IJPST
Indonesian Journal of Pharmaceutical Science and Technology

Research Article
Vol. 12 Suppl. 2 (2025), 90-97
https://doi.org/10.24198/ijpst.v12s2.59646

Hypoglycaemic Properties of The Combination of *Hibiscus sabdariffa L.* and *Stevia rebaudiana Bertoni* in Hyperglycaemic Rats

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Abstract
Roselle (*Hibiscus sabdariffa L.*) and stevia (*Stevia rebaudiana Bertoni*) are known for high content of phenolic compounds and flavonoids which are beneficial in glycemic control. This study aimed to determine the phytochemical content and evaluate the hypoglycemic effects of a combined aqueous extract of *H. sabdariffa* and *S. rebaudiana* in a 3:1 ratio on diabetic rats. Aqueous extracts were prepared and subjected to quantitative analysis to determine total phenolic and flavonoid contents utilizing the Folin-Ciocalteu and aluminum chloride colorimetric assays, respectively. Diabetes was experimentally induced in male Wistar rats via intraperitoneal administration of alloxan (235 mg/kgBW). Rats in the test groups received 500 or 1000 mg/kgBW of the combination, with blood glucose levels measured on the 0th, 7th, and 14th days post-treatment using a glucometer. *H. sabdariffa*, *S. rebaudiana*, and their combination contained total phenolic contents of 102.75, 188.053, and 118.856 mgGAE/g, respectively, and flavonoid contents of 3.099, 6.987, and 4.791 mgQE/g, subsequently. Administration of the combination extract at 1000 mg/kgBW showed the highest hypoglycemic effect, reducing blood glucose levels by 35.98% on the 14th day compared to the negative control ($p<0.05$). The combination of *H. sabdariffa* and *S. rebaudiana* extracts demonstrates significant hypoglycemic activity, attributed to their phenolic and flavonoid content.

Keywords: Alloxan-induced rats, blood glucose levels, diabetes, Roselle calyx (*Hibiscus sabdariffa L.*), Stevia leaves (*Stevia rebaudiana Bertoni*)

Article History:
Submitted 5 December 2024
Revised 23 January 2025
Accepted 25 January 2025
Published 14 February 2025

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Citation:
Febriyanti, R.M, Putri , R.N., Diantini A., Levita J. Hypoglycaemic Properties of The Combination of Hibiscus sabdariffa L. and Stevia rebaudiana Bertoni in Hyperglycaemic Rats. Indonesian Journal of Pharmaceutical Science and Technology. 2025: Vol.12 Suppl.2 : 90-97.

Aktivitas Hipoglikemik Kombinasi *Hibiscus sabdariffa L.* dan *Stevia rebaudiana* Bertoni pada Tikus Hiperglikemia

Abstrak
Rosela (*Hibiscus sabdariffa Linn.*) dan Stevia (*Stevia rebaudiana B.*) diketahui mengandung senyawa fenolik dan flavonoid yang berperan sebagai antioksidan dan bermanfaat dalam menurunkan kadar gula darah. Penelitian ini bertujuan untuk mengetahui kandungan fitokimia dan efek kombinasi ekstrak air *H. sabdariffa* dan *S. rebaudiana* dengan perbandingan 3:1 terhadap penurunan kadar glukosa darah tikus diabetes yang diinduksi aloksan. Ekstrak air dianalisis kandungan fenolik dan flavonoid total dengan menggunakan metode Folin-Ciocalteu dan aluminium klorida secara berturut-turut. Induksi diabetes pada tikus Wistar Jantan dilakukan dengan injeksi aloksan intraperitoneal (235 mg/kgBB). Tikus pada kelompok uji menerima 500 atau 1000 mg/kgBB ekstrak kombinasi, dengan kadar glukosa darah diukur pada hari ke-0, ke-7, dan ke-14 pasca perawatan menggunakan glukometer. Ekstrak *H. sabdariffa, S. rebaudiana*, dan kombinasinya mengandung total kandungan fenolik masing-masing 102,75 mgGAE/g, 188,053 mgGAE/g, dan 118,856 mgGAE/g, serta kandungan flavonoid masing-masing 3,099 mgQE/g, 6,987 mgQE/g, dan 4,791 mgQE/g. Pemberian ekstrak kombinasi pada dosis 1000 mg/kgBB menunjukkan efek hipoglikemik tertinggi, menurunkan kadar glukosa darah sebesar 35,98% pada hari ke-14 dibandingkan dengan kelompok kontrol negatif ($p<0,05$). Kombinasi ekstrak *H. sabdariffa* dan *S. rebaudiana* menunjukkan aktivitas hipoglikemik yang signifikan yang diduga dari kandungan fenolik dan flavonoidnya.

