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RESEARCH ARTICLE

# Quantitative analysis of flavonoids and phenolics extracted from *Diplazium esculentum* (Retz.) Sw. for their anti-hyperglycemic potential

Silvera Devi, Tiara Mulyani Rambah, Mhd. Muslim Syaifullah, Mukhlis, Itnawita, Rudi Hendra
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Riau, Indonesia

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**Correspondence**
Rudi Hendra
Department of Chemistry
Faculty of Mathematics and Natural Sciences
Universitas Riau
*Rudi.hendra@lecturer.unri.ac.id*

## Abstract

**Background:** Hyperglycemia arises from excessive blood glucose due to starch hydrolysis by α-amylase and α-glucosidase. Drugs like acarbose inhibit these enzymes to manage hyperglycemia, but long-term use poses risks. Fern species *Diplazium esculentum* metabolites show promise as acarbose alternatives. **Methods:** Sequential extraction of secondary metabolites from *D. esculentum* involved solvents in successive stages: hexane, ethyl acetate, and water. Subsequently, an assessment of phenolic and flavonoid concentrations within the ethyl acetate and aqueous extracts was conducted. Furthermore, the investigation encompassed the evaluation of % inhibition and $IC_{50}$ values pertaining to the activities of α-amylase and α-glucosidase. **Results:** The findings indicated that the flavonoid concentrations in the ethyl acetate and aqueous extracts were 146.90 ± 27.20 µg quercetin equivalent per gram of fresh weight (eq./g FW) and 101.95 ± 40.62 µg quercetin eq./g FW, respectively. In terms of total phenolic content, the ethyl acetate extract contained 121.25 ± 8.29 µg of gallic acid eq./g FW, whereas the aqueous extract had 109.90 ± 7.22 µg gallic acid eq./g FW. The $IC_{50}$ values demonstrated that the aqueous extract had moderate inhibitory effects on α-amylase with a value of 108.05 µg/mL, while acarbose exhibited very strong inhibition with a value of 3.12. **Conclusion:** Ferns are relatively ineffective in treating hyperglycemia.

## Introduction

Diabetes mellitus (DM), also known as hyperglycemia, is a major global health concern. According to the International Diabetes Federation in 2019, Indonesia is expected to see an increase in diabetes cases, with projections reaching 13.7 million by 2030, up from 10.7 million in 2019. The pathology of hyperglycemia is intricately linked to an increase in blood glucose levels that exceeds the normal physiological limits (Ardiany *et al*., 2021). Enzymatic activities, particularly α-amylase and α-glucosidase in the small intestine, are critical in glucose metabolism. These enzymes hydrolyse dietary starch to produce glucose, which enters the bloodstream (Ćorković *et al*., 2022). Current therapeutic strategies, such as acarbose administration, have been developed to inhibit these enzymes, thereby moderating the rise in blood glucose levels (Kaur *et al*., 2021).

Among the numerous natural resources studied for therapeutic properties, ferns, particularly the species *Diplazium esculentum*, which grows in humid tropical regions, have received attention. *D. esculentum*, colloquially known as paku sayur in Indonesia, serves both dietary and medicinal purposes. Historically, this plant has been used in traditional medicine to treat a variety of ailments ranging from respiratory disorders like asthma and coughs to metabolic and inflammatory conditions, like diabetes and rheumatism (Cao *et al*., 2017). Recent pharmacological studies have given its traditional applications a scientific twist. Chai *et al*. (2015) identified *D. esculentum*'s α-glucosidase inhibitory properties, revealing an $IC_{50}$ value of 6.85 ± 0.08 µg/mL. This potency outperformed even well-

known inhibitors like myricetin. Semwal *et al*. (2021) discovered a diverse profile of secondary metabolites in *D. esculentum*, which demonstrated significant inhibition of both α-amylase and α-glucosidase activities.

This study reports on the extraction of total phenolic and flavonoid content from polar fractions, followed by α-amylase and α-glucosidase activities within the *Diplazium esculentum* (Retz.) Sw. species.

## Methods

*Diplazium esculentum* (Retz.) Sw. was collected in Pekanbaru, Indonesia (0.539166, 101.448843) (Figure 1). The species was identified by the Head of the Botany Laboratory in the Department of Biology at Riau University. The accepted name of the plant species was verified on World Flora Online. The samples were freeze-dried, ground into a fine powder, and then stored in a refrigerator until further analysis was conducted. Phytochemical analysis was performed to determine the content of secondary metabolites from the species.

