Research Article

Antioxidant Activity and Alpha-Glucosidase Inhibitory Activity of the Combination of Cat's Whiskers Herb and Stevia Leaf

Nursafira Khairunnisa Ismail[1], Tzazkia Febriyana Akbar[1], Dhiffa Namira Alifia Putri[1], Soraya Riyanti[1], Ari Sri Windyaswari[1] and Fahrauk Faramayuda[1*]

[1]*Faculty of Pharmacy, Jenderal Achmad Yani University (UNJANI), Cimahi, Indonesia.*

Abstract | Indonesia ranks fifth worldwide in the prevalence of diabetes mellitus. Oxidative stress is a major contributing factor to this condition and its associated complications. Therefore, there is an urgent necessity for natural antioxidant agents to mitigate this issue. Certain plants, commonly used in traditional medicine, have been scientifically proven to be natural antioxidants and antidiabetic agents, including cat's whiskers (*Orthosiphon aristatus* (Blume) Miq.) and stevia (*Stevia rebaudiana* Bertoni). This research aims to evaluate the radical scavenging activity and antidiabetic effects of the combination of aqueous extracts of cat's whiskers herb white-purple variety and stevia leaves. The antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay with a UV-Visible spectrophotometer at 516 nm. In parallel, the antidiabetic activity was assessed against the alpha-glucosidase enzyme using an ELISA reader at 405 nm. The results of the radical scavenging activity assay conducted using the DPPH reagent on the combination of aqueous extracts from cat's whiskers herb and stevia leaves in formulations F1 (1:1), F2 (2:1), and F3 (3:1) demonstrate IC_{50} values of 48.06, 45.55, and 37.41 µg/mL, respectively. In terms of inhibitory activity against the alpha-glucosidase enzyme in F1 (1:1), F2 (2:1), and F3 (3:1), demonstrate IC_{50} values of 114.90, 228.78, and 459.81 µg/mL, respectively. According to the results of the DPPH radical scavenging assay, the most potent antioxidant activity was observed in Formula 3 (3:1), while Formula 1 (1:1) demonstrated the highest efficacy in inhibiting the alpha-glucosidase enzyme.

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***Correspondence** | Fahrauk Faramayuda, Faculty of Pharmacy, Jenderal Achmad Yani University (UNJANI), Cimahi, Indonesia; **Email:** ramayuda.f@gmail.com
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Introduction

In recent years, there has been a significant rise in the global prevalence of diabetes mellitus. According to the International Diabetes Federation (IDF), Indonesia ranks fifth among nations with the highest number of individuals affected by diabetes. In 2021, it was reported that 19.5 million Indonesians were diagnosed with diabetes, and this number is projected to increase to 28.6 million by the year 2045 (International Diabetes Federation, 2021).

Diabetes mellitus is a chronic metabolic disorder characterised by elevated blood glucose levels, attributable to either inadequate insulin secretion, diminished insulin receptor sensitivity, or a combination of both (Wells *et al*., 2015). A crucial contributor to the etiology of diabetes mellitus and its related complications is oxidative stress, which impairs insulin secretion by pancreatic beta cells (Triandita *et al*., 2016). This oxidative stress results from an imbalance between the production of free radicals and the body's antioxidant defences (Anggraini, 2020). Therefore, it is imperative to employ natural antioxidant compounds to mitigate this issue, as the utilisation of synthetic antioxidants may pose carcinogenic risks (Ngibad and Lestari, 2020). Several plants, traditionally employed and scientifically validated for their roles as natural antioxidants and antidiabetic agents, include the cat's whiskers and stevia.

A significant factor contributing to the development of diabetes and its associated complications is oxidative stress, which impairs insulin secretion from pancreatic beta cells (Konda *et al*., 2019). Oxidative stress originates from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defences (Mustafa *et al*., 2021). Therefore, the incorporation of natural antioxidant agents is essential for mitigating oxidative stress, especially considering that synthetic antioxidants may pose potential risks (Kim *et al*., 2018). Traditional medicinal plants, including *Orthosiphon aristatus* (cat's whiskers) and stevia, have been recognised for their antioxidant and antidiabetic properties.

Natural antioxidants derived from plants have demonstrated significant antidiabetic properties. For example, the methanolic extract of *Euphorbia helioscopia* has been identified to contain phenolic compounds that exhibit a positive correlation with both antioxidant and antidiabetic activities (Mustafa *et al*., 2022). Similarly, the antioxidant properties of cat's whiskers are associated with its high flavonoid content, which plays a vital role in neutralising free radicals and reducing oxidative stress (Baishya *et al*., 2018). Furthermore, research indicates that plant-based therapies can effectively lower blood glucose levels and improve lipid profiles in diabetic models, making them promising alternatives to traditional synthetic medications (Salama *et al*., 2021). The therapeutic effectiveness of cat's whiskers and other medicinal plants is further validated by their ability to augment the body's antioxidant capacity. For instance, extracts from these plants have been shown to enhance the activity of various antioxidant enzymes, thereby improving the oxidative balance in individuals with diabetes (Araújo *et al*., 2020).