Kata Kunci: diabetes, kadar glukosa darah, rosela (*Hibiscus sabdariffa Linn.*), stevia (*Stevia rebaudiana B.*), tikus yang diinduksi aloksan

1. Introduction

Diabetes mellitus (DM), a disease characterized by elevated blood glucose levels, is a worldwide public health problem.[1,2] According to the International Diabetes Federation (IDF), the global prevalence of diabetes in 2021 was 10.5%, with 537 million people aged 20-79 years affected and caused 6.7 million deaths. The global diabetes rate is projected to increase, reaching 643 million by 2030 and 783 million by 2045.[3] Indonesia ranked fifth in the world, following China, India, Pakistan, and the United States, with 19.5 million diabetes cases in 2021.[4] These epidemiological data underscore that diabetes is a global health problem, affecting millions worldwide and necessitating profound and comprehensive management.

In individuals with diabetes, impaired insulin production or resistance to insulin leads to leads to hyperglycemia, characterized by elevated blood glucose levels, which, if inadequately managed, may precipitate a range of systemic complications, including cardiovascular, renal, and neurological disorders.[5,6] Effective management of diabetes often necessitates lifelong administration of antidiabetic pharmacotherapies to maintain glycemic control and mitigate the risk of long-term complications. However, long-term use of these drugs can cause undesirable side effects and incur significant and escalating costs.[7–11] Consequently, there is a need for alternative traditional medicines derived from natural ingredients that are more readily available and considered safer, thereby potentially reducing side effects and medical costs.

Roselle calyxes (*Hibiscus sabdariffa L.*) has been reported to have hypoglycaemic properties.[12,13] H. sabdariffa contains anthocyanin pigments, a flavonoid type that acts as antioxidants and gives the flower its red color.[14,15] One of the anthocyanin compounds in *H. sabdariffa*, cyanidin-3-glucoside, helps stabilize blood glucose levels and improve insulin sensitivity. Additionally, flavonoid compounds in *H. sabdariffa* inhibit α-amylase activity, enhance the function and integrity of pancreatic β-cells by counteracting free radicals, and increase protection against insulin resistance.[16,17] In previous research by Hamadjida et al. (2023), the test group receiving an aqueous extract of *H. sabdariffa* at doses of 100, 200, and 400 mg/kgBW demonstrated a significant blood glucose levels reduction in a dose-dependent manner and accompanied by a decrease of blood lipids.[18]

Another plant that has been reported to have antidiabetic properties is stevia (*Stevia rebaudiana Bertoni*). *S. rebaudiana* leaves are a low-calorie natural sweetener often used as a sugar substitute.[19,20] Analysis of *S. rebaudiana* leaf extract has shown that the main components, rebaudioside, and stevioside, are about 300 times sweeter than regular sucrose. Both compounds have the same steviol groups that act on glucose transporters.[21–23] The diterpene glycosides in S. rebaudiana can also repair damage to pancreatic β-cells and stimulate insulin secretion through direct action on these cells.[24]

The effect of plants on blood sugar levels can be observed by measuring blood glucose levels in test animals, such as Wistar rats, which are commonly used in experimental research due to their relatively fast metabolism. Test animals can be induced into a diabetic state using chemicals such as alloxan, streptozotocin, diacetyl, adrenaline, glucagon, and EDTA. [25,26] Alloxan is frequently used as a diabetogen because it can induce permanent hyperglycemia within two to three days by damaging pancreatic β-cells, thereby disrupting insulin production.[27]

Based on previous research, it is evident that *H. sabdariffa* and *S. rebaudiana*. Therefore, this study will use a combination of these two plants to achieve a more effective reduction in blood glucose levels than a single preparation and determine an effective dose based on prior research.