**Figure 1: *Diplazium esculentum* (Retz.) Sw**

### *Extraction*

Approximately 500 g of the sample was extracted using the maceration method with methanol for 24 hours. This process was repeated three times until the maceration no longer yielded a green extract. The crude extract was then concentrated using a rotary evaporator. Subsequently, it was partitioned with n-hexane and ethyl acetate to produce n-hexane, ethyl acetate, and water extracts (Hendra *et al*., 2020; Khodijah *et al*., 2023). Following this, the concentrations of flavonoids and phenolics in each extract were determined. Additionally, inhibition and $IC_{50}$ tests for the α-amylase and α-glucosidase enzymes were conducted.

### *Determination of total flavonoid content*

The total flavonoid content was determined using a modified colorimetric method involving aluminum chloride ($AlCl_3$). To each 100 µL of the extract (both ethyl acetate and water extracts), 1 mL of 5% $NaNO_2$ and 1 mL of 10% $AlCl_3$ were added. The solution was incubated for 30 minutes. Subsequently, 1 mL of 4% NaOH was introduced, followed by another 40 minute incubation. Absorbance was then measured at λ 510 nm, with quercetin serving as the standard (Almurdani *et al*., 2020).

### *Determination of total phenolic content*

The total phenolic content of the extract was quantified using a modified method with the Folin-Ciocalteu reagent. For each 100 µL of the extract (both ethyl acetate and water), 1 mL of 7% $Na_2CO_3$ and 1 mL of Folin-Ciocalteu reagent were added. After a 30 minute incubation, the absorbance was measured at λ 765 nm. Gallic acid served as the standard (Almurdani *et al*., 2020).

### *In vitro α-amylase inhibition*

The inhibition test for the $\alpha$-amylase enzyme was conducted based on a method involving a starch substrate and the 3,5-dinitrosalicylic acid (DNS) reagent, as described by Santoso *et al* (2022). In this method, the DNS reagent reacts with the reducing ends of broken-down starch chains, resulting in a coloured product. The intensity of this colour, indicative of the enzymatic activity, was quantified by measuring its absorbance using a UV-Vis spectrophotometer at λ 540 nm.

### *In vitro α-glucosidase inhibition*

The inhibition test for the $\alpha$-glucosidase enzyme was conducted using the substrate p-nitrophenyl-$\alpha$-D-glucopyranoside (p-NPG), as detailed by Khodijah *et al*. (2022) and El Ridhasya *et al* (2020). In this assay, the $\alpha$-glucosidase enzyme acts on p-NPG, which is initially colourless, converting it to p-nitrophenyl that exhibits a yellow hue. The intensity of this resultant yellow colour, which reflects the enzymatic activity, was quantified by measuring its absorbance with a microplate reader set to λ 405 nm.

### *Data analysis*

Total levels of flavonoids and phenolics were calculated using the linear line equation y = ax and the quercetin and gallic acid calibration curves for flavonoids and gallic acid for phenolics, respectively. Using the formula's absorbance value, calculate the percent

inhibition of the extract against α-amylase and α-glucosidase:

$$\% \text{ Inhibition} = \frac{(B_1 - B_0) - (S_1 - S_0)}{B_1 - B_0} \text{ x } 100\ \%$$

Where:

($B_1$-$B_0$) = Absorbance does not contain sample

($S_1$-$S_0$) = Absorbance of sample

The $IC_{50}$ (or inhibitory concentration of 50%) value of a sample is the value at which the activity of the α-amylase and α-glucosidase enzymes is inhibited by 50%. The ln values of several extract concentrations and % inhibition are plotted on the X (Ln concentration) and Y (% inhibition) axes.

## Results

The fern sample was taxonomically identified as the species *Diplazium esculentum* (Retz.) Sw. The data is presented in Table I and Figure 1. Results from the phytochemical tests are also available in Table II.

**Table I: Taxonomic identification results**

| Kingdom | Plantae |
|---|---|
| Phylum | Pteridophytes |
| Class | Polypodiopsida |
| Order | Polypodiales |
| Family | Athyriaceae |
| Genus | Diplazium |
| Species | ***Diplazium esculentum* (Retz.) Sw** |

**Table II: Phytochemical from *Diplazium esculentum* (Retz.) Sw.**

| Secondary metabolites | Reagent | Reaction colour | Results |
|---|---|---|---|
| Alkaloids | Mayer Dragendorff | Yellow/orange | - |
| Terpenoids | Liebermann Burchard | Green | - |
| Steroids | | Green | + |
| Saponins | $H_2O$ | Foamy | + |
| Flavonoids | Mg-HCl | Green | + |
| Phenolics | $FeCl_3$ | Blackish-green | + |