Cat's whiskers is a botanical species rich in antioxidant compounds and exhibits antidiabetic properties (Juliani *et al*., 2016; Mohamed *et al*., 2015). In Indonesia, three types of cat's whiskers are cultivated, such as white, white-purple, and purple varieties (Faramayuda *et al*., 2021a; Faramayuda *et al*., 2022a). The white-purple variety remains underutilised despite evidence that it contains 1.95% sinensetin (Faramayuda *et al*., 2023; Faramayuda *et al*., 2021c). Moreover, the eupatorin content is notably higher in the white-purple variety than in the other variety (Faramayuda *et al*., 2021b). Research indicates that the sinensetin compounds in cat's whiskers can lower blood glucose levels by inhibiting the conversion of carbohydrates into glucose, as demonstrated by their inhibitory effects on the alpha-glucosidase enzyme (Faramayuda *et al*., 2021a; Mohamed *et al*., 2015). In contrast, stevia leaves are commonly used as a sucrose alternative for individuals with diabetes, owing to their diterpene glycosides, which exhibit significant antihyperglycemic effects (Jan *et al*., 2021). Previous research has shown that the aqueous extracts of both cat's whiskers and stevia leaves can effectively reduce DPPH free radicals, with IC_{50} values of 95.05 µg/mL and 83.45 µg/mL, respectively (Ahda *et al*., 2023; Borgo *et al*., 2021). Additionally, the aqueous extract of stevia leaves and the methanol extract of cat's whiskers have been shown to inhibit the alpha-glucosidase enzyme, with IC_{50} values of 596.77 µg/mL and 465.83 µg/mL, respectively (Juliani *et al*., 2016; Ruiz-Ruiz *et al*., 2015).

Both plants exhibit substantial antioxidant and antidiabetic properties. It is optimistic that their combination will generate a synergistic effect, thereby enhancing their effectiveness. Consequently, the results of this *in vitro* study may serve as a foundation for future pre-clinical trials and could potentially facilitate the development of innovative traditional medicines that function as natural antioxidants and antidiabetic agents.

Material and Methods

The white-purple variety of cat's whiskers (*Orthosi-*

phon aristatus (Blume) Miq.) was obtained from PT Holistic Bio Medicine, located in Purwakarta Regency at an altitude of 290 m above sea level (MASL). Stevia leaves (*Stevia rebaudiana* Bertoni) were obtained from Bumi Herbal Dago, located in Cimenyan, Bandung, at an altitude of 1,200-1,350 MASL. The following materials were also used: DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) (Smart Lab), quercetin (Sigma Aldrich), water for injection (WFI), alpha-glucosidase enzyme derived from *Saccharomyces cerevisiae* (Sigma Aldrich), bovine serum albumin (Sigma Aldrich), acarbose (PT. Dexa Medica), p-nitrophenyl-α-D-glucopyranoside substrate (PNPG) (Sigma Aldrich), potassium dihydrogen phosphate, sodium hydroxide, sodium carbonate, distilled water, toluene, magnesium powder, hydrochloric acid, ammonia, chloroform, chloral hydrate, Mayer's reagent, Dragendorff's reagent, gelatin, sulfuric acid, $FeCl_3$, citric acid, boric acid, $AlCl_3$, amyl alcohol, ether, analytical balance (Shimadzu), parchment paper, microscope, blender, laboratory glassware, aluminium foil, furnace, desiccator, hot plate, ash-free filter paper, oven, spatula, infusion pan, thermometer, micropipette, micropipette tips, eppendorf tubes, ice gel, ice box, 96-well plates, pH meter (Hanna HI98107), refrigerator, silica gel thin-layer chromatography plates F254 (Merck), freeze dryer (Biobase), incubator (UN30, Memmert), UV-Visible spectrophotometer (Shimadzu), ELISA reader (Tecan Infinite M200 Pro).

Methods

Plant identification and sample preparation

The taxonomic classification of cat's whiskers white-purple variety (*Orthosiphon aristatus* (Blume) Miq.) and stevia leaves (*Stevia rebaudiana* Bertoni) was conducted at the Herbarium Jatinangoriense, Department of Biology at FMIPA Padjadjaran University, thereby confirming the authenticity of the samples. Samples of the cat's whiskers herb and stevia leaves were cleaned, washed, and dried. The drying process for the cat's whiskers herb was conducted under indirect sunlight for approximately five days. Conversely, the stevia leaves were dried in a drying cabinet at approximately 70°C for 10-12 hours. The dried plant material was then ground into a powder using a blender and stored in an airtight container protected from light.

Sample standardisation and phytochemical screening

Sample standardisation was conducted based on specific and non-specific parameters, followed by phytochemical screening of dried plant material and extracts to identify the presence of secondary metabolites.

Extraction

Cat's whiskers herb white-purple variety and stevia leaves were extracted separately using the infusion method (100 g sample in 1 L water), heated at 90°C for 15 minutes and filtered. The process was repeated twice, and the filtrates were then freeze-dried to obtain dry extracts.

Thin layer chromatography

The extracts of the cat's whiskers herb white-purple variety and stevia leaves were analysed by Thin Layer Chromatography (TLC) on a silica gel 60 F254 and eluted with an appropriate mobile phase, and visualised under UV light (254 and 366 nm) before and after spraying with 0.2% DPPH and another specific reagent.

Antioxidant activity assay with DPPH reagent

The radical scavenging activity was evaluated using the DPPH reagent (50 µg/mL in methanol). 2 mL of the DPPH solution was pipetted into a vial and mixed with 2 mL of methanol. The mixture was incubated for 30 minutes in a dark environment, protected from sunlight. The absorbance was measured using a UV-Visible spectrophotometer at wavelengths 400-800 nm. Quercetin, as the reference standard, was tested at concentrations of 1, 1.5, 2, 2.5, 3, and 3.5 µg/mL. The assays were performed on the combination extract of cat's whiskers herb white-purple variety and stevia leaves in formula 1 at concentrations of 10, 20, 30, 40, 50, and 60 µg/mL; formula 2 at concentrations of 20, 30, 40, 50, 60, and 70 µg/mL; and formula 3 at concentrations of 10, 20, 30, 40, 50, and 60 µg/mL. Absorbance measurements were taken at the maximum wavelength of DPPH at 516 nm using a UV-Visible spectrophotometer in triplicate (Windyaswari *et al.*, 2018). The percentage inhibition was calculated using the following equation:

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

The IC_{50} value was calculated using the linear regression equation (y = bx + a), where the x-axis

represents sample concentration and the y-axis represents percentage inhibition. The antioxidant measurement data were analysed using SPSS version 29.0.2.0 to determine any differences in the IC_{50} values generated by each combination formula of cat's whiskers herb extract and stevia leaf extract using one-way ANOVA. Differences in the data were analyzed using the Tukey test.

