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α-Glucosidase Inhibitory and Antioxidant Activities of Purified Fractions from Methanolic Extract of *Justicia gendarussa*

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ABSTRACT. *Justicia gendarussa* Burm. f. (gandarusa) is an indigenous Indonesian medicinal plant with potential antidiabetic properties. This study aimed to investigate the *α*-glucosidase inhibitory activity of bioactive compounds present in purified fractions derived from the methanolic extract of gandarusa through column chromatography. Dried leaf powder was sequentially extracted using *n*-hexane, ethyl acetate, and methanol. The methanolic extract was further fractionated by column chromatography employing an optimized eluent system (*n*-hexane:ethyl acetate, 6:4 v/v). The crude extract and eight purified fractions were concentrated and evaluated for antioxidant activity using thin-layer chromatography bioautography, as well as for in vitro *α*-glucosidase inhibitory activity. The results demonstrated that bioassay-guided fractionation produced fractions with enhanced antioxidant capacity and greater *α*-glucosidase inhibitory effects compared to the crude extract. Among all fractions, fraction 2 exhibited the strongest inhibitory activity, as indicated by its lowest IC_{50} value (99.34 ± 10.70 ppm). One-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) confirmed that its inhibitory effect was significantly different from those of the other fractions and the reference drug acarbose. These findings indicate that purified fractions of gandarusa demonstrate promising potential for further development as natural antidiabetic agents.

INTRODUCTION

Diabetes mellitus (DM) is a long-term metabolic disorder marked by persistent high blood sugar levels due to inadequate insulin secretion, ineffective insulin action, or a combination of both (Gaw *et al*., 2013). The International Diabetes Federation (IDF) reported that in 2021, approximately 537 million adults aged 20 – 79 years were living with diabetes. This number is expected to increase to 643 million by 2030 and 783 million by 2045. The growing prevalence of DM is strongly associated with unhealthy lifestyles, including poor dietary habits and physical inactivity, which contribute to obesity and insulin resistance. In Indonesia, the number of people with DM has risen significantly, from 19.5 million to 28.6 million in recent years (Magliano and Boyko, 2021). People with DM face a significant threat of experiencing both microvascular and macrovascular complications, which can severely impair quality of life. These challenges emphasize the urgency of searching for effective strategies to prevent and manage diabetes (Fajriyah *et al*., 2020; Wicaksono, 2015).

One of the common strategies for managing type 2 diabetes mellitus is to block enzymes that break down carbohydrates, particularly *α*-glucosidase. This enzyme catalyzes the conversion of carbohydrates into glucose in the small intestine. By inhibiting *α*-glucosidase, the absorption of glucose is slowed, thereby reducing postprandial hyperglycemia (Hossain *et al*., 2020). Clinically, *α*-glucosidase inhibitors, such as acarbose and miglitol, have been shown to be effective in controlling blood glucose levels (Zeng *et al*., 2016). However, some findings reported that synthetic drugs are associated with several effects, including undesirable gastrointestinal disturbances (NIDDK, 2021). This has spurred interest in exploring natural plant-based compounds that may offer similar therapeutic benefits with fewer adverse effects.

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In recent years, herbal remedies have gained increasing attention as alternative or complementary treatments for managing diabetes. *Justicia gendarussa* Burm. f. (gandarusa), a medicinal plant native to Indonesia, has traditionally been used for various therapeutic purposes, including as an antidiabetic treatment (Jain *et al*., 2024; Kavitha *et al*., 2016). Extracts from *J. gendarussa* leaves have demonstrated moderate antidiabetic and antioxidant activities, highlighting their potential as a natural source for diabetes therapy (Nurfachri, 2020; Wulansari *et al*., 2025). Kavitha *et al*. (2016) and Sarian *et al*. (2017) reported that the presence of flavonoids and phenolic acids may contribute to the pharmacological properties of plants. Furthermore, previous studies have shown that purification techniques, such as column chromatography, can enhance the biological activity of phytochemicals (Dwicesaria, 2023; Sukib and Kusmiyati, 2011).

