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Post-harvest and Extraction Conditions for the Optimum Alpha Glucosidase Inhibitory Activity of *Stenochlaena palustris*

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Abstract. *Stenochlaena palustris* has been studied for its anti-diabetic potency related to its alpha-glucosidase inhibitory (AGI) activity. This study aimed to evaluate the impact of post-harvest and extraction conditions on the AGI activity of *S. palustris* and isolate the AGI bioactive principal component. AGI activity was calculated based on the inhibition of *S. palustris* samples against *in vitro* rat intestinal maltase and sucrase hydrolytic activities in enzymatic reactions. The selected extracts were subjected to activity-guided fractionation using liquid-liquid sequential separation, followed by silica N60 column chromatography. This study showed that post-harvest treatment significantly protected the AGI activity of *S. palustris*, while its optimum extract condition was observed with methanol and a smaller particle size (< 250 µm) at a sample to solvent ratio of 1:20 (w/v) for 24 h. Further fractionation, followed by Liquid chromatography-mass spectrometry (LC-MS) and proton nuclear magnetic resonance (NMR) evaluation of the *S. palustris* extracts from optimum post-harvest and extraction conditions, also resulted in the identification of kaempferol 3-O-β-glucopyranoside (astragalin) as the responsible bioactive AGI compound. This study's findings are expected to contribute to further study and utilization of *S. palustris* as an anti-diabetic agent based on its AGI activity.

Keywords: Alpha-glucosidase inhibitory activities; Diabetic; Kaempferol 3-O-β-glucopyranoside; Kelakai; *Stenochlaena palustris*

1. Introduction

Kelakai (*Stenochlaena palustris*; Figure 1) is an endemic fern used as food and traditional medicine by the Dayak ethnic society in central Kalimantan, Indonesia. *S. palustris* is also found and used for medicinal treatments in other countries (Ponnusamy et al., 2013; Neamsuvan et al., 2015). Extracts of this plant's mature (Chai et al., 2015) and young fronds (Leng, 2016) possess potent, high natural alpha-glucosidase inhibitory (AGI) activity associated with hyperglycemia treatment.

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Figure 1 Left to right: (a) field; (b) young leaves; and (c) mature leaves of *Stenochlaena palustris*

The activities of *S. palustris* against AGI enzymes, along with its long consumption history, strongly encourage the plant's utilization as a functional food ingredient for diabetes management, especially given the rising awareness of self-monitoring and self-controlling blood sugar levels (Dewi et al., 2017). Common challenges in the development and production of functional food from natural products are maintaining AGI activity consistency and standardization during harvesting and processing (Widiputri et al., 2020). Extraction conditions, such as type of solvents and solvent to solid ratio, have been known to affect extraction efficiency (Dianursanti et al., 2020; Widiputri et al., 2020). To support the utilization of *S. palustris* as a functional ingredient for diabetes management, it is important to select post-harvest and extraction treatments for optimum and consistent AGI activity.

Various phenolic compound derivatives, such as flavonoids, anthocyanins, proanthocyanidins (condensed tannins), and hydroxycinnamic acids (Chai et al., 2012; Chai et al., 2015; Chear et al., 2016 Rahmawati et al., 2017), have been identified in the extracts of *S. palustris*. However, the compounds responsible for this plant species' AGI activity have not yet been confirmed. Identifying the major bioactive compounds responsible for AGI activity is also important in providing guidelines that the plant's principal activity compounds remain during harvesting and processing.

Previous studies have revealed that the water fraction of methanol extract in the mature (Chai et al., 2015) and young fronds (Leng, 2016) of *S. palustris* possess potent, high natural AGI activity commonly associated with hyperglycemia treatment. The first objective of this study is thus to select *S. palustris* post-harvest and extraction treatments for optimum and consistent AGI activity. Furthermore, to provide guidelines for the principal activity compounds, this study aims to identify the major bioactive compounds responsible for this plant's AGI activity using activity-guided fractionation and instrumental analysis of the isolated compounds.