2. Materials and Methods

2.1. Materials and Instruments

The materials used in this study included dried calyces of *Hibiscus sabdariffa L.* (Roselle) and leaves of Stevia rebaudiana Bertoni (Stevia), which were collected from local suppliers in West Java, Indonesia. The identity of the plants was confirmed by taxonomists at the Department of Biology, Faculty of Mathematics and Science, Universitas Padjadjaran, and voucher specimens were deposited in the university's herbarium for reference. The alloxan monohydrate used for diabetes induction was obtained from Sigma-Aldrich, USA. Instruments included a UV-Vis spectrophotometer (Shimadzu UV-1800) and Accu-Chek™ Blood Glucose Meter.

2.2. Sample Preparation

H. sabdariffa calyces and *S. rebaudiana* leaves were powdered using a mechanical grinder. Powdered *H. sabdariffa* calyxes and *S. rebaudiana* were dissolved in hot water at a ratio of 2 grams per 50 mL, stirred for 5 minutes, filtered, and freeze-dried.

2.3. Determination of Total Phenolic Content

The total phenolic content was determined using the Folin-Ciocalteu method with gallic acid as the standard compound, analyzed via a UV-Vis spectrophotometer. Initially, 10 mg of gallic acid was dissolved in 25 mL of methanol p.a. Serial dilutions were performed to obtain concentrations of 5, 15, 30, 50, 70, and 100 µg/mL. For each concentration, 1 mL of the gallic acid standard solution was pipetted and mixed with 5 mL of 7.5% Folin-Ciocalteu reagent. The mixture was shaken and left to stand for 8 minutes, followed by adding 4 mL of 1% NaOH and shaken until homogeneous. The prepared solution was then shaken and left to stand for 1 hour, and its absorbance was measured at 765 nm. A calibration curve was generated by plotting the absorbance against gallic acid concentration, producing the regression equation $y=0.003x-0.011$ with an $R^2=0.9993$.

For the sample analysis, 200 mg of *H. sabdariffa* extract, *S. rebaudiana* extract, and their combination (3:1) were dissolved in 25 mL of methanol p.a. Subsequently, 1 mL aliquot of the sample was combined with 5 mL of a 7.5% Folin-Ciocalteu reagent solution. The mixture was shaken, and allow to stand for 8 minutes. Then, 4 mL of 1% NaOH was added, and the mixture was shaken until homogeneous. The total phenolic content (TPC) was quantified and expressed in terms of gallic acid equivalents (mg GAE/g extract).

2.4. Determination of Total Flavonoid Content

The total flavonoid content was determined by the complex formation method using aluminum chloride (AlCl3) with quercetin as the standard, analyzed via a UV-Vis spectrophotometer. Initially, 10 mg of quercetin was dissolved in 25 mL of ethanol p.a. A series of dilutions were then prepared to obtain 25, 50, 75, and 100 µg/mL. For each concentration, 0.5 mL of quercetin solution was combined with 1.5 mL of ethanol, 0.1 mL of 10% AlCl3, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. The resultant mixture was incubated for 30 minutes at room temperature, and absorbance was subsequently measured at 434 nm to determine the flavonoid content. A calibration curve was constructed by plotting the absorbance against quercetin concentration, yielding the regression equation $y=0.0036x-0.007$ with an $R^2=0.994$.

For the sample analysis, 200 mg of *H. sabdariffa* extract, *S. rebaudiana* extract, and their combination (3:1) were dissolved in 25 mL of ethanol p.a. and treated similarly. Absorbance readings were corrected by subtracting the absorbance of control solutions (ethanol and reagents without samples). The total flavonoid content was calculated by applying the regression equation obtained from the calibration curve, with the results expressed as quercetin equivalents (mg QE/g extract). To ensure precision and reproducibility, each analysis was conducted in triplicate

2.5. Development of Alloxan-Induced Diabetes White Rats

Before conducting the animal study, ethical clearance was submitted to the Ethical Committee. This study has been approved by the Research Ethics Commission of Universitas Padjadjaran with approval number 525/UN6.KEP/EC/2024.