Antidiabetic activity assay against alpha-glucosidase enzyme

Antidiabetic activity was assessed according to Etsassala *et al.*, (2020), with minor modifications. The testing solution consisted of a sample solution, a control solution (without an inhibitor, either acarbose or extract), and a blank solution (without enzyme) which served as a correction factor. The antidiabetic activity was evaluated by testing acarbose as the reference standard at concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 µg/mL. Subsequently, the combination extract of cat's whiskers herb white-purple variety and stevia leaves was prepared in three formulas: F1 (1:1), F2 (2:1), and F3 (3:1). For each formula, 50 mg of combination extract was dissolved in a 25 mL volumetric flask with Water For Injection (WFI) to obtain a stock solution of 2000 µg/mL and then diluted with phosphate buffer (pH 6.8) to obtain several concentrations. The antidiabetic assays were performed on Formula 1 (1:1) at concentrations of 31.25, 62.5, 250, 500, and 1000 µg/mL; Formula 2 (2:1) at concentrations of 31.25, 62.5, 125, 500, and 1000 µg/mL; Formula 3 (3:1) at concentrations of 62.5, 125, 250, 500, and 1000 µg/mL. Alpha-glucosidase inhibitory activity was assessed by pipetting 20 µL of the acarbose or each extract combination at various test concentrations into a 96-well plate. Subsequently, 50 µL of phosphate-buffered saline (pH 6.8) was added, followed by 10 µL of alpha-glucosidase enzyme (1 U/mL), and the mixture was incubated at 37°C for 10 minutes. Next, 20 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) substrate was added, and the mixture was incubated at 37°C for 20 minutes. After incubation, 50 µL of 0.1 M sodium carbonate (Na_2CO_3) was added as a stopping solution to cease the enzymatic activity. The absorbance was measured using ELISA reader at 405 nm, and all measurements were conducted in triplicate. The percentage inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

IC_{50} values were calculated using GraphPad Prism 10.2.3 (GraphPad Software, Inc., La Jolla, CA, USA). A smaller IC_{50} value indicates greater inhibitory activity against the alpha-glucosidase enzyme (Sulastri *et al.*, 2021).

Results and Discussion

Standardisation was conducted across multiple parameters, as shown in Table 1. These evaluations aimed to verify the quality of the samples.

The determination of water content in the dried plant material was performed using the azeotropic distillation method. The results showed that the water content in the dried plant material of cat's whiskers herb white-purple variety was 3.80 ± 0.53% (v/w), whereas the water content in dried stevia leaves was 0.83 ± 0.29% (v/w) (Table 1). These findings indicate that the water content of both dried plant materials meets the standards established in the Indonesian Herbal Pharmacopoeia Edition II, which specifies an acceptable water content below 10% (Ministry of Health of the Republic of Indonesia, 2017).

Table 1: *Result of Sample Standardization*

Parameter	Result (%)	
	Cat's whiskers herb white-purple variety	Stevia
Water content (%v/w)	3.80 ± 0.53	0.83 ± 0.29
Loss on drying (%w/w)	4.90 ± 0.01	3.40 ± 0.59
Water soluble extract content (%w/w)	28.42 ± 0.65	39.42 ±0.30
Ethanol soluble extract content (%w/w)	5.09 ± 0.11	33.45 ±0.51
Total ash content (%w/w)	12.34 ± 0.14	9.26 ± 0.19
Water soluble ash content (%w/w)	7.64 ± 0.61	4.36 ± 0.08
Acid insoluble ash content (%w/w)	4.10 ± 0.20	2.75 ± 0.07

Table 2: *Phytochemical screening result*

Compound group	Reagent	Cat's whiskers herb white-purple variety		Stevia	
		Dried plant material	Extract	Dried plant material	Extract
Alkaloids	Dragendorff	-	-	-	-
	Mayer	-	-	-	-
Flavonoids	Amyl alcohol	+	+	+	+
Polyphenol	$FeCl_3$	+	+	+	+
Tannins	Gelatin 1%	-	-	-	-
	Steasny	-	-	-	-
Saponins	HCl 2N	+	+	+	+
Quinone	KOH 5%	+	+	+	+
Steroids-triterpenoids	Liebermann Bourchard	+	+	+	+
Monoterpenoids- sesquiterpenoids	Vanilin 10%- sulfate	+	+	+	+

The loss on drying for the dried plant material of cat's whiskers herb white-purple variety was 4.90 ± 0.01% (w/w), while the stevia leaf dried plant material was 3.40 ± 0.59% (w/w) (Table 1). Based on these results, it can be observed that the loss on drying of both dried plant materials exceeds the water content. This discrepancy may be attributed to the fact that, during the drying process, not only is water evaporated, but volatile compounds and essential oils may also be lost due to the heating involved (Sutriandi *et al*., 2016).
The determination of both water-soluble and ethanol-soluble extract content aims to quantify the compounds in dried plant material that can be extracted with water and ethanol. The water-soluble extract content in the cat's whiskers herb white-purple variety was 28.42 ± 0.65% (w/w), while in stevia leaf was 39.42 ± 0.30% (w/w). Conversely, the ethanol-soluble extract content in the cat's whiskers herb was 5.09 ± 0.11% (w/w), while in the stevia leaf was 33.45 ± 0.51% (w/w) (Table 1). These results suggest that the compounds in the dried plant material of the cat's whiskers herb white-purple variety and stevia leaves are more soluble in water than in ethanol.

