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PHYTOCHEMICAL SCREENING AND A-AMYLASE INHIBITORY ACTIVITY OF ORTHOSIPHON STAMINEUS AND ANNONA MURICATA LEAF ETHANOLIC FRACTIONS

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Abstract

Background: Orthosiphon stamineus and Annona muricata are medicinal plants traditionally used for diabetes management because they contain bioactive compounds capable of inhibiting carbohydrate-hydrolyzing enzymes.

Objective: This study investigated the phytochemical composition and in vitro α-amylase inhibitory activity of the ethanolic leaf fractions of *O. stamineus* and *A. muricata*, individually and in combination.

Methods: Leaf powders were extracted by methanol maceration, followed by liquid–liquid partitioning using n-hexane and 96% ethanol to obtain the ethanolic fractions. Phytochemical screening and an in vitro experimental study of fractions to α-amylase inhibition assays were performed individually and in combinations, with acarbose as the positive control. Data were analyzed using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$).

Results: Both ethanolic fractions contained alkaloids, flavonoids, and tannins. *O. stamineus* showed the highest α-amylase inhibitory activity (64.67%), followed by *A. muricata* (26.98%) and the 2:1 combination (59.74%), whereas the 1:1 combination exhibited the lowest inhibition (12.68%). Significant differences were observed among all treatments ($p < 0.05$).

Conclusions: *O. stamineus* demonstrated greater α-amylase inhibitory activity than *A. muricata*. Combining the two extracts did not improve α-amylase inhibition compared with *O. stamineus* alone under the experimental conditions evaluated.

Cite this Article

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia due to defects in insulin secretion, insulin action, or both. The current global prevalence continues to rise, placing substantial burdens on healthcare systems and emphasizing the need for novel, cost-effective treatment strategies. Meanwhile, the management of type 2 diabetes mellitus (T2DM) often involves pharmacological agents, which may be costly or have side effects, and thus complementary approaches, especially from medicinal plants, are increasingly being explored (1). According to the *World Health Organization* (2024), over 500 million adults are currently living with diabetes, and this number is projected to exceed 700 million by 2045, with the majority of cases occurring in low- and middle-income countries (2). Type 2 diabetes mellitus (T2DM) accounts for approximately 90–95% of cases and is closely linked to lifestyle factors, insulin resistance, and impaired β-cell function (3).

Conventional treatments for diabetes, including insulin therapy and oral hypoglycemic agents (e.g., metformin, sulfonylureas, acarbose), effectively reduce blood glucose but may be associated with side effects such as gastrointestinal discomfort, hypoglycemia, and reduced patient compliance. Consequently, there is a growing interest in natural products and medicinal plants as complementary or alternative antidiabetic therapies due to their accessibility, cost-effectiveness, and multitarget mechanisms of action (4,5)

Two Southeast Asian medicinal plants, *Orthosiphon stamineus* (commonly known as "cat's whiskers" in English or *kumis kucing* in Indonesian) and *Annona muricata* (commonly known as soursop) have drawn particular attention for their antidiabetic potential. *Orthosiphon stamineus* is widely used in traditional Southeast Asian medicine for the treatment of diabetes, hypertension, and renal disorders. Phytochemical investigations indicate that *Orthosiphon stamineus* contains flavonoids (e.g., sinensetin), rosmarinic acid, and other phenolic compounds that exert antioxidant, anti-inflammatory, and carbohydrate-digesting enzyme-modulating activities. For example, in vitro studies showed that a 50 % ethanolic extract of *O. stamineus* and its isolated flavonoid sinensetin inhibited α-glucosidase and α-amylase with IC_{50} values of 4.63 mg/mL and 36.70 mg/mL (extract) and 0.66 mg/mL and 1.13 mg/mL (sinensetin), respectively (6,7). A systematic review confirmed that *O. stamineus* exerts antidiabetic effects through multiple mechanisms, including α-amylase/α-glucosidase inhibition, antioxidant/anti-inflammatory effects, improving insulin secretion and sensitivity, and regulating lipid metabolism (7,8).

Similarly, *Annona muricata* shows promise in antidiabetic research. In vitro studies have reported that *A. muricata* fruit part extracts inhibited α-amylase and α-glucosidase enzymes dose-dependently; for example, pericarp extract had an α-amylase IC_{50} of 0.46 mg/mL in one study (9). Further, leaf extracts exhibited α-amylase inhibitory activities in vitro (e.g., almost 53 % inhibition for hydromethanol extracts) and in silico docking analyses supported plausible binding of its phytochemicals to α-amylase (10,11). In addition, one study specifically reported that an aqueous extract of *A. muricata* peel inhibited both α-amylase and α-glucosidase more effectively than acarbose in vivo and in vitro, and improved insulin levels, lipid profile, and anti-inflammatory markers in alloxan-induced diabetic rats (12).