2. Methods

2.1. Materials

For this study, around 15 kg of wild *S. palustris* plants were excavated from a 5×5 m^2 plot of peat land in Tumbang Nusa, Central Kalimantan, Indonesia (2°26'10.5" S, 114°10'24.8" E) during the monsoon season. The sample included young and old fronds, collected 15 cm from the tip of the branch. The plant materials collected were identified and deposited at the Herbarium Bogoriense, Research Center for Biology, Cibinong, Indonesia (No. 252/IPH.1.01/If.07/II/2019).

2.2. Stenochlaena palustris Extraction

The samples were separated into two batches of 7 kg each. One batch, *Stenochlaena palustris* 0 (SP0), was stored in an insulated container. While the other batch, *Stenochlaena palustris* 1 (SP1), was first grouped into 30 sprigs per group, giving 76 groups, with their cut ends covered with wet cotton, tissue, and cling wrap, tied with rubber bands, then

wrapped with either plastic or banana leaves, and then stacked in an insulated container filled with frozen ice gel. Both SP0 and SP1 were stored in the insulated container for 2 days during transportation to the laboratory facilities to receive further treatment, including washing, oven drying at 40°C, and chopping.

Both SP0 and SP1 were extracted at a ratio of 1:10 (m/v) using deionized water, 50% aqueous methanol (50% methanol), and methanol as solvents. Then, the mixtures were agitated using a twist mixer (50 rpm, 24 h at room temperature), centrifuged (6000 rpm for 20 min), and concentrated with a rotary evaporator at 35°C, followed by vacuum drying until they arrived at a constant weight. The water extract was then freeze-dried. All extracts were subjected to an AGI activity assay with sucrose and maltose as substrates. The solvent used in the extract with the highest AGI activity was used for further evaluation of the extraction conditions' effects on AGI activity. The evaluated extraction conditions were particle size, extraction ratio, and extraction time. Optimum extraction conditions were further applied in extracting *S. palustris* for the fractionation and evaluation of its principal AGI activity component.

2.3. Stenochlaena palustris Fractionation

Methanolic extract of the *S. palustris* went through two level fractionations, as shown in Figure 2. Activity-guided fractionation was performed via AGI activity evaluation for each vacuum-dried fraction (35°C). Selected extract powder went through liquid-liquid fractionation (n-hexane, ethyl acetate, butanol, and water, ratio 1:50 (w/v)). The fraction with the highest AGI activity was further fractionated using silica N60 gel column chromatography, resulting in six fraction groups (f1–f6). The fraction with the highest AGI activity from the second fractionation was evaluated using liquid chromatography-mass spectrometry (LC-MS) at a 200 µl/min flow rate with gradient eluent for the first 30 min (start – 95:5 water:methanol, end – 5:95 water:methanol) and static eluent (5:95 water:methanol) for another 5 min. Proton nuclear magnetic resonance (^{1}H NMR) was done using deuterated methanol-d4 (CD3OD-d4) as a solvent at 400 MHz.