***Total flavonoid and phenolic content***

The total flavonoid content in *D. esculentum* was represented as µg quercetin equivalent per gram of fresh weight (µg quercetin eq./g FW). Similarly, the total phenolic content of *D. esculentum* was given in terms of µg gallic acid equivalent per gram of fresh weight (µg gallic acid eq./g FW). The ethyl acetate extract had a flavonoid content of 146.90 ± 27.2 µg quercetin eq./g FW, whereas the water extract exhibited 101.95 ± 40.6 µg quercetin eq./g FW. Additionally, the ethyl acetate extract displayed a phenolic content of 121.25 ± 8.29 µg gallic acid eq./g FW, and the water extract contained 109.90 ± 7.22 µg gallic acid eq./g FW.

***In vitro α-amylase and α-glucosidase inhibitions***

The inhibitory activity of the extracts against the α-amylase and α-glucosidase enzymes is expressed as the sample's $IC_{50}$ value, with acarbose serving as the positive control. Table III displays the results of the inhibition test for each ethyl acetate extract, water, and acarbose.

**Table III: α-amylase and α-glucosidase inhibition of *Diplazium esculentum* (Retz.) Sw.**

| Sample | $IC_{50}$ (µg/mL) | |
|---|---|---|
| | α-amylase | α-glucosidase |
| Ethyl acetate extract | 238.54 | 160.53 |
| Water extract | 108.05 | >500 |
| Acarbose | 3.12 | 90.84 |

## Discussion

The taxonomic analysis of the fern is presented thoroughly in Table I. The species is identified as *D. esculentum* within the genus Diplazium. In a concurrent study, the phytochemical constituents of *D. esculentum* are listed in Table II. Notably, it contains a large number of secondary metabolites, including steroids, saponins, flavonoids, and phenolics. Cao *et al*. (2017) found in their study that the *D. esculentum* plant contains a variety of secondary metabolites, including alkaloids, flavonoids, phenols, saponins, steroids, and tannins.

*D. esculentum* is typically macerated in methanol for extraction. Methanol is admired for its universal solvent properties, which allow it to dissolve a wide range of secondary metabolites in plants, whether they are polar, non-polar, or semi-polar. Methanol's intrinsic hydroxyl group attracts polar components due to its hydrophilic nature, whereas its methyl group attracts non-polar plant constituents due to its hydrophobic nature (Iloki-Assanga *et al*., 2015). To protect the secondary metabolites, the macerate is concentrated using a rotary evaporator at 45°C. When the solvent has

been concentrated, it is allowed to evaporate at room temperature, yielding a concentrated macerate.

The macerate is then partitioned with n-hexane solvent. This procedure is critical in separating non-polar compounds while adhering to the scientific principle that similar compounds dissolve in each other. The concentrated substance is extracted again with ethyl acetate, yielding two distinct extracts: ethyl acetate and water. The ethyl acetate extract is concentrated using a rotary evaporator after it has been separated, yielding 14.3 g of viscous extract. In contrast, the aqueous extract was freeze-dried, yielding 10.6 g of dry extract.

Surprisingly, both the ethyl acetate and aqueous extracts are thought to contain flavonoids and phenolics. These compounds are well known for their ability to inhibit α-amylase and α-glucosidase activities. As a result, there is an effort to determine the total flavonoid and phenolic content of each extract, as well as their efficacy in inhibiting these activities.

The colorimetric aluminum chloride ($AlCl_3$) method is used to quantify total flavonoids, with quercetin serving as a control. According to Chang *et al*. (2002) this is due to its structural composition, specifically the keto group on the C-4 atom and hydroxyl groups on the C-3 and C-5 atoms. This method works on the basis of the formation of an $AlCl_3$ complex with these groups in flavones and flavonols. Furthermore, $AlCl_3$ interacts with and forms stable complexes with orthohydroxyl groups found in flavonoids' A or B rings.

Simultaneously, the total phenolic content is determined using the Folin-Ciocalteu method. This evaluation makes use of the oxidation-reduction reaction. Polyphenols have been shown to convert the Folin-Ciocalteu reagent (phosphomolybdic acid and phosphotungstic acid) into molybdenum-tungsten. When samples are introduced, tungstate molybdate, a component of the Folin-Ciocalteu reagent, oxidises them. As Rover *et al*. (2013) observed this causes a colour transition from yellow to blue. Gallic acid is the gold standard for this method due to its stability. In an alkaline medium, the inherent hydroxy groups in phenolic compounds interact with the Folin-Ciocalteu, converting them into phenolic ions. These ions, in turn, initiate a reduction reaction that results in the formation of a molybdenum-tungsten blue complex, the intensity of which can be measured using UV-Vis spectrophotometry.