Although the antidiabetic properties of *Orthosiphon stamineus* and *Annona muricata* have been extensively investigated individually, studies evaluating the combined use of these medicinal plants remain scarce. Previous studies have primarily focused on the inhibitory activity of individual plant extracts against carbohydrate-hydrolysing enzymes, particularly α-amylase and α-glucosidase, or on their antioxidant and antihyperglycaemic activities in experimental models (6–12). A recent systematic review by Wang et al. reported that *O. stamineus* exerts antidiabetic effects through multiple mechanisms, including inhibition of α-amylase and α-glucosidase, antioxidant and anti-inflammatory activities, improvement of insulin secretion and sensitivity, and regulation of lipid metabolism (7). However, the authors also emphasized the need for further studies to identify the pharmacologically active constituents and their mechanisms of action. Likewise, a comprehensive review by Zubaidi et al. concluded that *A. muricata* possesses considerable antidiabetic potential through enzyme inhibition and glucose homeostasis regulation, while highlighting the lack of studies investigating its molecular mechanisms and combinations with other medicinal plants (13).

The rationale for combining *O. stamineus* and *A. muricata* is based on their distinct yet complementary phytochemical profiles. *O. stamineus* is characterized by abundant polymethoxylated flavonoids, including sinensetin and eupatorin, together with phenolic acids such as rosmarinic acid, whereas *A. muricata* contains flavonoids, phenolic compounds, acetogenins, and alkaloids that have been associated with antidiabetic, antioxidant, and anti-inflammatory activities. Since these phytochemicals differ in their chemical structures and reported biological activities, evaluating their combined effects may provide insight into how interactions among bioactive constituents influence α-amylase inhibition. Importantly, combinations of plant extracts may result in increased, unchanged, or reduced biological activity; therefore, their effects should be determined experimentally rather than assumed.

To the best of our knowledge, no previous study has specifically evaluated the α-amylase inhibitory activity of combined ethanolic leaf fractions of *O. stamineus* and *A. muricata* under comparable in vitro conditions. Previous investigations have largely examined each species separately, whereas evidence regarding their combined activity remains unavailable. Therefore, the novelty of the present study lies in evaluating the α-amylase inhibitory activity of individual and combined ethanolic leaf fractions of *O. stamineus* and *A. muricata* while simultaneously characterizing their phytochemical constituents. By comparing different combination ratios with the individual extracts, this study provides new evidence regarding whether combining these two medicinal plants modifies α-amylase inhibitory activity and contributes to the development of plant-based complementary strategies for postprandial glucose management.

MATERIALS AND METHODS

Equipments

The equipment used in this study included standard laboratory glassware, a blender, a separating funnel, micropipettes (20, 500, and 1000 µL; Accumax), an analytical balance (Durascale), a rotary evaporator (Biobase), a UV–Vis spectrophotometer (Shimadzu), and a water bath (Memmert AH-6).

Materials

The materials used were acarbose tablets (50 mg produced by Kimia Farma), aluminum foil, 1% starch solution, distilled water, *Orthosiphon stamineus* (cat's whiskers) leaves, *Annona muricata* (soursop) leaves, $FeCl_3$ solution (5%), HCl p.a (Merck), H_2SO_4 p.a (Merck), Whatman filter paper No. 1, methanol (Merck), magnesium powder (Merck), Mayer's reagent, 3,5-dinitrosalicylic acid (DNS) (Merck), and α-amylase enzyme (Xi'an Best Bio Tech Co. Ltd Batch Number BH20240120DF).

Plant Material Preparation

Fresh *O. stamineus* and *A. muricata* leaves were collected from Sembalun Village, East Lombok, Indonesia. The plant materials were authenticated and verified as *O. stamineus* and *A. muricata* by a botanist from Science Laboratory, Universitas Islam Negeri Mataram. The leaves were washed thoroughly, cut into 1–2 cm pieces, and shade-dried under sunlight for seven days while covered with a black cloth to prevent photodegradation of phytochemicals. The dried leaves were then ground into a fine powder with a blender and stored in airtight containers for extraction.

Extraction Procedure

The powdered leaves of *Orthosiphon stamineus* (250 g) and *Annona muricata* (300 g) were separately extracted by maceration using methanol as the extraction solvent. Each powdered sample was immersed in methanol at a plant material-to-solvent ratio of 1:4 (w/v) and macerated for 24 h at room temperature (25 ± 2°C) with occasional stirring. After filtration through Whatman No. 1 filter paper, the marc was re-macerated twice under identical conditions until the filtrate became nearly colorless to ensure exhaustive extraction. Methanol, a polar organic solvent, efficiently extracts polar and moderately polar compounds such as alkaloids, flavonoids, saponins, and phenolics. The combined filtrates were concentrated under reduced pressure using a rotary evaporator (Biobase, China) at 50°C, followed by evaporation in a water bath to remove residual solvent and obtain the crude methanolic extracts. Methanol extraction resulted in a higher yield for soursop leaves (13.30%) than for cat's whiskers leaves (9.47%).

Fractination

The crude methanolic extracts were subjected to liquid–liquid partitioning to obtain the ethanol fractions. Briefly, 8 g of *A. muricata* crude extract was suspended in 40 mL of distilled water (1:5, w/v). The suspension was transferred into a separatory funnel and partitioned with *n*-hexane (16 mL) to remove non-polar constituents. The partitioning process was repeated three times, and the non-polar fractions were discarded. The remaining fraction was subsequently processed to obtain the ethanol fraction, which was concentrated in a water bath until a constant weight was achieved. Similarly, 5 g of *O. stamineus* crude extract was suspended in 25 mL of distilled water and subjected to the same fractionation procedure using *n*-hexane (10 mL). The resulting ethanol fractions were concentrated and stored at 4°C until further analysis.