In this study, the α-glucosidase inhibitory and antioxidant activities of purified fractions of *J. gendarussa* obtained through column chromatography were investigated. The extraction process involved sequential organic solvents to isolate a broad range of phytochemicals. Following purification, the antioxidant activity was evaluated by bioautographic thin-layer chromatography, whereas the α-glucosidase inhibitory activity was determined by spectrophotometric assay. The findings of this study provide insight into the possibility of using *J. gendarussa* as a natural antidiabetic agent, which may offer an alternative approach to conventional diabetes therapy with fewer adverse effects.

RESEARCH METHODS

The plant material (*Justicia gendarussa* Burm. f.) leaves were obtained from Biopharmaca Research Center, IPB University. The collected material was dried and processed into simplicia prior to extraction. The solvents used in this study included *n*-hexane, ethyl acetate, and methanol. Other materials comprised thin-layer chromatography (TLC) silica gel 60 F_{254} (Merck), cellulose powder (Merck), silica gel 60 (Merck, 70 – 230 mesh), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich), α-glucosidase enzyme (maltase, 100 U/mg, Sisco Research Laboratories), *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) (Sigma Aldrich), Na_2CO_3, phosphate buffer (0.1 M, pH 6.8), acarbose (Abcam), and trolox (Sigma-Aldrich). The instruments used in this research included a rotary evaporator (Labtech), semi-automatic TLC equipment consisting of a CAMAG Linomat V (linked to a nitrogen gas cylinder) and a Reprostar 3 documentation device operated using WinCATS software (version 1.2.3), a column chromatography setup, and a microplate spectrophotometer (Epoch, BioTek).

Determination of Moisture Content

The moisture content was analyzed using the gravimetric method (Vonna *et al*., 2021). Briefly, 2 g of powdered leaves were placed in a pre-weighed porcelain dish. The dish containing the sample was then dried in an oven at 105 °C for 3 hours to ensure complete removal of moisture. Following the drying process, the dish was allowed to cool in a desiccator to avoid moisture reabsorption from the air. After cooling, the dish was reweighed. The moisture content was subsequently determined using Equation 1.

$$\text{Moisture content} = \frac{\text{Initial weight (g)} - \text{Final weight (g)}}{\text{Initial weight (g)}} \times 100\% \quad (1)$$

Extraction

Powdered *J. gendarussa* leaves (80 mesh) were extracted through a multistage maceration method utilizing *n*-hexane, ethyl acetate, and methanol, as described by Prasetyo *et al*. (2022). A 10 g portion of the powdered leaves was immersed in 100 mL of *n*-hexane at ambient temperature for 24 hours. After filtration, the residue was successively remacerated with 100 mL of ethyl acetate and 100 mL of methanol, each for 24 hours. The methanol filtrate was concentrated under reduced pressure using a rotary evaporator (60 rpm, 40 °C). The extraction yield was calculated as the ratio of the dried extract weight to the initial sample weight. All extractions were performed in triplicate (n= 3) to ensure reproducibility, and the yield was determined using Equation 2.

$$\text{Yield extract} = \frac{\text{Dry extract weight (g)}}{\text{Initial weight (g)} \times (1 - \text{Moisture content})} \times 100\% \quad (2)$$

Optimization of Purification Solvent

Solvent optimization was performed using thin-layer chromatography (TLC) following the method of (Fauziah, 2023). A 10 µL sample was applied onto a TLC plate using a CAMAG Linomat V semiautomatic applicator at a spotting speed of 80 nL/s and a bandwidth of 8 mm. The mobile phase consisted of various

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combinations of *n*-hexane, ethyl acetate, and methanol. After development, the eluted spots were visualized under UV light at 254 nm and 366 nm. The migration distance of each spot was measured and the retention factor (R_f) was calculated using Equation 3.

$$\text{Retention factor } (Rf) = \frac{\text{Distance of compound (cm)}}{\text{Distance of solvent (cm)}} \quad (3)$$

Purification of Gandarusa Extract

The methanol extract of *J. gendarussa* leaves was purified using a gradient elution system consisting of various combinations of *n*-hexane, ethyl acetate, and methanol, following the methods of Syaefudin *et al*. (2022) and Dwicesaria (2023). A total 1.22 g of crude methanol extract was dissolved in methanol (5 mL) and mixed with 4.88 g of cellulose powder to obtain a homogeneous mixture, which was then evaporated under vacuum to remove the solvent.