Thirty healthy male Wistar rats (120-160 g, aged 3-4 months) were used in this experiment. Rats were obtained from the Faculty of Pharmacy, Institut Teknologi Bandung. Rats were divided into five groups (each group consisting of six rats), namely normal control group (normal healthy rats without alloxan induction), negative control group (alloxan-induced diabetes rats, given 0.5% NaCMC); test group I, alloxan-induced diabetes rats, given 500 mg/kgBW of extract combination); test group II (alloxan-induced diabetes rats, given 1000 mg/kgBW of extract combination); positive control group (alloxan-induced diabetes rats, given 0.45 mg/kgBW of glibenclamide). group II – V were then injected with 235 mg/kgBW of alloxan intraperitoneally.

Three days post-induction, fasting blood glucose levels were quantified to confirm the effective induction of diabetes mellitus. Only rats exhibiting fasting blood glucose levels of 250 mg/dL or above were classified as diabetic and subsequently included in the study. [18]

2.6. Administration of Extract Preparations

The freeze-dried combination of *H. sabdariffa L.* and *S. rebaudiana* was dissolved in hot water at 73°C–74°C. For the 500 mg/kgBW dose, 4.6 g of the powdered extract was dissolved in 480 mL of hot water, while for the 1000 mg/kgBW dose, 9.6 g of the powdered extract was dissolved in 480 mL of hot water. Alloxan-induced diabetic rats were then subsequently treated under the designated groups. The administration of extracts/drugs was carried out in each group once a day for 14 days orally.

2.7. Blood glucose level measurements

Blood glucose levels were quantified using an Accu-check™ Blood Glucose Meter AGM-2100 accompanied by the corresponding test strips. Following a 12-hour overnight fast, blood samples

were obtained via tail vein puncture. Blood glucose level assessments were performed at four designated time points: baseline (D0, prior to extract/drug administration) and subsequently on days 1, 7, and 14 following the induction of diabetes mellitus.

2.8. Data Analysis

The blood glucose levels (pre- and post-treatment) were analyzed using a repeated-measures analysis of variance (ANOVA), with measurements taken on days 1, 7, and 14 post-induction serving as the basis for between-group comparisons. Upon detecting significant overall differences, post-hoc pairwise analyses were performed utilizing the Bonferroni correction to delineate specific group contrasts. The normality of the dataset was confirmed via the Shapiro-Wilk test, while Levene's test was employed to verify homogeneity of variances. All statistical analyses were conducted using SPSS software (version 30), with statistical significance defined as $p < 0.05$.

3. Result

In the present study, plant materials were prepared as freeze-dried water extract with the extract yield is presented in the Table 1 and Figure 1.

Figure 2a depicts the total phenolic content in each *H. sabdariffa* and *S. rebaudiana* water extract, as well as their combination. The highest total phenol content was found in the *S. rebaudiana* extract, measuring 188.053 ± 0.0005 mg GAE/g extract. This was followed by the combination extract, which contained 118.856 ± 0.001 mg GAE/g extract. The lowest total phenol content was observed in the *H. sabdariffa* extract, with a 102.751 ± 0.001 mg GAE/g extract. The incorporation of S. rebaudiana leaves into the combined extracts enhanced the total phenolic content of *H. sabdariffa* in a single preparation, which may potentially augment the antidiabetic activity of the mixture.

Consistent with the total phenol findings, the *S. rebaudiana* extract contains the highest total flavonoid content. Figure 2b depicts the total flavonoid content in each aqueous extract of *H. sabdariffa, S. rebaudiana,* and their combination. The highest total flavonoid content was found in the S. rebaudiana leaf extract, measuring 6.987 ± 0.001 mg GAE/g extract. This was followed by the combination extract, which contained 4.791 ± 0.002 mg GAE/g extract. The lowest total flavonoid content was observed in the extract of *H. sabdariffa,* with a value of 3.099 ± 0.001 mg GAE/g extract.

The antidiabetic activity of a combination of H. *sabdariffa* and *S. rebaudiana* in a 3:1 ratio was investigated for its effect on reducing blood glucose levels in diabetic rats. Table 2 and Figure 3 present the results of blood glucose level measurements in each test group before and after treatment. The ANOVA test in this study was conducted with a confidence level of 95%. Based on the ANOVA test results, the statistical analysis confirmed significant differences in blood glucose levels among the treatment group.