Phytochemical Investigation

Alkaloids. Alkaloid screening was conducted using Mayer's Reagent as prepared by Shaikh and Patil (2020). A few mL filtrate + 1-2 drops of Mayer's reagent (along the sides of the test tube). A positive result was obtained if a creamy white/yellow precipitate was produced (14).

Flavonoids. Flavonoid screening was conducted using Shinoda's test/Mg-Hydrochloride reduction test. The plant extract is dissolved in 5 mL of alcohol and added to fragments of a magnesium ribbon and a few drops of concentrated HCl. Positive results were obtained if a crimson colored solution (flavonal glycosides) was produced (14).

Tannins. 1 mL of plant extract is reacted with 10% iron (III) chloride solution; the presence of tannin is indicated by the formation of a dark blue or greenish black color (15).

Saponins. A 0.5 g plant extract was added with 2mL water (vigorously shaken). A positive result was obtained if the persistent foam was stable for 10 minutes (14).

Terpenoids. 2 mL of chloroform was added to 5 mL of plant extract and 3 mL of concentrated H_2SO_4, and boiled on a water bath. Positive results were obtained if a grey colored solution was produced (14).

Preparation of Positive Control (Acarbose) Solution

Acarbose served as the positive control. Acarbose tablets (50 mg) were finely ground, and the powder was accurately weighed and dissolved in distilled water to obtain a 1000 ppm stock solution. The acarbose stock solution was diluted to obtain a concentration of 200 ppm acarbose solution.

Preparation of Blank Solution

A blank solution without a sample served as the negative control. A blank solution was prepared by mixing 600 µL of α-amylase enzyme with 1200 µL of phosphate buffer (pH 6.9), 1000 µL of 1% starch solution, 2000 µL of phosphate buffer, and 1000 µL of DNS reagent dissolved in 2000 µL of phosphate.

α-Amylase Inhibitory Activity Assay

The antidiabetic potential of the ethanol fractions and their combinations was evaluated using the α-amylase inhibitory assay. Ethanolic fractions of *O. stamineus* and *A. muricata* were dissolved in distilled water to prepare 500 ppm solutions. Samples were tested individually and in combinations at different ratios: 1:0, 0:1, 1:1, 1:2, and 2:1 (v/v). However, acarbose as a positive control has been tested at a concentration of 200 ppm. The ethanolic fractions were tested at 500 ppm because they are complex mixtures with potentially lower α-amylase inhibitory potency than acarbose, requiring a higher concentration to achieve measurable activity. In contrast, acarbose is a purified and potent inhibitor, so 200 ppm was sufficient as a positive control while preventing complete enzyme inhibition.

For each assay, 100 µL of sample solution was mixed with 600 µL of α-amylase enzyme (dissolved in 1200 µL phosphate buffer) and incubated at 25°C for 5 minutes. Next, 1000 µL of 1% starch solution and 2000 µL of phosphate buffer were added and incubated for an additional 5 minutes. The reaction was terminated by adding 1000 µL of DNS reagent dissolved in 2000 µL phosphate buffer. The mixture was then heated in a water bath for 5

minutes and immediately cooled. The absorbance was recorded at 540 nm. Acarbose served as the positive control, while a reaction mixture without a sample served as the negative control.

The percentage inhibition of α-amylase was calculated using the following formula:

$$\text{Inhibtion } (\%) = \frac{Ac-As}{Ac}\ x\ 100\%$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

Based on Webb's fractional product method some phytochemical and enzyme inhibition studies report a Synergy Index (SI) based on the expected additive effect.

The expected inhibition is:

$$E = A + B - \left(\frac{AB}{100}\right)$$

Where:

A = % Inhibition of extract *O. Stamineus*

B = % Inhibition of extract *A. muricata*

Then, the synergy index (SI) is:

$$SI = \frac{O}{E}$$

Where:

O = Observed inhibition combination

E = Expected additive inhibition

SI > 1 : Synergistic

S1 =1 : Additive

SI < 1: Antagonistic

Statistical Analysis

All experiments were performed in triplicate (n = 3), and the results are expressed as mean ± standard deviation (SD). Prior to comparative analysis, the assumptions of normality and homogeneity of variances were evaluated using the Shapiro–Wilk test and Levene's test, respectively. Data that met these assumptions were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test for multiple pairwise comparisons among treatment groups. Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). A two-tailed *p*-value of less than 0.05 was considered statistically significant.

This study was conducted exclusively using plant materials and in vitro biochemical assays. No human participants, human tissues, experimental animals, or animal-derived procedures requiring ethical approval were involved. Therefore, ethical approval was not required for this study in accordance with the institutional guidelines.

RESULTS AND DISCUSSIONS

The Preparation of Simplicia

The dried raw plant material used in herbal medicine production is a critical step that influences both the stability and the bioactive-constituent integrity of the finished herbal product. In the present study, *Orthosiphon stamineus* (cat's whiskers) and *Annona muricata* (soursop) leaves were subjected to open-sun drying for seven days, under a black cloth covering to limit UV exposure, followed by gravimetric determination of moisture loss. The

results showed water losses of 79.1 % (residual moisture ~19.9 %) for *O. stamineus*, and 75.0 % (residual moisture ~25.0 %) for *A. muricata*, indicating substantial drying but still exceeding the often-recommended <10 % residual moisture threshold as shown in **Table 1**. While direct sun-drying is widely practiced, especially in rural and resource-limited settings due to its low cost and simplicity, it remains vulnerable to both environmental variability, for instance, solar intensity and ambient humidity, as well as contamination such as dust, insects, and microbial ingress. For example, Abidin et al. (2022) found that applying a super-absorbent polymer dryer with controlled temperature achieved moisture content reduction to ≤10 % within 18-26 h, much faster and more controlled than open-sun methods (16). More broadly, Eapen et al. (2025) review novel drying techniques (microwave, vacuum, hybrid) and emphasise that conventional sun-drying may compromise key phytochemicals via oxidation or photolysis if not well-controlled (17).