The data reveals an intriguing distinction: the ethyl acetate extract contains more flavonoids than phenolics, whereas the aqueous extract exhibits the opposite trend. This disparity is due to polarity differences between phenolic compounds and flavonoids. Because it is semi-polar, ethyl acetate can attract both types of compounds. Water, on the other hand, attracts polar compounds because it is polar. Because phenolics have a higher polarity than flavonoids, they dissolve more easily in polar solvents such as water. Flavonoids, on the other hand, prefer semi-polar solvents such as ethyl acetate.

Figure 2 depicts the extracts' inhibitory potential. The ethyl acetate extract inhibits α-amylase and α-glucosidase activities by 52.2% and 50.8%, respectively. At a concentration of 250 µg/mL, the aqueous extract inhibits α-amylase at a higher rate of 58.5%, but α-glucosidase at a much lower rate of 11.2%. When compared to acarbose, a positive control, both extracts fall short. Acarbose inhibits α-amylase 83.8% and α-glucosidase 95.5%. Furthermore, when compared to Semwal *et al*. (2021), these extracts have a noticeably lower inhibitory capacity. This highlights the fact that the indigenous *D. esculentum* may not be an ideal substitute for hyperglycemia medications.

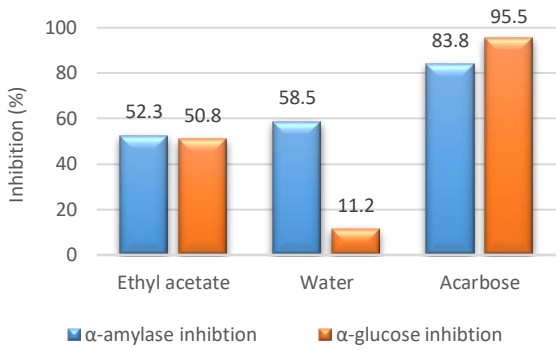


**Figure 2: Percentage of inhibition of extracts and acarbose against α-amylase and α-glucosidase at 250 µg/mL**

In order to investigate the complexities, α-amylase activity inhibition can be measured using the Dinitrosalicylic Acid (DNS) method. This method is based on the idea that α-amylase degrades starch into maltose. This maltose then converts DNS to 3-amino-5-nitrosalicylic acid, giving the product a distinct reddish-orange colour. The vibrancy of this colour, measured at 540 nm, correlates with the amount of maltose broken down (Papoutsis *et al*., 2021).

The activity of the α-glucosidase enzyme is measured using a colourless substrate called p-nitrophenyl—glucopyranoside (p-NPG). When the enzyme breaks it down, it turns into p-nitrophenol, a yellow substance (Papoutsis *et al*., 2021). Because of its role in regulating blood sugar in diabetics, acarbose emerges as a leading positive control. It works by competitively inhibiting the

α-glucosidase enzyme, mimicking the intermediate stage of the substrate (Rosak *et al*., 2012).

Table III graphically displays the $IC_{50}$ values for the extracts in relation to α-amylase and α-glucosidase activities, demonstrating their potency. The $IC_{50}$ value of the ethyl acetate extract for α-amylase is 238.54 µg/mL and for α-glucosidase is 160.53 µg/mL. In comparison, the aqueous extract has enzyme concentrations of 108.05 µg/mL and >500 µg/mL, respectively. When compared to acarbose, which has an $IC_{50}$ of 3.12 µg/mL for α-amylase and an incredible 90.84 µg/mL for α-glucosidase, the difference in efficacy between the extracts and acarbose becomes clear. This variation in potency can be explained by the different constituents found in each extract, which influence the overall inhibitory effect on the enzymes.

## Conclusion

The ethyl acetate extract contains more flavonoids and phenolic compounds than the aqueous extract. When comparing the $IC_{50}$ values of both extracts for α-amylase and α-glucosidase activities, the aqueous extract is significantly more potent for α-amylase but significantly less potent for α-glucosidase. In contrast, in terms of enzyme inhibition, acarbose outperforms both extracts, demonstrating its superior efficacy. The differences in inhibitory capacity between these extracts are likely attributable to their distinct chemical constituents, which modulate enzyme activity significantly.

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