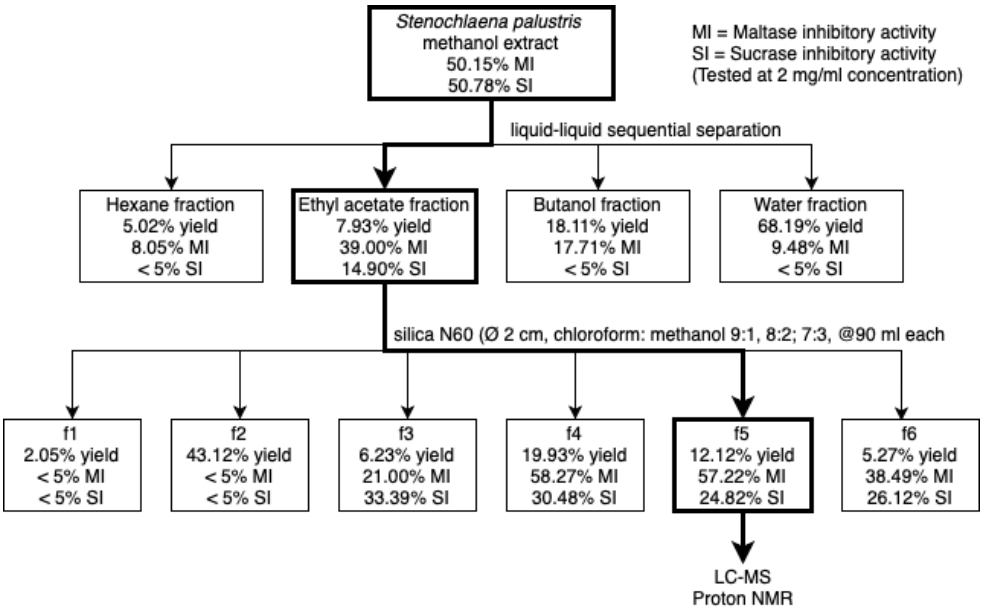


Figure 2 Fractionation scheme of the *Stenochlaena palustris* leaves' methanol extract

2.4. Alpha Glucosidase Inhibitory Activity Evaluation

The AGI activity evaluation was conducted according to Gunawan-Puteri and Kawabata (2010) with a slight modification in the centrifugation conditions. Rat intestinal glucosidase

was extracted from commercial intestinal acetone powder. This powder measured 0.1 g and was mixed with 2 ml of a 0.1 M potassium phosphate buffer (pH 6.9). The mixture was shaken on a seesaw shaker for 5 min, then centrifuged at 15,000 rpm. Afterward, the supernatant was recovered and stored on ice, as well as considered glucosidase possessing the AGI activity to hydrolyze maltose and sucrose.

Each sample was tested for AGI activity with two sample replications (duplo). For elution, 50% dimethyl sulfoxide (DMSO) was used to dissolve the samples until they reached 10 times the intended testing concentration. First, 5 µl of sample solution was added to a 0.2 ml polymerase chain reaction (PCR) tube labeled either sample or sample blank, while 5 µl of 50% DMSO was added to similarly sized tubes labeled either control or control blank. Then, 25 µl of substrate in the 50% DMSO solution (2.88 mg/ml of maltose for the maltase inhibition assay, 21.90 mg/ml sucrose for the sucrase inhibition assay) was added to each tube and agitated with a vortex. The pre-incubated (37°C, 5 min) mixtures in the sample and control tubes were given 20 µl of the rat intestinal glucosidase solution, while those in the blanks were given 20 µl of potassium phosphate buffer. The mixtures were further incubated at 37°C (20 min for the maltase inhibition assay, 25 min for the sucrose inhibition assay) to allow glucosidase hydrolytic activity to take place, and 150 µl of Tris-HCl solution (pH 7) was added to stop the reactions. All mixtures were then passed through a short Cosmosil column made from a shortened Pasteur pipette, cotton, and 1 mg of Cosmosil 75C18-OPN gel. Samples of the filtered mixtures were taken (20 µl for the maltase inhibition assay, 30 µl for the sucrose inhibition assay), mixed with 200 µl of a glucose CII test-kit Wako® solution in a 96 well plate, and incubated (37°C, 5 min), with absorbance measured at 505 nm. AGI activity was evaluated based on inhibition against sugar hydrolysis and calculated using Equation 1, where A is absorbance. The experiment's results were statistically analyzed using analysis of variance, followed by a Tukey honestly significant difference (HSD) post-hoc test in OpenSTAT.

$$\%Inhibition = \frac{\left((A_{control} - A_{control\ blank}) - (A_{sample} - A_{sample\ blank})\right)}{(A_{control} - A_{control\ blank})} \times 100\% \quad (1)$$