Drying under direct sunlight is widely recognized as an effective and accessible technique for moisture reduction; however, it can pose challenges to phytochemical preservation. According to Eapen et al. (2025), exposure to high light intensity and fluctuating temperatures can lead to oxidation and photolysis of thermolabile compounds such as flavonoids and polyphenols, potentially diminishing the bioactivity of herbal materials (17). Similarly, Safrina et al. (2023) reported that prolonged drying at high ambient temperatures significantly decreased total phenolic content and antioxidant capacity in medicinal plant simplicia. The partial shading approach employed in this study, using a black cloth barrier, was therefore a practical strategy to balance moisture removal and protection of photoactive constituents (18).

The residual moisture levels of around 20-25 % observed here, although perhaps acceptable in the short term as no fungal growth was noted during storage, exceed many pharmacognostic thresholds. Several herbal-quality standards stipulate that simplicia should contain no more than ~10 % moisture to minimise microbial proliferation, hydrolytic degradation, and loss of volatile or heat-labile compounds (19). Elevated moisture may thus reduce shelf-life, increase risk of microbial contamination, or degrade active constituents over time. Indeed, Safrina et al. (2023) reported that different drying methods affect the drying-rate constant and moisture content of medicinal flower simplicia, and observed that delayed drying increased the risk of degradation (18).

The retained green coloration of both dried leaf samples suggested minimal chlorophyll degradation and satisfactory pigment stability. This observation corresponds with the findings of Zhang et al. (2023), who demonstrated that moderate sunlight drying preserved color integrity and α-amylase inhibitory activity in *Syringa pubescens* flowers when compared with oven drying at high temperatures (20). Furthermore, the stable appearance and absence of microbial growth indicate that, despite moisture levels above 10%, the water activity may have been sufficiently low to inhibit microbial proliferation a factor that merits further investigation through direct measurement. To enhance the physicochemical quality of simplicia, the implementation of improved drying technologies such as solar dryers, forced-air convection systems, or hybrid drying (combining solar and electrical heat) is recommended. These methods have been shown to achieve faster drying, maintain phytochemical integrity, and yield moisture contents below 10% (16,17). From a pharmacological standpoint, maintaining phytochemical stability during drying is critical, as both *O. stamineus* and *A. muricata* contain compounds that contribute to α-amylase

inhibitory activity and antidiabetic effects. Loss of phenolic or flavonoid components during drying could reduce enzyme inhibition efficacy and overall therapeutic potential.

Table 1. Result of simplicia preparation

No.	Sample	Initial Weight (g)	Final Weight (g)	Water Loss (%)	Color
1.	Soursop leaves	1200	300	75.00	Green
2.	Cat's whiskers leaves	1200	250	79.10	Green

Extraction

Methanol extracts of *Annona muricata* (soursop) and *Orthosiphon stamineus* (cat's whiskers) leaves were obtained through the maceration extraction method. From 300 g of soursop simplicia, 40.00 g of thick extract was produced, corresponding to a yield of 13.30%, while 250 g of cat's whiskers simplicia yielded 23.68 g of thick extract, equivalent to 9.47% as shown in **Table 2**. The difference in extract yields between the two samples can be attributed to variations in phytochemical composition, solvent–solute interactions, and matrix structure. Methanol, a polar organic solvent, efficiently extracts polar and moderately polar compounds such as alkaloids, flavonoids, saponins, and phenolics. The higher yield obtained from soursop leaves suggests that their bioactive constituents exhibit greater polarity compatibility and solubility in methanol compared to those of cat's whiskers leaves. Similar findings were reported by Plaskova et al. (2023), who noted that solvent polarity, extraction duration, and particle size significantly affect yield and metabolite recovery during maceration-based extraction (21). In comparison, Faramayuda et al. (2021) found that a 96% ethanol extract of cat's whiskers leaves yielded 13.42%, higher than the 9.47% yield obtained with methanol in this study. This difference likely reflects variations in solvent polarity and the types of phytochemicals dissolved under each extraction condition. Ethanol, being less polar than methanol, can extract a wider spectrum of semi-polar compounds, potentially resulting in higher yields (22). Additionally, the physical composition of the plant materials contributes to extraction efficiency. Previous reports indicate that *A. muricata* leaves contain approximately 40–50% crude fiber and 10–15% protein (23), while *O. stamineus* leaves consist of 40.13% crude fiber, 2.13% fat, 10.35% water, and 20.15% cellulose (24).

The maceration process involves passive diffusion of the solvent through the plant cell walls, swelling of the protoplasm, and dissolution of metabolites with similar polarity to the solvent. Hence, polar metabolites are efficiently extracted by polar solvents, while non-polar components tend to remain undissolved. Extraction efficiency is also influenced by maceration parameters such as solvent-to-solid ratio, temperature, agitation, and extraction duration. A 2024 study by Mungwari et al. highlighted that optimizing these variables significantly improves extraction yield and selectivity for phytochemical recovery (25).