The mean relative blood glucose levels (%) across different test groups showed distinct patterns throughout the treatment. Day 0 of each group was measured as baseline blood glucose level (100%). By Day 1, after alloxan induction (235 mg/kg BW), a marked increase in blood glucose levels was observed in all groups except the normal control group, which remained stable. The negative control group significantly increased to 338.35%, while test groups I and II showed even higher levels at 466.04% and 470.40%, respectively. The positive control group recorded 359.50%, significantly lower than test groups I and II. By day 7, the treatment groups' blood glucose levels began to decline. By day 14, continued improvement was observed, with test group II (311.98%) and the positive control group (197.10%) maintaining significantly lower glucose levels than the negative control group (487.39%, $p<0.05$). These results suggest that the interventions were effective in reducing blood glucose levels

Table 1. Extraction Results

Sample	Dried Plants weight (gram)	Extract weight (gram)	Extract yield (%)
H. sabdariffa	12	5.51	45.92
S. rebaudiana	12	3.79	31.58
Combination of H. sabdariffa and S. rebaudiana	480	166.72	34.73

A B C

Figure 1. Freeze-dried water extract (A) *H. sabdariffa* (B) *S. rebaudiana* and (C) Combination of *H. sabdariffa* and *S. rebaudiana*

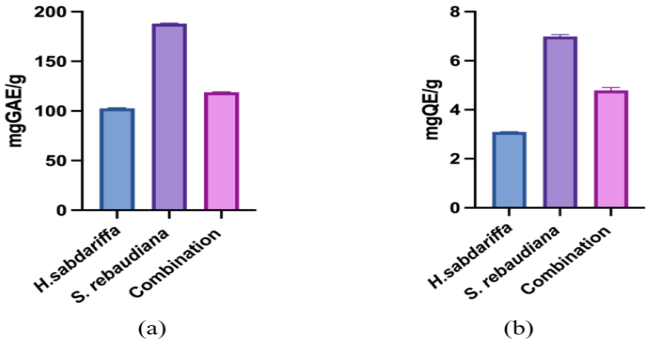

Figure 2. (a) Total Phenolic Content and (b) Total Flavonoid Content of H. sabdariffa extract, S. rebaudiana extract, and their combination. Bars are expressed as mean ± SD (n = 3).

4. Discussion

The findings of this study demonstrate the significant antidiabetic properties of the combination of *H. sabdariffa* and *S. rebaudiana* extracts in a 3:1 ratio. The hypoglycemic effect was evident from the substantial reduction in blood glucose levels in the diabetic test group, as observed over the 14-day treatment period. These results are consistent with previous studies reporting the individual antidiabetic effects of *H. sabdariffa* and *S. rebaudiana.*[28–30] Additionally, this study highlights the enhanced efficacy of their combination, supporting the hypothesis that polyherbal formulations can provide synergistic benefits in diabetes management. The significant reductions observed in blood glucose levels indicate the potential of this combination as a complementary therapeutic strategy for glycemic control.

The observed hypoglycemic effect may be attributed to the synergistic action of *H. sabdariffa* and *S. rebaudiana*. *H. sabdariffa* is known for its polyphenolic compounds, which exhibit antioxidant and glucose-lowering properties.[30–32] Moreover, *S. rebaudiana* contains bioactive compounds like stevioside, which enhance insulin sensitivity and regulate glucose metabolism.[29,33] The freeze-dried extracts likely preserved these bioactive compounds, maximizing their therapeutic potential.