3. Results and Discussion

3.1. Impact of Harvesting Technique and Extraction Solvent

AGI compounds in plants are secondary metabolites that are highly dependent on various environmental factors, such as light, temperature, soil water, soil fertility, and salinity. Accordingly, if one of these factors changes, it may alter the content of secondary metabolites as well (Yang et al., 2018). *S. palustris* can remain fresh up to 2 days after harvesting before it wilts. Restricted access to moisture after harvesting enhances the degenerative process of senescence. Browning in *S. palustris* is an example of a change that became noticeable on the fourth day after harvesting and even more obvious during the seventh day after harvesting. Aside from browning, shriveling and weight loss were also core problems in the post-harvest period (Nicholas et al., 2013). Slowing down the rate of senescence by storing the fronds at a low temperature is a common practice to delay color changes and moisture loss. In this study, one batch of the sample received additional post-harvesting treatments (SP1). The cut ends of the ferns were covered with wet cotton, then wrapped with either plastic or banana leaves to preserve moisture and thereby delay drought-caused senescence.

Statistical analysis showed SP1 had significantly higher AGI activity and extract yields (Table 1). SP1 was extracted using 50% methanol and showed more than 50% inhibitory activity against both sucrase and maltase hydrolysis. Meanwhile, only the methanolic extract of SP0 showed an inhibitory effect at a weaker level (33.72%). The data further

indicated that the methanol solvent had better extraction capabilities when extracting the principal AGI components from *S. palustris*. The extract yield from SP1 was also 4.4 to 5.6 times greater than the SP0 extract yield with the same solvents. SP0 and SP1 consistently showed methanol could better extract principal AGI components. SP1, which received additional post-harvest treatment, also featured better AGI activity in comparison to SP1 collectively. Previous studies have shown the influence of environmental factors (Quan and Liang, 2017; Yang et al., 2018), age and part of plant (Chen et al., 2018), and growing area (Chai et al., 2015; Leng, 2016) on the AGI activity of *S. palustris*. However, this is the first study to show the significant impact of post-harvest treatment on the plant's AGI activity.

Table 1 Impact of post-harvest treatment and extraction solvent on alpha glucosidase inhibitory (AGI) activity in *Stenochlaena palustris*

Post-harvest treatment	Extraction solvent	AGI activity (10 mg/ml)*		Extraction yield (%)*
		Maltase	Sucrase	
Standard collection (SP0)	Distilled water	< 5%	< 5%	1.35 ± 0.67[a]
	50% methanol	< 5%	< 5%	6.23 ± 3.11[b]
	Methanol	33.72 ± 2.51[a]	< 5%	2.06 ± 0.53[a]
Collection with post-harvest treatment (SP1)	Distilled water	24.76 ± 3.59[a]	28.13 ± 1.13[a]	6.00 ± 1.24[b]
	50% methanol	54.84 ± 1.50[b]	51.07 ± 9.60[b]	28.05 ± 0.91[d]
	Methanol	50.15 ± 2.43[b]	50.78 ± 7.28[b]	11.60 ± 1.12[c]

*Results are shown as mean ± standard deviation. Different letters in the same column represent a statistical difference at $p < 0.05$

The solubility of all compounds was affected by both their own nature and their solvents (Chebil et al., 2007). Water and methanol have different polarities and affinities, so they will extract different types and/or amounts of compounds. As such, the water extract of *S. palustris* had a clear brown color, its methanolic extract a deep green color—which might be caused by the chlorophyll known to easily dissolve in methanol—and the 50% aqueous methanol extract a cloudy brownish green color (Figure 3).

Figure 3 Left to right: (a) water, methanolic, and 50% aqueous methanolic extracts of *Stenochlaena palustris* leaves; (b) Fine powder, coarse powder, and un-milled samples of *Stenochlaena palustris* leaves

SP1's methanolic and 50% methanolic extracts exhibited higher AGI activity than its water extract (Table 1). Methanol has the ability to extract more secondary metabolites from plants, which were the target compounds in this analysis (Abarca-Vargas et al., 2016; Namvar et al., 2017). Previous research conducted on *S. palustris* also indicated that alkaloid and phenolic groups were possible compounds responsible for the plant's AGI activity (Chai et al., 2015; Leng, 2016). The 50% methanolic extract had more yield and similar activity to the methanolic extract. However, the water content in the solvent reduces evaporation efficiency and increases the possible contamination of inactive materials dissolved in water. Therefore, methanol was selected as a solvent for further stages of this study.