The chromatography column (2.7 cm diameter, 30 cm height) was packed with silica gel as the stationary phase to a height of 16 cm using the dry packing method. The solvent system was gradually introduced while maintaining the outlet valve open and vacuum pressure at a flow rate of 1.22 ± 0.3 mL/s to prevent air bubbles and dry spots. Eight fractions were collected and subsequently concentrated using a rotary vacuum evaporator at 60 rpm and 40 °C. The yield of each fraction was calculated using Equation 4.

$$\text{Fraction yield} = \frac{\text{Final weight (g)}}{\text{Initial weight (g)}} \times 100\% \quad (4)$$

TLC-Bioautographic Assay

The TLC-bioautography method was employed to identify antioxidant compounds in the sample, with Trolox as the positive control based on Gu *et al*. (2009). The crude methanol extract and eight fractions obtained from the chromatography column were spotted onto TLC plates and developed using the selected mobile phase from the previous step, *n*-hexane:ethyl acetate (6:4, v/v). After separation, the plates were sprayed with a 0.04% (v/v) DPPH solution in methanol. As the plates were air-dried, yellow spots appeared against a purple background, indicating antioxidant-active compounds. The TLC plates were also examined under UV light at 254 and 366 nm to detect UV-active components.

α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was determined using a spectrophotometric assay, as described by Sancheti *et al*. (2009). The reaction setup consisted of a blank, standard (acarbose), and test samples, as shown in Table 1. Briefly, 10 µL of the test solution (crude extract, column chromatographic fractions, acarbose, or DMSO) was mixed with 50 µL of 0.1 M phosphate buffer (pH 6.8) and 25 µL of 10 mM *p*-NPG as the substrate. The reaction began with the addition of 25 µL of α-glucosidase solution (0.4 U/mL in 0.1 M phosphate buffer, pH 6.8). This was followed by a 30-minute incubation at 37 °C, and the reaction was stopped by introducing 100 µL of 0.2 M Na_2CO_3. Samples were tested at various concentrations (15.625, 31.25, 62.5, 125, 250, 400, 500, and 1000 ppm), and all assays were performed in triplicate (n= 3).

Table 1. Composition of test solutions for α-glucosidase inhibition assay.

Reagent	Blank (µL)		Standard (µL)		Sample (µL)	
	Negative Control	Blank	Negative Control	Standard	Negative Control	Sample
DMSO	10	10	-	-	-	-
Acarbose	-	-	10	10	-	-
Sample	-	-	-	-	10	10
Phosphate buffer	75	50	75	50	75	50
p-NPG	25	25	25	25	25	25
Enzyme	-	25	-	25	-	25
Incubate (37 °C; 30 minutes)						
Na_2CO_3	100	100	100	100	100	100
Total volume	210	210	210	210	210	210

The enzymatic hydrolysis of the substrate was monitored by measuring the released of *p*-nitrophenol in the reaction mixture at 410 nm using a microplate spectrophotometer. The sample concentrations that resulted in 50%

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inhibition of α-glucosidase activity was defined as the IC_{50} value. The percentage of enzyme inhibition was calculated using Equation 5.

$$\% \text{ Inhibition} = \left(\frac{\text{Abs. negative control} - \text{Abs. sample}}{\text{Abs. negative control}}\right) \times 100\% \quad (5)$$

Data Analysis

Quantitative data were expressed as mean ± standard deviation (SD) from triplicate measurements (n= 3). The percentage of α-glucosidase inhibition and antioxidant activity were calculated based on absorbance values obtained from the assays. IC_{50} values were determined using linear regression analysis in Microsoft Excel. Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS) version 29 for Windows. To identify significant group differences at a 95% confidence level, a one-way analysis of variance (ANOVA) was utilized, and Tukey's multiple range test was applied for post hoc comparisons when $p < 0.05$.

RESULTS AND DISCUSSION

Moisture Content and Yield of *J. gendarussa* Extract

The moisture content of powdered *J. gendarussa* leaves was determined to ensure quality and stability of the raw material, following the standards of the Indonesian Ministry of Health (2000). Excessive moisture can promote microbial growth, such as mold, which may reduce simplisia quality and alter its chemical composition (Vonna *et al*., 2021). The sample showed an average moisture content of 7.75 ± 0.002%, within the acceptable limit (≤ 10%) set by the Ministry of Health, confirming that the simplisia met the required quality standards for herbal materials.