The determination of total ash content provides an overview of the residual substances remaining post-spinning, including physiological and non-physiological ash (Department of Health of the Republic of Indonesia, 2000). The total ash content in the dried plant material of cat's whiskers herb white-purple variety was 12.34 ± 0.14% (w/w), whereas in dried stevia leaf was 9.26 ± 0.19% (w/w) (Table 1). The quantification of water-soluble ash content provides insights into the presence of ash derived from water-soluble salts, such as sodium (Na) and magnesium (Mg). The water-soluble ash content in the dried plant material of cat's whiskers herb white-purple variety was 7.64 ± 0.61% (w/w), while in dried stevia leaf was 4.36 ± 0.08% (w/w) (Table 1). The determination of acid-insoluble ash content assesses the presence of silica derived from soil or sand (Utami *et al*., 2020). The acid-insoluble ash content in the dried plant material of cat's whiskers herb white-purple variety was 4.10 ± 0.20% (w/w), while in dried stevia leaf was 2.75 ± 0.07% (w/w) (Table 1).

The phytochemical screening of dried plant material and extracts aims to provide insights into the secondary metabolites present, as shown in Table 2.

The findings from the phytochemical screening (Table 2) indicate that both dried plant material and extracts of the cat's whiskers herb white-purple variety and stevia leaves contain flavonoid, polyphenol, saponin, quinone, steroids-triterpenoid, and monoterpenoid-sesquiterpenoid. However, they do not contain alkaloid or tannin. These results are consistent with the findings of phytochemical screenings conducted by Das *et al*., (2022) and Faramayuda *et al*., (2022b).

The extraction process for cat's whiskers herb and stevia leaves was conducted using a hot extraction method, specifically the infusion technique, with water as the solvent. The obtained extracts were subsequently dried using a freeze dryer to obtain a stable dry extract. This method was chosen because it aligns with the traditional use of both plants. The yield of the cat's whiskers herb extract was 9.68%, whereas the stevia leaf extract yield was 10.61%.

The qualitative analysis by Thin Layer Chromatography (TLC) used silica gel 60 F254 as the stationary phase, with an eluent of ethyl acetate:methanol:water (15:3:2). The eluted TLC plate was subsequently sprayed with 0.2% DPPH spray reagent and another specific spray reagent, as shown in Figure 1, Figure 2, and Figure 3. Observation of the TLC plate under UV light at 254 nm resulted in fluorescence on the plate, with the sample appearing as a dark spot. Spots that were not visible under UV 254 nm could be observed under UV 366 nm.

To monitor the cat's whiskers herb extract, a cytoborate spray reagent was used to detect flavonoid compounds, which were characterized by bright blue, blue, light blue, and bluish-yellow spots under UV 366 nm with Rf 0.45 and 0.83 (Figure 1). The $AlCl_3$ spray reagent was also used to detect flavonoid compounds, which were characterized by greenish-yellow or blue spots under UV 366 nm with Rf 0.49 (Figure 1). Meanwhile, the stevia leaf extract showed greenish-yellow to blue spots with Rf 0.23, 0.45, and 0.72 (Figure 2).

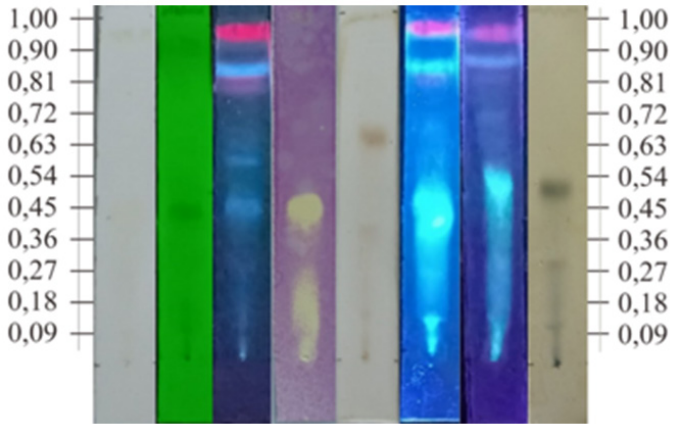

Figure 1: *TLC pattern of cat's whisker herb extract. Visual (a); Under UV 254 nm (b); Under UV 366 nm (c); Visually observed with 0.2% DPPH spray reagent (d); Observation with H_2SO_4 spray reagent after heating visually (e); Observation with cytoborate spray reagent after heating under UV 366 nm (f); Observation with $AlCl_3$ spray reagent after heating under UV 366 nm (g); Visually observed with $FeCl_3$ spray reagent after heating (h)*

The $FeCl_3$ spray reagent was used to detect polyphenolic compounds, which were characterized by yellow, green, brown, blue, red, and black spots. In the cat's whiskers herb extract, black spots were observed at Rf 0.27 and 0.49 (Figure 1). In the stevia leaf extract, black spots were observed at Rf 0.18 and 0.36 (Figure 2).

The H_2SO_4 spray reagent was used to detect organic compounds, with visual observation revealing brownish-pink spots. The TLC plate was heated prior to observation. The brownish-red spots indicate the presence of organic compounds. In the cat's whiskers herb extract, a brownish-red spot was observed at Rf 0.63 (Figure 1). In the stevia leaf extract, brownish-red spots appeared at Rf 0.18 and 0.36 (Figure 2).

Vanillin sulfate spray reagent was used to detect monoterpenoid-sesquiterpenoid compounds, characterized by purple and blue spots. The TLC plate was heated before observation. In stevia leaves, a blue spot was identified at Rf 0.6 (Figure 2).