3.2. Impact of Extraction Conditions

The impact of the extraction conditions (particle size, extraction ratio, and extraction time) on AGI activity was observed in SP0's methanolic extract (Table 2). Particle size has been shown to affect the extracted solid yield from *S. palustris* (Wijaya et al., 2017) and the AGI activity of other plants (Çam and İçyer, 2013). Extraction ratio and time have also been studied for their impact on the AGI activity of other plants (Gunawan-Puteri et al., 2016). In this study, particle size significantly affected both the activity and yield of the extracts ($p<0.05$). Extraction ratio only affected yield, while extraction time did not have a significant impact ($p<0.05$; Table 2).

Table 2 Impact of dominant particle size, sample to solvent ratio, and extraction time on the alpha glucosidase inhibitory (AGI) activity of *Stenochlaena palustris*

Dominant particle size (µm)	Sample to solvent ratio (w/v)	Extraction time (h)	Maltase inhibitory activity 10 mg/ml (%)	Extract yield (%)
> 5600 (un-milled)	1:10	24	33.11 ± 2.06	1.35 ± 0.08
		48	31.67 ± 1.60	1.68 ± 0.03
		72	28.59 ± 2.01	1.56 ± 0.12
	1:20	24	31.17 ± 2.02	1.98 ± 0.09
		48	27.62 ± 1.52	2.19 ± 0.15
		72	27.47 ± 1.49	2.04 ± 0.12
	1:30	24	26.11 ± 1.47	2.62 ± 0.07
		48	35.58 ± 3.62	3.04 ± 0.53
		72	36.02 ± 2.43	2.12 ± 0.06
630–2000 (coarse powder)	1:10	24	33.15 ± 1.18	2.36 ± 0.22
		48	34.21 ± 0.97	2.79 ± 0.28
		72	39.05 ± 2.11	2.48 ± 0.49
	1:20	24	38.31 ± 1.92	2.68 ± 0.18
		48	33.15 ± 1.90	3.50 ± 0.16
		72	31.11 ± 1.45	4.12 ± 1.33
	1:30	24	28.17 ± 1.62	3.68 ± 0.20
		48	30.34 ± 1.46	4.04 ± 0.69
		72	32.57 ± 1.16	4.82 ± 2.66
< 250 (fine powder)	1:10	24	36.18 ± 2.56	2.64 ± 0.34
		48	33.24 ± 0.99	3.09 ± 0.15
		72	29.91 ± 1.36	2.58 ± 0.44
	1:20	24	30.75 ± 2.79	3.28 ± 0.06
		48	31.86 ± 2.41	4.31 ± 0.76
		72	40.69 ± 1.51	4.05 ± 1.38
	1:30	24	37.72 ± 1.61	4.51 ± 0.30
		48	37.89 ± 1.42	4.30 ± 0.61
		72	39.47 ± 0.78	3.79 ± 0.65
Marginal mean extract AGI activity and yield from various particle sizes				
> 5600 (un-milled)			30.82 ± 2.02[a]	2.06 ± 0.14[a]
630–2000 (coarse powder)			33.34 ± 1.53[b]	3.39 ± 0.69[b]
< 250 (fine powder)			35.30 ± 1.71[c]	3.62 ± 0.52[b]
Marginal mean extract AGI activity and yield from various extraction ratios				
	1:10		33.23 ± 1.65[a]	2.28 ± 0.24[a]
	1:20		32.46 ± 1.89[a]	3.13 ± 0.47[b]
	1:30		33.76 ± 1.73[a]	3.66 ± 0.64[b]
Marginal mean extract AGI activity and yield from various extraction times				
		24	32.74 ± 1.91[a]	2.79 ± 0.17[a]
		48	32.84 ± 1.77[a]	3.22 ± 0.37[a]
		72	33.88 ± 1.59[a]	3.06 ± 0.81[a]