Overall, this study shows that methanol extraction resulted in a higher yield for soursop leaves (13.30%) than for cat's whiskers leaves (9.47%), reflecting differences in both the polarity of phytoconstituents and structural characteristics of the plant materials. Bhadange et al. (2024) reported that solvent composition optimization can substantially increase extraction efficiency and metabolite recovery (26).

Table 2. Results of the extraction of cat's whiskers (*Orthosiphon stamineus*) and soursop (*Annona muricata*) leaves

No.	Sample	Weight of Simplicia (g)	Weight of Thick Extract (g)	Yield (%)
1.	*Soursop* (*Annona muricata*) leaves	300	40.00	13.30
2.	*Cat's whiskers* (*Orthosiphon stamineus*) leaves	250	23.68	9.47

Fractionation

The fractionation of the methanol extracts from *Annona muricata* (soursop) and *Orthosiphon stamineus* (cat's whiskers) leaves was achieved via liquid–liquid separation using 96% ethanol as solvent. From an extract mass of 8 g for soursop leaves, a thick fraction weighing 1.42 g was obtained, yielding 17.75%. For cat's whiskers leaves, 5 g of extract afforded a thick fraction of 0.80 g, corresponding to a yield of 16.00% as present in Table 3. The relatively high yields for both herbals indicate that a large proportion of semi-polar and polar constituents are readily partitionable into a high-polarity ethanol fraction under the applied conditions. The principle of liquid–liquid fractionation, "like dissolves like," underlies this result, solvents of higher polarity preferentially dissolve compounds of similar polarity from the plant extract matrix (27).

Table 3. Results of fractionation of cat's whiskers (*Orthosiphon stamineus*) and soursop (*Annona muricata*) leaves

No.	Sample	Weight of Extract (g)	Weight of Thick Ethanol Fraction (g)	Yield (%)
1.	*Soursop* (*Annona muricata*) leaves	8	1.42	17.75
2.	*Cat's whiskers* (*Orthosiphon stamineus*) leaves	5	0.80	16.00

In the present case, using 96% ethanol favours the extraction of polar to semi-polar metabolites (e.g., flavonoids, glycosides, phenolics), which aligns with the moderate to high yields observed. Comparison of the two species reveals that *A. muricata* (17.75%) yielded slightly more fraction material than *O. stamineus* (16.00%). This difference, though modest, may reflect subtle distinctions in the chemical composition of each plant's extract, specifically the proportion of polar and semi-polar metabolites available for solubilization in 96% ethanol as well as matrix structural features influencing solvent penetration and partitioning efficiency.

Several factors may govern these yield outcomes: solvent polarity and miscibility, solvent-to-solid (or to extract) ratio, phase contact time, temperature, and the intrinsic plant matrix (including grain/powder size, cell wall composition, fiber/cellulose content). For instance, earlier work has emphasized that the choice of solvent and fractionation sequence strongly influences both yield and phytochemical profile recovered (27,28). The review by Susanti et al. (2024) highlights that separation methods for phenolic compounds often require careful selection of solvent systems to maximise recovery of target metabolites (29).

In light of these parameters, the results suggest that the 96% ethanol phase likely captured a large share of accessible polar/semi-polar components, but may not have optimally captured less-polar constituents, which could remain in more non-polar fractions (if performed) or in the residual matrix. For future work, extending the fractionation sequence to include lower polarity solvents (e.g., ethyl acetate, chloroform) may permit separation of non-polar and mid-polar components and improve overall phytochemical partitioning. The comprehensive review on liquid–liquid and solid–liquid partitioning methods emphasizes this multi-solvent strategy as beneficial for full phytochemical profiling (27,30).

In conclusion, the fractionation yields of 17.75% for soursop and 16.00% for cat's whiskers leaves indicate successful partitioning of extractable polar/semi-polar metabolites into the 96% ethanol fraction. These values are consistent with expected solvent-solute polarity matching and confirm that the chosen fractionation protocol is viable for targeting these herbals. Optimization of solvent polarity gradients and fractionation parameters may further enhance selectivity and yield of bioactive fractions.

Phytochemical Investigation

Preliminary phytochemical screening represents a critical step in elucidating the spectrum of secondary metabolites present in plant extracts. Such metabolites, including alkaloids, flavonoids, tannins, saponins, and terpenoids, often play key roles in biological activity and therapeutic potential (31,32). Although phytochemical screening cannot elucidate the chemical structures of isolated constituents, it forms the basis for phytochemical research. In the present study, the fractionated extract of *Annona muricata* (soursop) leaves revealed the presence of alkaloids, flavonoids, and tannins, whereas saponins and terpenoids were not detected, as shown in Table 4. These findings partially align with the earlier work of Safarini et al. (2019), which reported flavonoids, saponins, and alkaloids in soursop leaves (33). The absence of saponins and terpenoid in our fractions may reflect differences in extraction/fractionation conditions, the selectivity of the solvent system, or matrix-specific metabolite distribution.