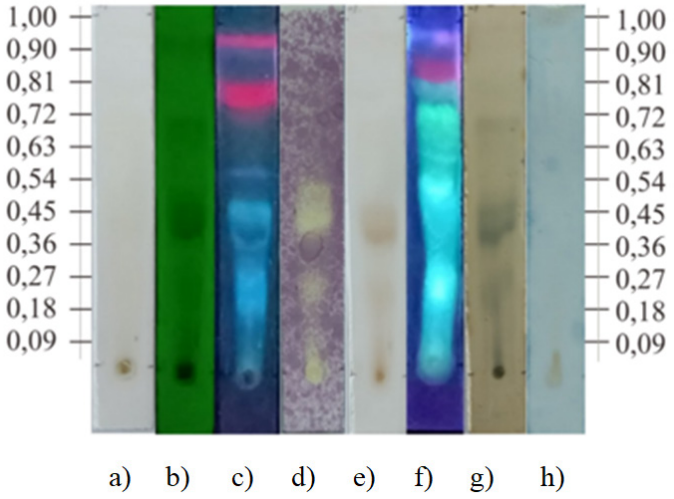

Figure 2: *TLC pattern of stevia leaf extract with silica gel 60 F_{254} stationary phase and ethyl acetate–methanol–water (15:3:2) mobile phase. Visual (a); Under UV 254 nm (b); Under UV 366 nm (c); Visually observed with 0.2% DPPH spray reagent (d); Visually observed with H_2SO_4 spray reagent after heating (e); Visually observed with $AlCl_3$ spray reagent after heating under UV 366 nm (f); Visually observed with $FeCl_3$ spray reagent after heating (g); Visually observed with vanillin sulfate spray reagent after heating (h)*

In the presence of a 0.2% DPPH spray reagent, compounds suspected of exhibiting antioxidant activity were identified by yellow spots against a purple background. In the cat's whiskers herb extract, yellow spots were observed at Rf 0.09, 0.18, and 0.45 (Figure 1), whereas in the stevia leaf extract, yellow spots appeared at Rf 0.23 and 0.45 (Figure 2).

The TLC monitoring of both cat's whiskers herb extract and stevia leaf extract indicated that both samples exhibited antioxidant activity due to the presence of yellow spots after being sprayed with the DPPH spray reagent. In addition, flavonoid and polyphenol group compounds were present in the extracts. The TLC examination of the reference sinensetin revealed a spot with Rf 0.84. The flavonoid identification of the cat's whiskers herb extract was observed at Rf 0.82 (Figure 3). The Rf value of the cat's whiskers herb extract matches that of the reference sinensetin, suggesting that the extract may contain sinensetin compounds.

The antioxidant activity of the combination of aqueous extracts of cat's whiskers herb white-purple variety and stevia leaves was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent. Antioxidant activity was quantified as IC_{50}. The IC_{50} value served as a measure of antioxidant activity, determined by linear regression of the calibration curve relating concentration to the percentage of DPPH inhibition, using the test sample as shown in Figure 4. The maximum wavelength of DPPH solution in methanol was identified at 516 nm. The results from the antioxidant activity assay of the combination of aqueous extracts of cat's whiskers herb white-purple variety and stevia leaves are shown in Table 3.

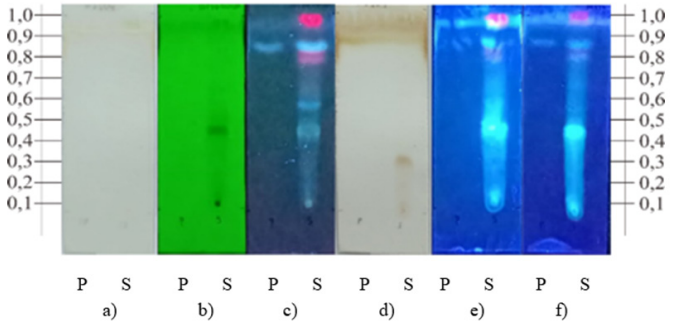

Figure 3: *TLC pattern of cat's whisker herb extract and reference of sinensetin with silica gel 60 F_{254} stationary phase and ethyl acetate–methanol–water (15:3:2) mobile phase. Visual (a); under UV 254 nm (b); under UV 366 nm (c); Visually observed with H_2SO_4 spray reagent after heating (d); Observation with Cytoborate spray reagent after heating under UV 366 nm (e); Observation with $AlCl_3$ spray reagent under UV 366 nm (f).*
Notes: P = Reference of sinensetin, S = Cat's whisker herb extract

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable, violet-colored free radical extensively used to assess the antioxidant activity of natural compounds. Compounds with antioxidant properties counteract DPPH free radicals by donating hydrogen or electrons, leading to the formation of diphenylpicrylhydrazine compounds (non-radical), which exhibit a color change from violet to yellow. The results of the antioxidant activity test against the reference compound (quercetin) yielded an IC_{50} value 2.51 ± 0.11 µg/mL, categorizing it as a very strong antioxidant (Table 3). In this study, quercetin was used as a standard reference due to its classification within the flavonoid group, specifically the flavonol subclass, which exhibits polar properties and demonstrates strong antioxidant activity (Cahyono *et al.*, 2020). The antioxidant activities of the combinations of cat's whiskers herb and stevia leaves in formula F1 (1:1), F2 (2:1), and F3 (3:1) were recorded as 48.06, 45.55, and 37.41 µg/mL, respectively (Table 3). The combination formula showing the most potent antioxidant activity was F3 (3:1), whereas F1 (1:1) exhibited the weakest antioxidant activity among the other formulas. This finding implies that an increased proportion of cat's whiskers herb extract in the combination correlates with enhanced antioxidant activity.