*Different letters in the same column from the same extraction condition represent statistical differences at

$p<0.05$

Further statistical analysis of samples with three particle sizes (Figure 3b) via a Tukey HSD post-hoc test showed that lowering particle size significantly increased extract AGI activity. Milling the samples into powder, whether coarse or fine, also significantly increased extract yield compared to un-milled samples. Extraction ratio did not show any significant impact on AGI activity in the *S. palustris* extracts. However, a Tukey HSD post-hoc test showed that the extract yield with a 1:20 extraction ratio (w/v) was significantly higher compared to that with a 1:10 ratio, but not significantly different from the yield with a 1:30 ratio. Therefore, for the following stage of principal component isolation, methanolic extraction was applied to SP1's fine *S. palustris* powder at the extraction ratio (w/v) 1:20. Since the extraction period did not show any influence on AGI activity or *S. palustris* extract yield, the shortest extraction period (24 h) was selected for efficiency.

3.3. Principal Component Isolation

Principal component isolation was conducted and produced four fractions. The four fractions were significantly different in yield and maltase hydrolytic activity (Table 3).

Table 3 Alpha glucosidase inhibitory (AGI) activity and yield of the four fractions resulting from principal component isolation

Sample (2 mg/mL)	Maltase inhibition (%)*	Sucrase inhibition (%)*	Yield (%)
Ethyl acetate fraction	39.00 ± 0.11^{b}	14.90 ± 6.09^{c}	7.93
Butanol fraction	17.71 ± 4.37^{a}	3.99 ± 5.64^{c}	18.11
Water fraction	9.48 ± 3.18^{a}	1.88 ± 2.65^{c}	68.19
Hexane fraction	8.05 ± 6.16^{a}	0.91 ± 1.28^{c}	5.02

*Results are shown as mean ± standard deviation. Different letters in the same column represent statistical differences at $p<0.05$

A Tukey HSD post-hoc test indicated that the ethyl acetate fraction had significantly higher maltase inhibitory activity compared to the other fractions and that all four fractions had significantly different yields: the highest was the water fraction, followed by the butanol, ethyl acetate, and hexane fractions. Ethyl acetate is known to dissolve alkaloids, polyphenols, tannins, flavonoids, terpenes, steroids, and quinones (Gangula et al., 2013; Mustarichie et al., 2017). The ethyl acetate *S. palustris* extract also contained high amounts of alkaloids, flavonoids, phenols, and saponins, moderate amounts of terpenoids, and a low number of glycosides and tannins (Arullappan et al., 2017; Saragih et al., 2017).

The ethyl acetate fraction was subjected to a second stage of fractionation using silica 60N column chromatography and three different eluents (chloroform:methanol (v/v) = 9:1, 8:2, and 7:3). Normal phase silica plate thin layer chromatography (TLC) was used to evaluate the extracted compounds, and those with separation profile similarity were grouped into similar fractions, resulting in six fractions named f1–f6 (Figure 4), whose AGI activity is shown in Table 4.

Table 4 Alpha glucosidase inhibitory (AGI) activity and yield of the four fractions resulting from principal component isolation

Sample (2 mg/ml)	Maltase inhibition (%)*	Sucrase inhibition (%)*
Ethyl acetate fraction	38.20 ± 1.76^{a}	21.35 ± 3.49^{a}
f1	0.07 ± 0.10^{b}	0.35 ± 0.49^{b}
f2	No inhibition	No inhibition
f3	21.00 ± 3.56^{c}	33.39 ± 1.73^{c}
f4	58.27 ± 0.13^{d}	$30.48 \pm 3.38^{a,c}$
f5	57.22 ± 1.63^{d}	$24.82 \pm 1.14^{a,c}$

f6	38.49 ± 0.93[a]	26.12 ± 2.30[a,c]