Table 4. Phytochemical investigation results of fractionated soursop (*Annona muricata*) and fractionated cat's whiskers (*O. stamineus*) leaves

No.	Secondary Metabolites	Investigation results	Information
1.	Alkaloids	+	A creamy white/yellow precipitate (14)
2.	Flavonoids	+	A pink to crimson solution (flavonal glycosides) (14)
3.	Tannins	+	Formation of dark blue or greenish black color (15)
4.	Saponins	-	There is no persistent foam for 10 minutes (14)
5.	Terpenoids	-	There is no formation of grey solution (14)

On the other hand, for *Orthosiphon stamineus* (cat's whiskers) leaves, the fraction exhibited alkaloids, flavonoids, and tannins, but not saponins and terpenoids, as shown in Table 4. These results are consistent with Nurcholis et al. (2022), who found flavonoids, terpenoids, tannins, and alkaloids in the ethanol leaf extract of *O. stamineus* (34). The present absence of terpenoids suggests that our fractionation protocol has selectively enriched

certain metabolite classes (alkaloids, flavonoids, and tannins) while excluding others, an outcome that may be purposeful if targeting those particular metabolite groups.

The variation between previously published profiles and the current findings underscores how extraction parameters (solvent polarity, fractionation scheme, plant part, geographic origin) influence the qualitative profile of secondary metabolites. Recent reviews emphasize that proper matching of solvent polarity to metabolite polarity, as well as the optimization of extraction and separation methodology, is required for comprehensive phytochemical recovery (35,36). Alkaloids, flavonoids, and tannins detected in both plant fractions are known to contribute significantly to antioxidant, anti-inflammatory, and other bioactive properties. Their detection confirms the suitability of the fractionation approach for isolating polar and semi-polar compounds of interest. At the same time, the absence of other classes (such as saponins and terpenoids) does not necessarily imply absence in the plant material, but rather reflects the partitioning behavior under the chosen solvent system.

α-Amylase Inhibitory Activity

The antidiabetic potential of the samples was evaluated through an *in vitro* α-amylase inhibition assay. This method, which measures enzyme activity outside a living system, determines the ability of a test extract to inhibit the α-amylase enzyme, an important enzyme in the hydrolysis of dietary starch into glucose. The reaction mixture's absorbance was measured at 540 nm using a UV-Vis spectrophotometer, as this wavelength corresponds to the optimal absorbance of glucose molecules (37). The assay was performed at a concentration of 500 ppm for the *O. stamineus* and *A. muricata* ethanolic fraction, and 200 ppm for the positive control (acarbose).

Table 5. α-Amylase inhibition of cat's whiskers and soursop ethanol fractionated leaf samples

No	Sample	Absorbance			α-Amylase Inhibition (%)			Average ±SD
		1	2	3	1	2	3	
1.	Cat's whiskers leaves (DKK)	0.257	0.256	0.247	64.56	64.51	64.95	64.67±0.24^{b}
2.	Soursop leaves (DS)	0.536	0.522	0.513	26.75	26.65	27.56	26.98±0.49^{d}
3.	DKK:DS (1:1)	0.629	0.624	0.621	11.96	12.50	13.59	12.68±0. 83^{f}
4.	DKK:DS (1:2)	0.399	0.396	0.391	44.70	44.62	45.27	45.03±0.35^{c}
5.	DKK:DS (2:1)	0.254	0.316	0.296	59.62	59.56	60.06	59.74±0.27^{a}
6.	Acarbose	0.227	0.202	0.247	68.48	68.43	68.83	68.58±0.21^{a}

Notes: DKK = Cat's whiskers leaves; DS = Soursop leaves; blank absorbances: 0.715 (Replication 1), 0.714 (Replication 2), 0.720 (Replication 3); fractionated sample tested at 500 ppm and acarbose at 200 ppm; $p < 0.05$ significant differences were observed among all treatments

Among all tested samples, the highest α-amylase inhibitory activity was observed in the positive control (acarbose, 68.58 %), followed closely by the single fraction of *Orthosiphon stamineus* (cat's whiskers) leaves (64.67 %). The 2:1 mixture of cat's whiskers to *Annona muricata* (soursop) leaves demonstrated substantial inhibition (59.74 %), while the 1:2

combination yielded 45.03 %. In contrast, the single soursop leaf extract and the 1:1 combination exhibited notably lower inhibitory activity (26.98 % and 12.68 %, respectively). These findings demonstrate that *O. stamineus* extract exhibits a stronger α-amylase inhibitory effect compared with *A. muricata*. This difference is likely associated with the higher concentration of bioactive alkaloids, flavonoids, and tannins in cat's whiskers, as revealed in the phytochemical screening results. The classes of compounds are known to inhibit α-amylase by distinct mechanisms. Tannins form stable complexes with protein residues at the enzyme's active site, preventing substrate access (38). Flavonoids interfere with enzymatic catalysis by binding to active and allosteric sites through hydrogen bonding and π–π interactions, thereby modulating enzyme conformation (39,40). In addition, alkaloids can inhibit α-amylase, but potency and mode (competitive, non-competitive, uncompetitive) depend strongly on specific structural features planar/heterocyclic aromatic systems, H-bonding groups, basic (protonatable) nitrogens, lipophilicity, steric bulk, and stereochemistry (5,41).