Table 3: *Results of the antioxidant activity of the combination of aqueous extracts of cat's whiskers herb white-purple variety and stevia leaves*

No	Sample	IC_{50} (µg/mL) ± SD	Category
1	Quercetin	2.51 ± 0.11*	Very strong
2	F1 Cat's whiskers herb : Stevia leaves (1:1)	48.06 ± 0.98*	Strong
3	F2 Cat's whiskers herb : Stevia leaves (2:1)	45.55 ± 0.67*	Strong
4	F3 Cat's whiskers herb : Stevia leaves (3:1)	37.41 ± 0.42*	Strong

Notes: : Significantly difference in p–value <0.05; n=3*

Cat's whiskers herb contains sinensetin, a flavonoid and polyphenolic compound believed to possess antioxidant activity. Additionally, stevia leaves contain flavonoid secondary metabolites that also display antioxidant activity. Flavonoids exert antioxidant activity by donating hydrogen ions, thus neutralising the harmful effects of radicals. Radicals oxidise flavonoids, forming more neutral and less reactive radicals. The greater the number of hydrogen atoms donated by secondary metabolites to DPPH, the greater the reduction of DPPH. The hydroxy group on the flavonoid B ring is thought to play a pivotal role in reducing the formation of free radicals by

donating a hydrogen, thereby stabilising them. The amount and type of secondary metabolites present in the test samples, along with their antioxidant properties, likely contribute to the observed variations in antioxidant activity (Leliqia *et al.*, 2020).

Research on a singular water extract of cat's whiskers leaves has classified its antioxidant activity as moderate, with an IC_{50} value of 95.05 µg/mL (Ahda *et al.*, 2023). Other studies on singular water extracts of stevia leaves have reported moderate antioxidant activity with an IC_{50} value of 641.26 µg/mL (Nuryandani *et al.*, 2024). The results of antioxidant activity from the combination of water extracts of cat's whiskers and stevia leaves indicate a synergistic effect, as the IC_{50} value is lower than that of the individual activities. A lower IC_{50} value indicates greater antioxidant activity.

The IC_{50} values obtained were statistically analysed using SPSS version 29.0.2.0 to ascertain if significant differences existed in the IC_{50} values produced across each formulation of the combination of cat's whiskers herb extract and stevia leaf extract, employing one-way ANOVA. The statistical analysis of one-way ANOVA for the three extract combinations with formulas 1:1, 2:1, and 3:1 revealed significant differences, with a p-value of 0.001 ($p < 0.05$). A Tukey test was subsequently conducted to identify significant differences among each formula. The results confirmed significant differences in each formula (1:1, 2:1, 3:1), as each sample was located in a distinct subset, indicating that each sample or formula possesses a significantly different IC_{50} value or antioxidant activity, demonstrating a notable effect of the combined treatment of cat's whiskers herb extract and stevia leaf extract on antioxidant activity.

The results of antidiabetic activity against the alpha-glucosidase enzyme of the combination of aqueous extracts of cat's whiskers herb white-purple variety and stevia leaves shown in Table 4 and Figure 5. The measurement of the inhibition of the alpha-glucosidase enzyme is based on the absorbance of p-nitrophenol from the hydrolysis of the substrate p-nitrophenyl-α-D-glucopyranoside (PNPG) by the alpha-glucosidase enzyme into yellow p-nitrophenol and D-glucose. The greater the sample's capacity to inhibit the activity of the alpha-glucosidase enzyme, the less p-nitrophenol is produced, resulting in a clearer solution and a lower absorbance reading.