The extraction yield indicates the efficiency of the multistage solvent extraction method in isolating secondary metabolites from *J. gendarussa*. In this study, multistage extraction was performed using *n*-hexane, ethyl acetate, and methanol was applied to separate bioactive compounds based on polarity. The *n*-hexane primarily extracts non-polar compounds such as steroids and terpenoids; ethyl acetate targets semi-polar compounds such as phenolics, terpenoids, and alkaloids; while methanol isolates polar compounds, including alkaloids, phenolics, and carotenoids (Edison *et al*., 2020). The crude methanolic extract showed a yield of 3.08 ± 0.37%, consistent with the 3.2% yield reported by Nurfachri (2020).

Eluent for Column Chromatography Purification

The selection of an appropriate eluent for column chromatography was optimized using TLC. Thirty solvent combinations with varying polarities were evaluated, including *n*-hexane:ethyl acetate, ethyl acetate:methanol, and *n*-hexane:methanol, with volume ratios ranging from 10:0 to 0:10 (v/v). The *n*-hexane:ethyl acetate mixture at a 6:4 (v/v) ratio proved to be the most effective eluent. This selection was based on the criteria outlined by Suminto *et al*. (2024), which include optimal R_f values (0.2 – 0.8), absence of overlapping or streaking spots, and clear separation of multiple distinct bands.

As shown in Figure 1 and Table 2, the *n*-hexane:ethyl acetate eluent (6:4, v/v) provided better separation of the methanolic extract than other ratios, producing clear bands with optimal R_f values of 0.72 at 254 nm and 0.79 at 366 nm (Koirewoa *et al*., 2012). In contrast, the 3:7 ratio resulted in band tailing with R_f values < 0.8, indicating poor resolution. Meanwhile, the 5:5 and 6:4 ratios showed excessive compound migration (R_f > 0.8), suggesting suboptimal separation (Wulandari, 2011).

Table 2. R_f values of *J. gendarussa* extract components separated by different *n*-hexane:ethyl acetate solvent systems in TLC analysis.

Sample	Solvent system (v/v)	254 nm		366 nm	
		No. bands	R_f values	No. bands	R_f values
a	7:3	4	0.56; 0.42; 0.27; 0.20	7	0.69; 0.64; 0.54; 0.42; 0.39; 0.28; 0.21
b	6:4	5	0.72; 0.64; 0.49; 0.42; 0.33	8	0.79; 0.76; 0.72; 0.59; 0.49; 0.43; 0.34; 0.26
c	5:5	2	0.93; 0.88	8	0.94; 0.89; 0.85; 0.77; 0.68; 0.40; 0.23; 0.13
d	4:6	4	0.90; 0.85; 0.75; 0.65	7	0.91; 0.87; 0.84; 0.75; 0.47; 0.31; 0.19

These findings align with previous reports emphasizing the effectiveness of *n*-hexane:ethyl acetate systems for separating nonpolar to semipolar phytochemicals (Chaudhari *et al*., 2012). The optimal resolution of bands using this solvent system underscores its suitability for fractionating complex plant extracts such as *J. gendarussa*,

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which is known to contain a wide range of bioactive secondary metabolites such as alkaloids (justridisamide A-D, benzoides A-D, lupeol), flavonoids (apigenin, quercetin, kaempferol, naringenin, apigenin), and saponins (gendarussin B-D) (Jain *et al*., 2024).

