated with a carbonyl group of phenolic structures that stabilize the binding forces to the active site of the α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the α-amylase enzyme. Elevated steeping temperatures and longer storage periods can easily cause the removal of the hydroxyl groups of phenolic compounds, which can reduce their ability to enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits stronger capability to obstruct the α-amylase enzyme.

α-glucosidase enzyme inhibition activity (GA). α-glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), thereby resulting in hyperglycemia (Nurcholis *et al.* 2014; Proenca *et al.* 2017). The ability of bioactive compounds to inhibit the α-glucosidase enzyme is used to determine their antidiabetic activity. This is supported by Werdani and Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent. Widyawati *et al.* (2020) found that the steeping of unstored *Pluchea* infusion at 95 °C for 5 min has an inhibitory effect on the α-glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage period. Steeping at 95 °C of the unstored *Pluchea* leaf infusion obtained the lowest inhibitory ability, *i.e.* 48.32 ± 1.27%, and the highest inhibitory activity was at 70 °C at 95.11 ± 0.70%. The results of a paired t-test showed that GA of *Pluchea* infusion was significantly different between steeping temperature and long storage. Figure 3 further shows that the ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. Data analysis in Table 2 showed that the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA, but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li *et al.* (2018) stated that flavonoid compounds can inhibit the action of the α-amylase and α-glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds such as rutin, myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid compounds is determined by the position and number of hydroxyl groups, the number of double bonds in rings A and B, and the heterocyclic ring in ring C. Tadera *et al.* (2006) and Zhang *et al.* (2014) also explained that flavonoid compounds of samples significantly inhibit the α-glucosidase enzyme activity.

The ability to inhibit the α-glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. Figure 3 also showed that the capability of *Pluchea* infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the α-glucosidase enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic acid residue, interacting ionic and hydrophobic with site other than the active site, and binding covalent with enzymes through an epoxy or aziridine group (Moorthy *et al.* 2012). Then, the mechanism of the α-amylase enzyme inhibitor includes blocking carbohydrates, thereby limiting the digestibility and absorption of carbohydrates, as well as blocking the active centers of several subsites of the enzyme (Gong *et al.* 2020).

Widyawati *et al.* (2017) stated that phenolic and non-phenolic compounds can inhibit the α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, which may occur in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Arsiningtyas *et al.* (2014) explained that the methyl-esterified quinic acid with the caffeic groups such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the α-glucosidase enzyme activity. The resulting analysis of

caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in stored *Pluchea* leaf infusion higher concentration than in unstored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the GA of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

This study showed that the increasing steeping temperature and storage period caused degradation of polyphenol compounds to produce simple phenolic compounds such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the TPC and TTC. The increase in the simple phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

CONCLUSION

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage periods. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unstored. TPC was highest in the stored and steeped at 95 °C and lowest in the unstored and steeped at 60 °C. Unstored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unstored samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unstored steeped samples, the tannin content was significantly the lowest in the samples steeped at 60 °C and the highest in the samples steeped at 95 °C.

The DPPH of the stored and steeped *Pluchea* leaf infusion was significantly higher than the unstored steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95 °C. The free radical scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels. The FRAP significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the unstored samples that were steeped at 60 °C, and the highest was exhibited in *Pluchea* samples that were stored for 5 yr and steeped at 95 °C. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging property, TPC, and TTC but inversely with TFC. The AA was generally found to be higher at lower steeping temperatures of the unstored *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The AA capacity of the *Pluchea* leaf infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH, and FRAP, but it was weakly and positively correlated significantly with TFC.

The ability of the *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unstored *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unstored sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. The GA was significantly strong and negative TPC, TTC, DPPH, and FRAP, and it was weakly and positively correlated significantly with TFC.

The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the presence of the bioactive compounds, antioxidant potential, and antidiabetic properties at different steeping temperatures and storage periods – including gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

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