Phenolic compounds have been extensively documented for their potent antioxidant properties, which are instrumental in mitigating oxidative stress in the body. These compounds exert their effects primarily by neutralizing reactive oxygen species (ROS), thereby preventing the oxidative damage that can compromise cellular integrity. In the context of diabetes mellitus, oxidative stress plays a critical role in the pathogenesis of the disease, particularly through the destruction of insulin-producing pancreatic beta cells.34–36 Therefore, increasing total phenolic levels through the combination of *H. sabdariffa* and S. rebaudiana extracts may offer enhanced protection to pancreatic cells, aiding in the maintenance of insulin production and control of blood sugar levels. This combination may also benefit through synergistic mechanisms, improving insulin sensitivity and cell glucose uptake.[37,38]

Several studies report that flavonoids can reduce blood glucose levels due to their antioxidant properties, which protect β-cells from damage and enhance insulin sensitivity.[39–41] Another antidiabetic mechanism of flavonoids is their ability to attenuate the activity of GLUT2, (Glucose Transporter type 2), the principal transporter responsible for intestinal glucose uptake. By inhibiting GLUT 2, flavonoids decrease blood glucose levels. This inhibition decreases the absorption of dietary glucose, thereby contributing to lower postprandial blood glucose levels. Consequently, the modulation of GLUT2 by flavonoids underscores their therapeutic potential in the management of

Table 2. (a) Results of mean relative blood glucose levels (%) in each test group after the treatment (n=6).

Day-	Normal Control Group	Negative Control Group	Test Group I	Test Group II	Positive Control Group
0	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
1	99.90 ± 8.09	338.35 ± 81.87	466.04 ± 156.02	470.40 ± 210.28	359.50 ± 93.63*
7	102.08±12.84	471.76 ± 69.56	433.27 ± 128.26	378.10 ± 57.45*	257.39 ± 63.48*
14	104.87± 11.61	487.39 ± 78.16	400.95 ± 122.38	311.98 ± 38.52*	197.10 ± 72.23*

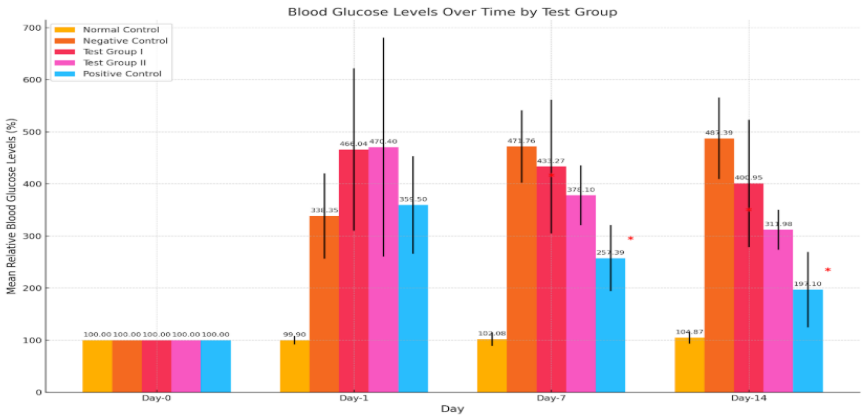

Figure 3. Mean relative blood glucose levels (%) in each group. The data were presented as the mean ± SD (n = 6). *p<0.05 shows a significant blood glucose level reduction difference compared to the negative control group.

hyperglycemia.[42] Additionally, flavonoids can inhibit phosphodiesterase, leading to an increase in cAMP in pancreatic beta cells. The elevated cAMP levels stimulate the release of protein kinase, enhancing insulin secretion, thereby increasing insulin production and reducing blood glucose levels.[42]

Finally, while the combination of *H. sabdariffa* and *S. rebaudiana* extracts shows promising potential for diabetes management, this study has several limitations. The sample size was limited, and the study was conducted exclusively on diabetic Wistar rats, which may not fully reflect the complex pathophysiology of diabetes in humans. Additionally, the 14-day study duration does not allow for assessing long-term efficacy and safety. The underlying mechanisms contributing to the synergistic effect of the extracts were not explored at the molecular level, leaving room for further mechanistic investigation.

5. Conclusion

This study demonstrates that combining *H. sabdariffa* and *S. rebaudiana* in a 3:1 ratio yields a freeze dried water extract that can effectively reduce blood glucose levels in alloxan nduced diabetic rats over a 14 day treatment period. Notably, the combination extract exhibited higher phenolic and flavonoid levels than *H. sabdariffa* alone, indicating the potential for augmented antidiabetic activity through synergistic effects.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

Acknowledgement

This study is funded by the Universitas Padjadjaran Internal Research Grant awarded to RMF.

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