*Results are shown as mean ± standard deviation. Different letters in the same column represent statistical differences at $p<0.05$

Fractions f1 and f2 showed no AGI activity and were thus excluded from the statistical evaluation of inhibitory activity against maltase and sucrase. Fractions f3–f6 were significantly different in their activity against maltase but not against sucrase. A Tukey HSD post-hoc test indicated that fractions f4 and f5 were significantly higher in maltase inhibitory activity compared to fractions f3 and f6. Though f5 had a significantly lower yield compared to f4, the TLC evaluation showed that f5 had better purity (Figure 4), so it was selected for the next purification and identification stage.

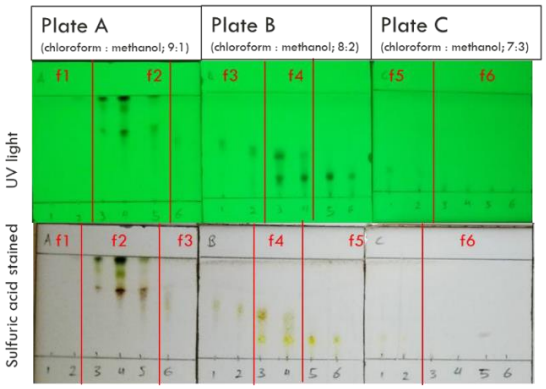
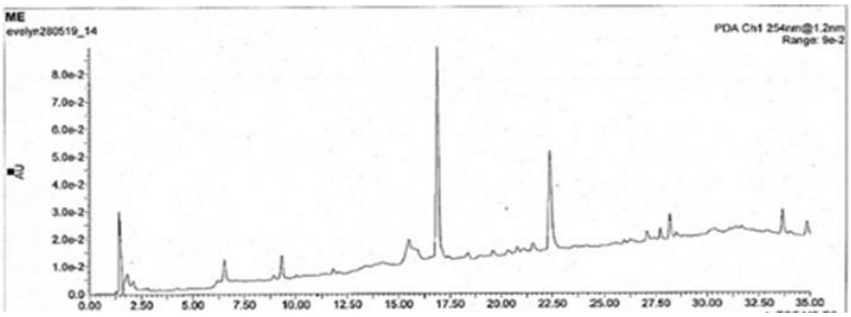


Figure 4 TLC profile of the fractions resulting from silica N60 column chromatography of the ethyl acetate fraction from the methanolic extract of *Stenochlaena palustris* leaves

Fraction f5 was subjected to liquid chromatography-mass spectroscopy (LC-MS), which revealed an active compound detected between minutes 16.85 and 17.06 (Figure 5c) that was also observed as a major peak in the original ethyl acetate fraction (Figure 5b) and even in the *S. palustris* leaves' original methanolic extract (Figure 5a). The mass spectrum evaluation showed matching m/z with kaempferol 3-O-β-glucopyranoside or astragalin (Figure 5d) with electrospray ionization-mass spectroscopy ESI-MS (negative): m/z 447 [M-H]-, 493 [M+FA-H]-, 895 [2M-H]-; and (positive): m/z 449 [M+H]+, 471 [M+Na]+, 897 [2M+H]+, 919 [2M+Na]+. Further, a ^{1}H NMR evaluation with CD3OD-d4 as a solvent from the isolated peak of fraction f5 showed a corresponding signal to astragalin ^{1}H NMR (500 MHz, CDCl3, rt): 8.05-8.03 (2H), 6.88-6.86 (2H), 6.38-6.31 (2H), 6.19 (2H), 5.25-5.23 (2H). Therefore, astragalin was confirmed as the principal AGI activity component in the *S. palustris* methanolic extract. Astragalin was previously found in *S. palustris*, along with other phytochemicals (Tan et al., 2013; Nurmilatina, 2017). Other studies have similarly shown that astragalin has high AGI activity (Choung et al., 2017), though none of these were conducted with *S. palustris* extracts.