The predominance of sinensetin and kaempferol in *O. stamineus* and kaempferol in *A. muricata* supports their respective roles in reducing glucose absorption and improving glycemic regulation (23,24). However, the more potent activity of cat's whiskers suggests that its poly-methoxylated flavones (such as sinensetin and eupatorin) exhibit higher affinity toward α-amylase. Recent studies confirm that such flavonoids and polyphenols possess strong inhibitory potential against both α-amylase and α-glucosidase, contributing to reduced post-prandial glucose levels (39,42). Moreover, synergistic inhibition observed in the 2:1 combination implies that cat's whiskers constituents dominate enzymatic binding when co-formulated, enhancing overall bioactivity. Conversely, reduced inhibition at higher soursop ratios may result from antagonistic or competitive binding effects between metabolite classes of differing polarity.

These findings demonstrate that *O. stamineus* exhibited superior α-amylase inhibitory activity compared to *A. muricata*. However, when both extracts were combined, the inhibitory activity decreased, indicating possible antagonistic interactions between their bioactive constituents. Phytochemical screening revealed that both *O. stamineus* and *A. muricata* fractions contained similar classes of secondary metabolites specifically alkaloids, flavonoid,s and tannins obtained through ethanol-based liquid-liquid fractionation. Despite this compositional similarity, the relative proportions, chemical structures, and binding affinities of individual metabolites differ between the two plants, leading to distinct biological effects.

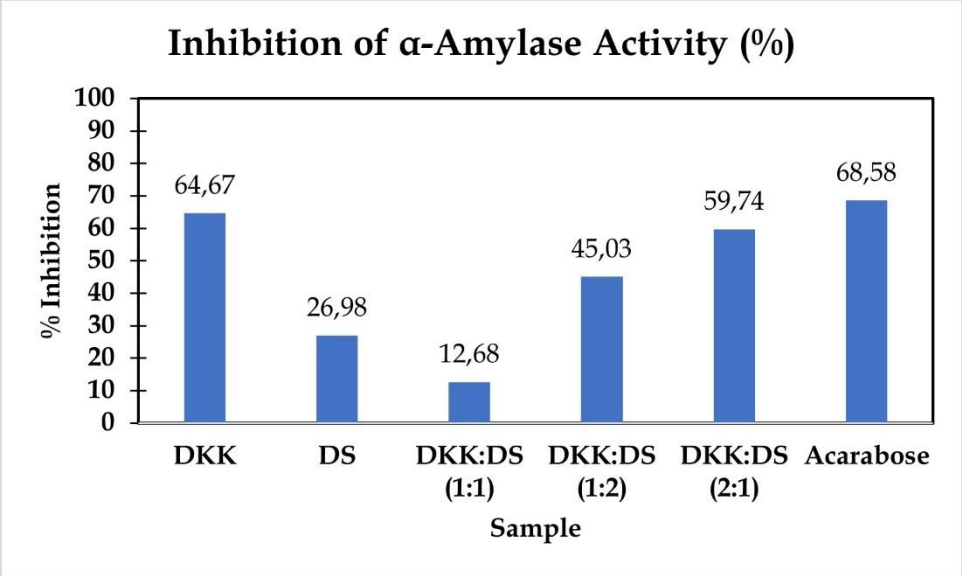

Figure 1. Inhibition of α-amylase activity (%) by fractions of cat's whiskers leaves (DKK), soursop leaves (DS), and their combinations compared with acarbose as a positive control

The stronger inhibition exhibited by *O. stamineus* may be attributed to its higher content of polymethoxylated flavones such as sinensetin, eupatorin, and kaempferol (24). These compounds are more lipophilic and thus can penetrate enzyme hydrophobic pockets more effectively, forming stable enzyme–flavonoid complexes (39,41). In contrast, *A. muricata* contains mainly kaempferol and quercetin derivatives (23,42), which, while bioactive, possess fewer methoxy groups and lower hydrophobicity, resulting in weaker binding affinity toward α-amylase (40). Additionally, the tannin composition of *O. stamineus* is dominated by condensed proanthocyanidins, which exhibit stronger protein-binding capacity than the hydrolysable tannins more commonly found in *A. muricata* (38). These structural and compositional differences explain why the two fractions, despite containing the same metabolite groups, display different levels of inhibitory activity.

Statistical analysis was performed using IBM SPSS Statistics version 26.0. Data are presented as mean ± SD (n = 3). The Shapiro–Wilk and Levene's tests confirmed normal distribution and homogeneity of variances ($p > 0.05$). In addition, one-way ANOVA revealed significant differences among treatments ($F = 7291.42$, $p < 0.001$). Tukey's post hoc test showed that all treatments differed significantly from one another ($p < 0.05$). Among the tested samples, acarbose exhibited the highest α-amylase inhibitory activity (68.58 ± 0.21%), followed by cat's whiskers leaf fraction (64.67 ± 0.24%) and the DKK:DS (2:1) combination (59.74 ± 0.27%). The DKK:DS (1:1) combination showed the lowest inhibitory activity (12.68 ± 0.83%), suggesting a possible antagonistic interaction between the fractions at this ratio.