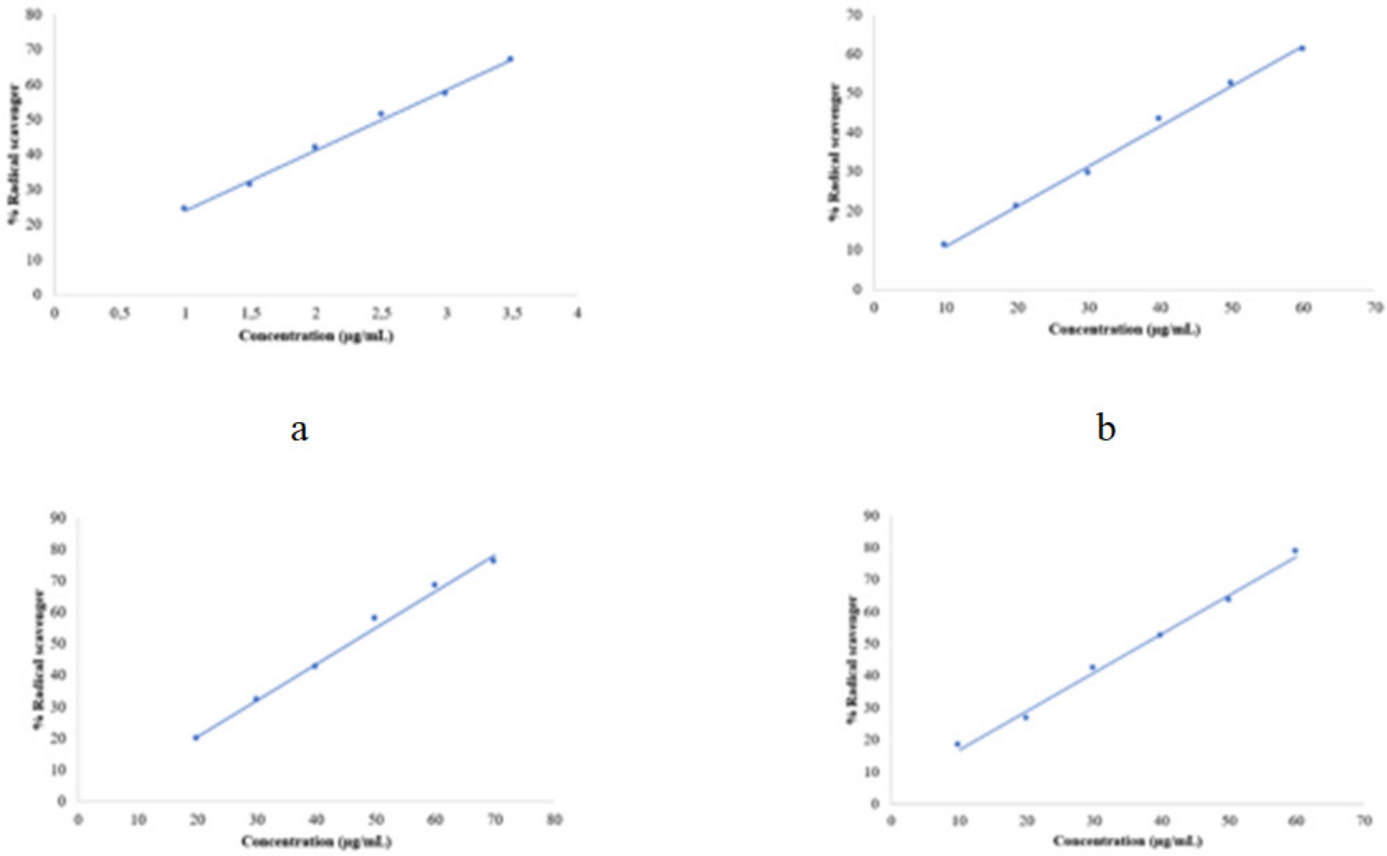

Figure 4: *Calibration Curve of DPPH. Quercetin (a); Formula 1 (1:1) (b); Formula 2 (2:1) (c); Formula 3 (3:1) (d)*

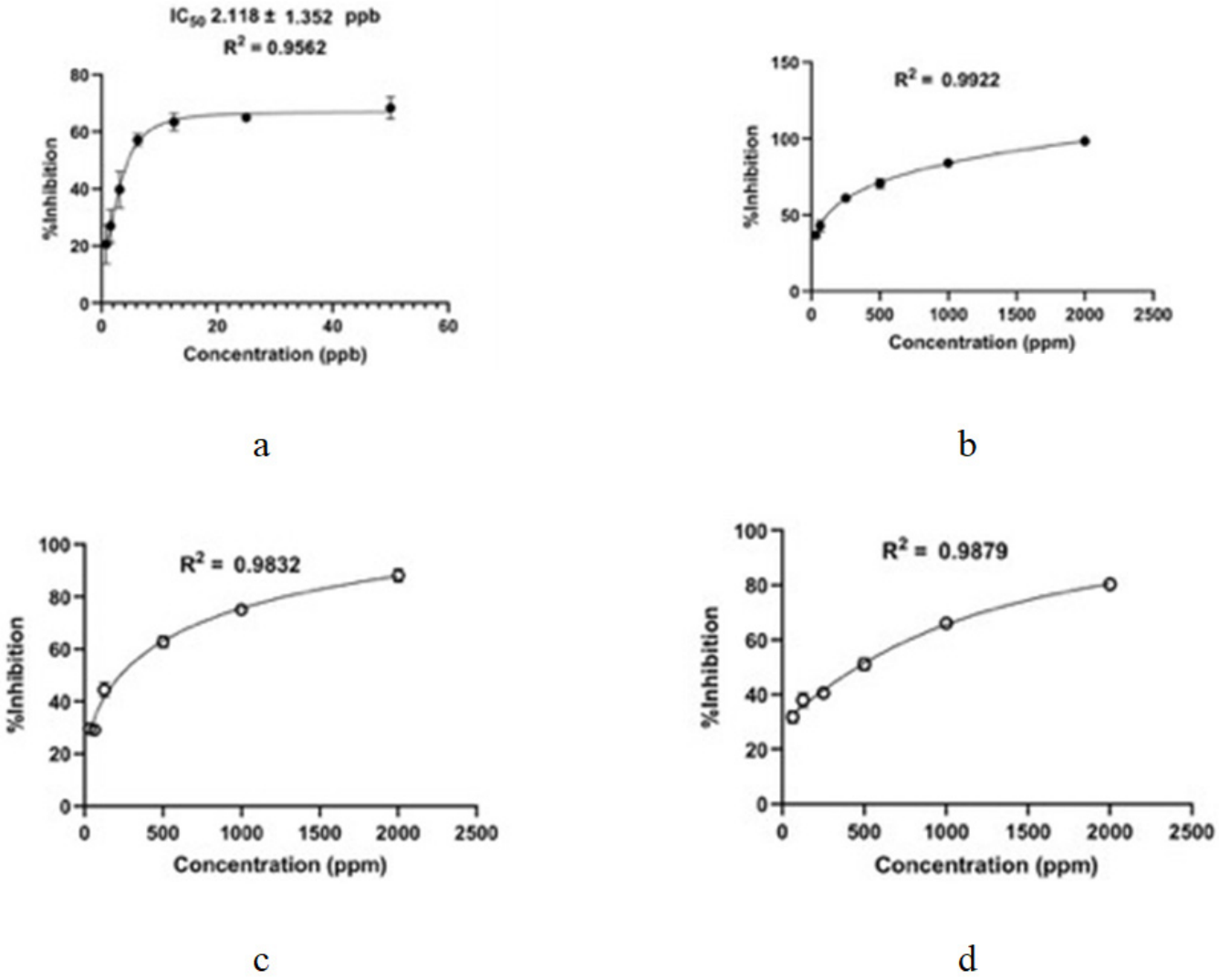

Figure 5: *Calibration Curve of Alpha-Glucosidase Inhibitory Activity. Acarbose (a); Formula 1 (1:1) (b); Formula 2 (2:1) (c); Formula 3 (3:1) (d)*