(a)



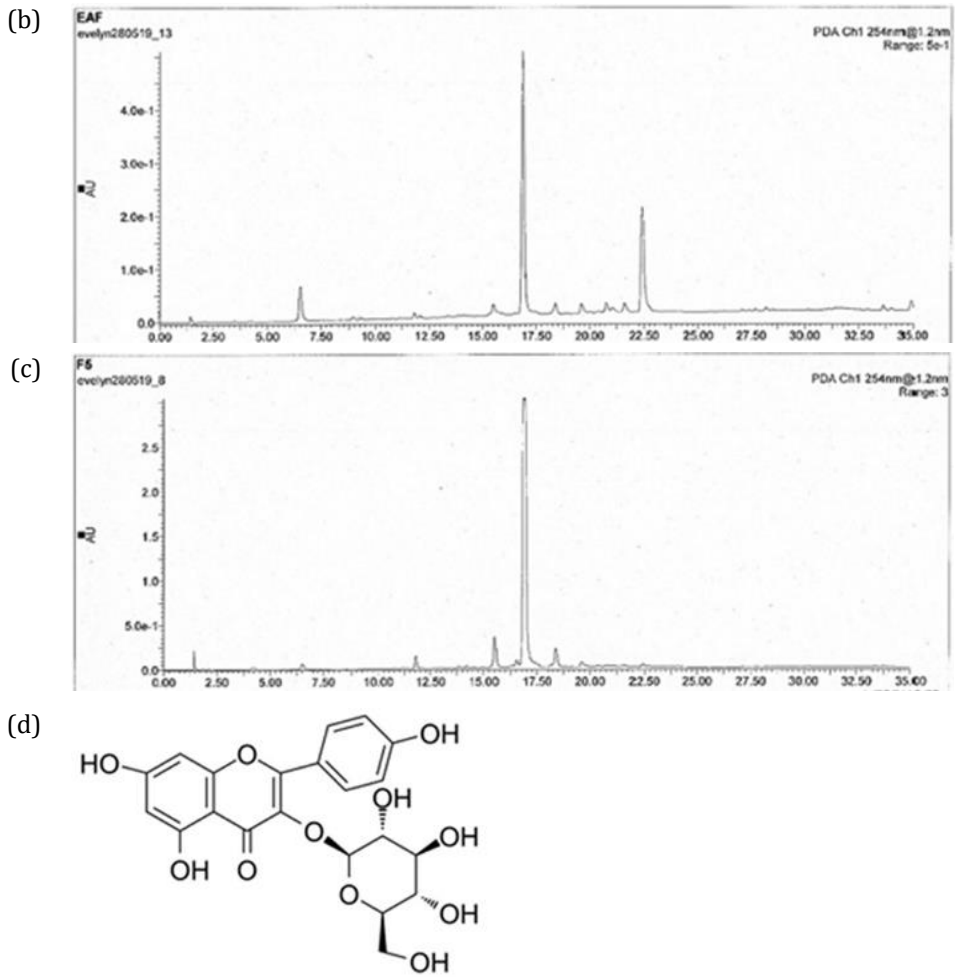


Figure 5 HPLC profile of *Stenochlaena palustris* leaves': (a) methanolic extract; (b) ethyl acetate fraction from the methanolic extract; and (c) fraction f5. (d) Chemical structure of kaempferol 3-O-β-glucopyranoside

4. Conclusions

This study revealed several important findings on the standardization and optimization of *S. palustris* extraction, specifically regarding post-harvest treatment and extraction conditions. Attempts to preserve moisture and delay the wilting of fresh *S. palustris* leaves was shown to also protect their AGI activity. The smaller particle size of dried *S. palustris* powder was shown to better facilitate extraction of the principal AGI component at a 1:20 (w/v) extraction ratio. This study also identified astragalin as the active compound responsible for the AGI activity in *S. palustris*. This study's findings are expected to contribute to the further study and utilization of *S. palustris* as a functional ingredient for diabetes management based on its AGI activity.

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