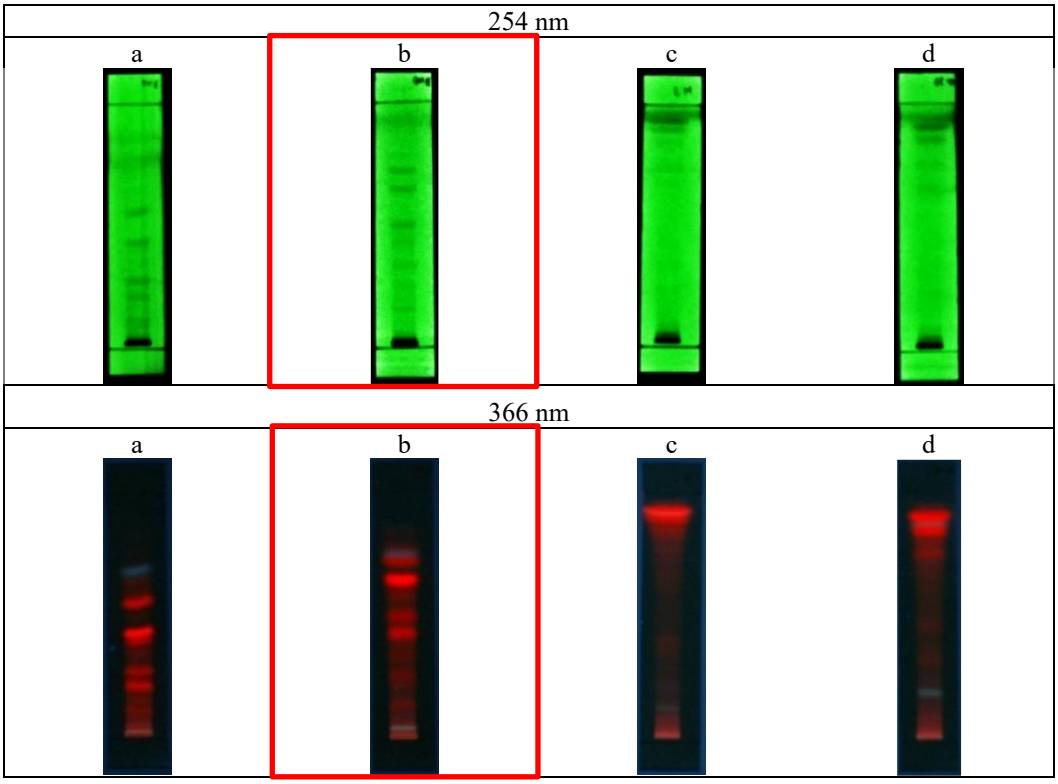


Figure 1. TLC profiles of *J. gendarussa* methanol extract visualized under UV light at 254 nm and 366 nm using different *n*-hexane:ethyl acetate solvent systems: (a) 7:3, (b) 6:4, (c) 5:5, and (d) 4:6 (v/v).

Purification of *J. gendarussa* Extract

The crude methanol extract was purified using column chromatography assisted by a vacuum pump, representing a modification of the conventional gravity-based method. This approach is particularly effective for processing large sample volumes, as it enhances separation efficiency and significantly reduces processing time (Heliawati, 2018). Elution with *n*-hexane:ethyl acetate (6:4, v/v) resulted in eight distinct fractions, within yields range of 1.80 – 36.33%, reflecting considerable variation in compound solubility and chemical composition (Figure 2). These differences in yield are likely due to heterogeneous nature of plant secondary metabolites and variations in the volume of eluent applied during the process (Lalopua, 2020).