We found that when *O. stamineus* and *A. muricata* fractions were combined, the α-amylase inhibitory activity decreased, suggesting an antagonistic interaction, particularly at a 1:1 (DKK:DS) combination compared to individual activity, as shown in Figure 1. In addition, the combination ratio markedly influenced α-amylase inhibitory activity. The DKK:DS (1:1) mixture exhibited strong antagonism (SI = 0.28), producing substantially lower inhibition than predicted from the individual activities of DKK and DS. In contrast,

the DKK:DS (1:2) and DKK:DS (2:1) combinations demonstrated synergistic interactions, with synergy indices of 1.14 and 1.15, respectively. Among the combinations tested, DKK:DS (2:1) showed the highest α-amylase inhibitory activity (59.74 ± 0.27%), suggesting that a higher proportion of cat's whiskers leaf fraction enhances the antidiabetic potential of the mixture. These findings indicate that the interaction between *Orthosiphon stamineus* and *Annona muricata* fractions is ratio-dependent, with higher proportions of DKK producing more favorable α-amylase inhibitory activity.

Table 6. Analysis of synergism, additivity, or antagonism of the combination *O. stamineus* and *A. muricata* to the individual fractions.

Treatment	Expected (%)	Observed (%)	Synergy Index	Interpretation
DKK:DS (1:1)	74.20	12.68	0.17	Antagonistic
DKK:DS (1:2)	74.20	45.03	0.61	Antagonistic
DKK:DS (2:1)	74.20	59.74	0.61	Antagonistic

Certain compounds in one extract compete for the same binding sites on the enzyme or form intermolecular complexes that reduce the bioavailability of active constituents. In this case, the higher polarity of *A. muricata* constituents—particularly glycosylated flavonoids and hydrolysable tannins—may interfere with the binding of the more hydrophobic flavonoids from *O. stamineus*. These polar compounds may occupy peripheral binding regions of α-amylase without effectively inhibiting catalytic activity, thereby preventing stronger inhibitors from accessing the active site. Additionally, some compounds in *A. muricata* (such as acetogenins and alkaloid traces) can interact non-specifically with polyphenols, reducing their free concentration and weakening overall enzyme inhibition (38,40,41).

The combination of *O. stamineus* and *A. muricata* exhibited lower α-amylase inhibitory activity than *O. stamineus* alone under the experimental conditions evaluated. Interactions between compounds of different polarity or redox potential can diminish inhibitory efficiency by altering enzyme conformation or forming inactive complexes (39). Therefore, the reduced α-amylase inhibition observed in the mixed fractions underscores the importance of optimizing extract ratios to achieve maximum pharmacological synergy. Overall, the α-amylase inhibition data corroborate the phytochemical screening results, confirming that flavonoids, tannins, and alkaloids play major roles in mediating the observed antidiabetic activity. The superior inhibitory effect of *O. stamineus* underscores its potential as a promising natural α-amylase inhibitor, warranting further isolation, kinetic modeling, and in vivo studies to validate its therapeutic potential.

CLINICAL IMPLICATION

The findings of this study demonstrate that *Orthosiphon stamineus* possesses substantial α-amylase inhibitory potential, suggesting its possible therapeutic application as a natural antidiabetic agent. The strong enzyme inhibition observed may help reduce postprandial glucose levels by delaying carbohydrate digestion and absorption. This mechanism is comparable to that of conventional α-amylase inhibitors such as acarbose but may offer fewer gastrointestinal side effects due to its plant-based origin. The results also support the potential integration of *O. stamineus* leaf extracts into functional foods or

phytopharmaceutical formulations for diabetes management. However, the antagonistic effect observed in combination with *Annona muricata* indicates that polyherbal formulations should be carefully optimized to avoid reduction of bioactivity due to chemical interactions among plant constituents.

LIMITATIONS

This study was limited to in vitro enzyme inhibition assays and qualitative phytochemical analysis. Therefore, the biological relevance of the observed α-amylase inhibition requires validation through in vivo studies and kinetic modeling to elucidate the specific mechanism of inhibition (competitive, noncompetitive, or uncompetitive). Quantitative analysis of individual bioactive compounds was not conducted, which limits the correlation between specific phytochemicals and inhibitory potency. Furthermore, only one concentration (500 ppm) was tested, preventing the determination of IC_{50} values for comparative potency assessment. Future research should employ dose-response studies, chromatographic isolation, and molecular docking to confirm structure–activity relationships and synergistic or antagonistic effects in combined extracts.

CONCLUSIONS

In summary, both *O. stamineus* and *A. muricata* fractions exhibited α-amylase inhibitory activity associated with the presence of flavonoids and tannins. However, *O. stamineus* exhibited higher α-amylase inhibitory activity than *A. muricata* under the tested conditions. Although both fractions share similar metabolite classes, structural variations and compositional ratios determine their relative potencies. When combined, their interactions likely produced lower α-amylase inhibitory, reducing total inhibition. These findings suggest that *O. stamineus* alone holds greater promise as a natural source of α-amylase inhibitors for antidiabetic treatments.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTOR CONTRIBUTIONS

Baiq Risma Fatmayanti served as the main author and principal writer by conceptualizing the study, designing the methodology, interpreting the data, and preparing the initial manuscript draft. Sri Wardiatul Fitri contributed as a co-author and data collector by conducting the experimental procedures, preparing the samples, and acquiring the research data. Arief Rafsanjani contributed as a co-author by reviewing the manuscript, analyzing the relevant literature, and validating the interpretation of the study findings. All authors reviewed, revised, and approved the final version of the manuscript for submission.

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DECLARATION OF ARTIFICIAL INTELLIGENCE USE

The authors utilized ChatGPT (OpenAI, version GPT-5) to enhance the language and grammar of the manuscript. The authors reviewed and verified the content to ensure accuracy and integrity.

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