Table 4: *The results of antidiabetic activity against the alpha-glucosidase enzyme of the combination of aqueous extracts of cat's whiskers herb white-purple variety and stevia leaves*

No	Sample	IC_{50} (µg/mL)
1	Acarbose	2.12
2	F1 Cat's whiskers herb : Stevia leaves (1:1)	114.90
3	F2 Cat's whiskers herb : Stevia leaves (2:1)	228.78
4	F3 Cat's whiskers herb : Stevia leaves (3:1)	459.81

The test results for alpha-glucosidase enzyme inhibitor activity against acarbose as a reference standard yielded an IC_{50} value of 2.12 µg/mL (Table 4). The most effective alpha-glucosidase enzyme inhibitor activity was found in Formula 1 (1:1), with an IC_{50} value of 114.90 µg/mL (Table 4). Meanwhile, Formula 2 (2:1) and Formula 3 (3:1) yielded IC_{50} values of 228.78 µg/mL and 459.81 µg/mL, respectively (Table 4).

Based on the results of alpha-glucosidase enzyme inhibitory activity against the combination of cat's whiskers herb extracts of the white-purple variety and stevia leaves, it can be concluded that a synergistic effect is present, as the IC_{50} values obtained from the three extract combination formulas are lower than those reported in previous studies that tested the activity of alpha-glucosidase enzyme inhibitors for each extract in individual. Cat's whiskers herb extract and stevia leaf water extract have been reported to inhibit alpha-glucosidase enzyme activity with IC_{50} values of 465.83 µg/mL and 596.77 µg/mL, respectively (Juliani *et al.*, 2016; Ruiz-Ruiz *et al.*, 2015).

The inhibitory activity against alpha-glucosidase is attributed to the compounds present in each extract. The sinensetin compound in cat's whiskers extract is believed to play a crucial role in inhibiting the alpha-glucosidase enzyme (Mohamed *et al.*, 2012).

Sinensetin exhibits antihyperglycemic effects by inhibiting glucose absorption in the small intestine (Mohamed *et al*., 2013). Sinensetin belongs to the flavonoid group, characterised by a polymethoxyflavone structure with five methoxy group substitutions. The presence of hydroxy groups (OH), methoxy groups, and carbonyl groups (C=O) is thought to enhance the inhibitory activity against alpha-glucosidase enzymes (Juliani *et al*., 2016).

Additionally, the stevioside compounds in stevia leaf extract are reported to contribute to the inhibition of alpha-glucosidase enzymes. Stevioside is a diterpene glycoside compound. Terpenoid compounds are recognised for their inhibitory activity against alpha-glucosidase, and the presence of sugar-binding groups (glycosides) allows stevioside to effectively interact with the active site of the alpha-glucosidase enzyme (Septiana *et al*., 2021).

Utilising a combination of extracts can enhance the inhibitory activity against alpha-glucosidase enzymes (Septiana *et al*., 2021). The compounds present in cat's whiskers herb extract and stevia leaf extract can occupy the active site of the enzyme. When the active site of the enzyme is fully occupied by compounds believed to inhibit alpha-glucosidase, the substrate, akin to carbohydrates from food, can no longer bind to the enzyme. Consequently, the breakdown process of complex carbohydrates into glucose is inhibited, leading to reduced glucose absorption (Simamora *et al*., 2019).

Flavonoids and phenolic compounds are well recognized for their diverse biological activities, which include antioxidant and enzyme-inhibitory properties. Wongsa *et al*., (2022) identified a correlation between the phenolic and flavonoid contents in various herbal infusions and their inhibitory effects on alpha-glucosidase, a key enzyme involved in carbohydrate metabolism.

Conclusions and Recommendations

The findings of this study demonstrate that the combination of cat's whiskers (*Orthosiphon aristatus*) white-purple variety and stevia leaf (*Stevia rebaudiana* Bertoni) extract exhibits synergistic bioactivity, highlighting the potential advantage of combining both plant extracts rather than using them individually. The most significant antioxidant activity was observed in formula 3 (3:1), with IC_{50} value of 37.41 ± 0.42 µg/mL. In contrast, the formula exhibiting the most potent alpha-glucosidase inhibitor activity was formula 1 (1:1), with IC_{50} value of 114.90 µg/mL. These results indicate that this combination extract exhibits a synergistic effect and have promising potential for further development as natural antioxidant and antidiabetic agents.

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Novelty Statement

While cat's whiskers and stevia have been individually studied for their medicinal properties, this research provides the first comprehensive evaluation of the synergistic potential from cat's whiskers herb white-purple variety (*Orthosiphon aristatus* (Blume) Miq.) and stevia leaf (*Stevia rebaudiana* Bertoni) when combined in specific ratios (1:1, 2:1, and 3:1). These findings establish a scientifically validated ratio for developing herbal medicine with potent synergistic of antioxidant and antidiabetic properties.

Authors' Contribution

Fahrauk Faramayuda: Study conception and design, drafting of the manuscript.
Nursafira Khairunnisa Ismail: Performed the anti-diabetic activity assay, drafting of the manuscript.
Tzazkia Febriyana Akbar: Performed the antioxidant activity assay.
Dhiffa Namira Alifia Putri: Sample preparation and standardisation.
Soraya Riyanti: Analysed the data.
Ari Sri Windyaswari: Interpretation of results.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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