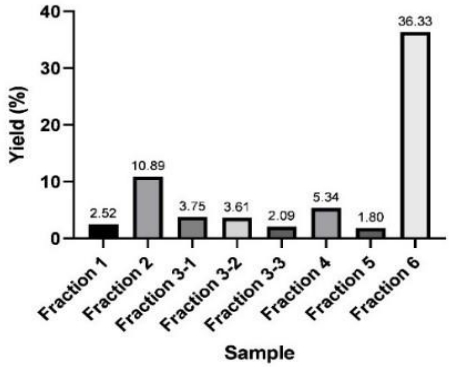


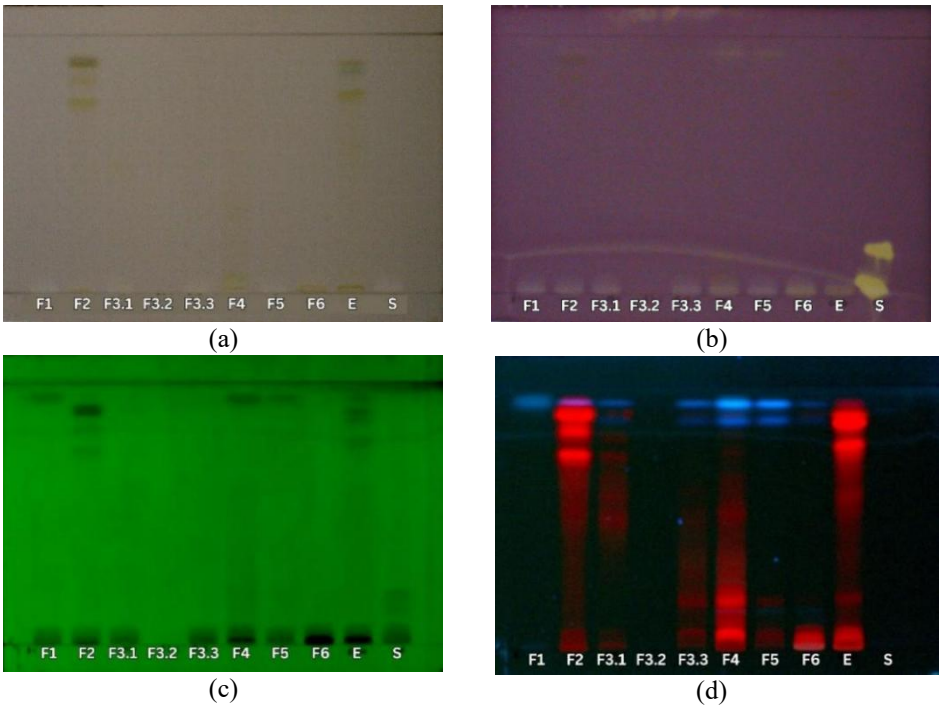
Figure 2. Yield distribution of fractions obtained from vacuum-assisted column chromatography purification of *J. gendarussa* methanol extract using *n*-hexane:ethyl acetate (6:4, v/v) as mobile phase.

TLC-Bioautographic Assay for Antioxidant Activity

The antioxidant activity of *J. gendarussa* was qualitatively evaluated using TLC-direct bioautography with 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, which produces intense coloration and enhances detection

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sensitivity (Sherma, 2018). After development with the optimized mobile phase, the TLC plates were immersed in DPPH solution. Antioxidant compounds were visualized as yellowish-white bands against a purple background (Gu *et al*., 2009).



Note:
F1-F6: fractions 1-6
F3.1-F3.3: subfractions 3.1-3.3
E: crude extract
S: Trolox standard

Figure 3. TLC profiles of *J. gendarussa* crude extract, purified fractions, and Trolox reference under various detection conditions: (a) visible light, (b) visible light after DPPH staining in methanol, (c) UV light at 254 nm, and (d) UV light at 366 nm.

As shown in Figure 3, all fractions except fraction 3.2 exhibited antioxidant activity, indicated by yellowish bands in the DPPH bioautography assay (Figure 3b), although with lower intensity than the Trolox standard, suggesting moderate activity (Setiawan *et al*., 2018). Under UV 366 nm (Figure 3d), there are approximately three distinct fluorescence colors observed. Red fluorescence is commonly associated with chlorophyll-related compounds due to their porphyrin structure (Bladt and Wagner, 2009; Zych and Pyka-Pająk, 2025). Blue fluorescent bands observed at higher R_f values may be attributed to phenolic-type compounds, while yellow–orange fluorescent bands at lower R_f values are often associated with flavonoid-like constituents or certain anthraquinones, which exhibit such emission due to their conjugated aromatic structures (Bladt and Wagner, 2009; Harborne, 1987). These assignments are tentative, as TLC characteristics do not provide definitive compound identification, but are consistent with previous reports on the phytochemical composition of *J. gendarussa* (Jain *et al*., 2024; Pal and Rahaman, 2015).

Overall, these findings highlight the utility of TLC-bioautography for rapid antioxidant screening and preliminary phytochemical profiling of medicinal plant extracts. This technique offers a practical and efficient approach for the preliminary screening of antioxidant compounds in complex plant extracts by simultaneously enabling compound separation and activity detection. Its simplicity and minimal reliance on advanced instrumentation make it especially valuable for the initial identification of bioactive constituents in natural products (Gaurav *et al*., 2020; Gu *et al*., 2009).

Inhibition of *α*-Glucosidase Activity

The *α*-glucosidase inhibition activity was quantitatively evaluated using *p*-nitrophenyl-*α*-D-glucopyranoside (*p*-NPG) as the substrate. This assay measures the enzymatic hydrolysis of *p*-NPG into yellow-colored *p*-nitrophenol, with inhibition indicated by a reduced absorbance at 400 – 410 nm (Fadhli *et al*., 2021). Initial screening of the crude extract and eight purified fractions revealed that only fractions 2 and 3.1 exhibited >50% inhibition at the tested concentrations, allowing reliable IC_{50} determination. The remaining fractions failed to reach this threshold and were excluded from further analysis. These findings align with previous reports indicating that purification enhances the *α*-glucosidase inhibitory activity of *J. gendarussa* compared to the crude extract (Sukib

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and Kusmiyati, 2011). Fraction 2 showed the strongest activity (IC_{50}= 99.34 ± 10.70 ppm), followed by fraction 3.1 (IC_{50}= 371.13 ± 25.65 ppm) (Figure 4). Statistical analysis (one-way ANOVA followed by Tukey's test, $p < 0.05$) confirmed significant differences between the active fractions.

Acarbose, a competitive inhibitor that delays intestinal glucose absorption, was used as a positive control. The IC_{50} value of acarbose in this study (1972.17 ± 140.14 ppm) is consistent with its mechanism of action, in which the valienamine-dideoxyglucose nitrogen bond resists enzymatic hydrolysis (Li *et al*., 2018), resulting in slow dissociation kinetics from *α*-glucosidase (Tsunoda *et al*., 2022). Variations in reported IC_{50} values for acarbose (996.02 – 1067.67 ppm) may result from differences in experimental conditions, such as enzyme sources, substrate concentrations, and incubation parameters (Dirir *et al*., 2022; Li *et al*., 2022; Rozenan *et al*., 2021).

The correlation between antioxidant activity and *α*-glucosidase inhibition observed in this study may be explained by the presence phenolic compounds, including flavonoid-like constituents, in *J. gendarussa* (Jain *et al*., 2024). Fraction 2, which exhibited distinct antioxidant bands in the TLC-bioautographic assay, also showed the lowest IC_{50} value (99.34 ± 10.70 ppm), indicating the strongest inhibitory activity. The presence of flavonoid-like compounds is tentatively suggested based on their fluorescence under UV 366 nm and DPPH reactivity, which are commonly associated with phenolic constituents in TLC analysis (Bladt and Wagner, 2009; Harborne, 1987). However, these observations do not constitute definitive compound identification. In earlier studies, it has been found that phenolic compounds, especially flavonoids, have shown dual functionality due to their hydroxyl groups, which enable free radical scavenging through hydrogen donation and α-glucosidase inhibition through hydrogen bonding and hydrophobic interactions at the enzyme active site (Sarian *et al*., 2017; Xu, 2010). Overall, the combined use of TLC-bioautography and enzymatic assays suggests that the active fractions may contain phenolic constituents with potential antidiabetic activity (Gu *et al*., 2009; Prastika, 2020; Xu, 2010).

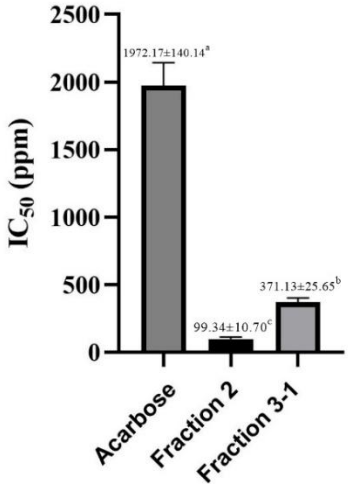


Figure 4. IC_{50} values of active *J. gendarussa* fractions (showing > 50% inhibition) with the standard acarbose. Different letters indicate significant differences (Tukey's test, $p < 0.05$).

CONCLUSION

Purification of *Justicia gendarussa* methanolic leaf extract using column chromatography yielded fractions with significant biological activity. Fraction 2 demonstrated the strongest *α*-glucosidase inhibitory activity, indicating significant antidiabetic potential. The antioxidant properties of the active fractions were further validated through TLC-bioautography, as shown by distinct DPPH radical scavenging activity. These findings highlight the potential of *J. gendarussa* leaf fractions as promising candidates for the development of antidiabetic herbal therapeutics.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in this research.

AUTHOR CONTRIBUTION

DND: Data Analysis, Investigation, Manuscript Drafting; SF: Conceptualization, Supervision; SS: Conceptualization, Supervision, Funding acquisition, Manuscript Review.

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DECLARATION OF GENERATIVE AI

During the preparation of this manuscript, the authors used Paperpal to refine the language and improve readability. Following the use of this tool, the authors thoroughly reviewed and revised the content as needed and take full responsibility for the content of the published article.

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