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Evaluation of antidiabetic and antibacterial activities of *Horsfieldia macrothyrsa* leaves extracts

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ABSTRACT

Cold maceration was used to extracting *Horsfieldia macrothyrsa* leaves with high lignan and alkaloid contents along with antidiabetic properties from methanol. This work examined the antidiabetic and antibacterial activities of methanol extract and other fractions from *H. macrothyrsa* leaves, utilizing α-glucosidase and antibacterial inhibition methods against some bacterial targets including *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus subtilis*. This study found that the methanol extract (HMM) and ethyl acetate fraction of *H. macrothyrsa* (HME) leaves have antidiabetic potential, with IC_{50} values of 7.318 ± 0.57 and 6.694 ± 0.44 µg/mL, respectively. Interestingly, only the ethyl acetate (HME) fraction exhibited antibacterial activity against *E. coli* and *S. aureus* with MIC/MBC values at concentrations of 0.31/0.31 mg/L and 0.63/0.63 mg/L, respectively. As a result, the HME fraction was separated into 13 fractions using a gravity column chromatography method. The results revealed that the HME column chromatography fractions, namely F11 and F12, had the highest antidiabetic values. The best inhibitory power against all bacteria tested has been shown by F2 and F3, which have an inhibitory capacity of 8.67± 0.47 mm against *S. aureus* and 9.00 ± 0.00 mm *B. subtilis* bacteria on with MIC/MB values of 0.15/>0.15 mg/L. The HME fraction contained several bioactive compouds including the 7-hydroxy-3-methoxyflavone-2'-carboxylic acid and *8-acetyl harpagide*, whereas the alkaloid group h*ad 4-[(3R)-3-[Bis((2R)-2-hydroxy-2-phenylethyl)amino]]benzamide* and 1,1–dimethylethyl 4-(4-aminobutyl)-1 piperidinecarboxylate, were also detected these compounds were tentatively identified using liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS). These findings suggest that *H. macrothyrsa* leaves may have dual advantages as a reducing agent suggesting potential antidiabetic activity of avoiding diabetes (under 25 µg/mL) and against bacteria *S. aureus* and *E. coli*.

Keywords: α-glucosidase; antibacterial; *Horsfieldia macrothyrsa*

1. INTRODUCTION

According to the World Health Organization (WHO, 2018), infectious diseases caused by harmful bacteria remain among the most detrimental to human health. Finding novel active compounds is the primary goal, as these hazardous bacteria have developed resistance to traditional and synthetic antibiotics. Mortality rates have increased as more bacterial species become resistant to various antibacterial treatments (Egharevba et al., 2019).

Furthermore, antibiotic misuse has accelerated the emergence of microorganisms resistant to numerous medications (Ventola, 2015). In recent decades, one of the most pressing concerns among researchers worldwide has been the progressive increase in drug resistance to conventional antibiotics (Wali et al., 2020). Additionally, diabetes mellitus is characterized as a chronic, non-communicable metabolic disorder marked by hyperglycaemia resulting from decreased insulin production and resistance to insulin's effects on specific organs. Diabetes affects about 422 million people globally due to a combination of environmental factors, including high-energy diets, sedentary lifestyles, and genetic predisposition (Prabha et al., 2018).

Horsfieldia is a prominent genus of woody plants in the *Myristicaceae* family, distributed throughout Southeast Asia. *Horsfieldia* species have been employed in traditional medicine to treat various conditions, including antidiabetic properties from *H. macrobotyrs* (Ramadhan & Phuwapraisirisan, 2015) and *H. motleyi* (Ramadhan et al., 2018), as well as antibacterial properties from *H. helwigii* (Khan et al., 2001) and *H. spicata* (Megawati et al., 2023). Traditionally, this plant has been used to flavor food, but several reports indicate potential medicinal properties. Notably, recent phytochemical investigations have revealed that *H. macrothyrsa* extracts demonstrate antidiabetic efficacy in the hexane fraction (Megawati et al., 2023). *H. macrothyrsa* is endemic to Sumatra and has not been subjected to any documented chemical analysis or medicinal applications. Further investigation into the chemical constituents of the leaf extracts is essential to elucidate the secondary metabolism of *H. macrothyrsa* leaves, which remain unexplored.

2. METERIALS AND METHODS

H. macrothyrsa leaves were collected from cultivated plants in Cibinong, West Java, Indonesia [GPS location: 6°29'20.1"S 106°51'14.6"E]. A voucher specimen (No.04-SHU-12-2023) was deposited at the Traditional Medicine Raw Material Standardization Laboratory, National Research and Innovation Agency (BRIN). The bacterial strains used in this study including Gram-positive (Staphylococcus aureus (FNCC-0047), Bacillus subtilis (FNCC-0059)) and Gram-negative (Escherichia coli (FNCC-0195), and Pseudomonas aeruginosa (ATCC 9027)) bacteria. All bacterial strains were obtained from the culture collection of the Food and Nutrition Study Program, Gadjah Mada University, Indonesia. The Materials used included α-glucosidase (0.124 unit/mL), 0.1 M phosphate buffer (pH 7.0), DMSO, 5 mM p-NPG (Wako, Japan), 0.1 M Na2CO3, n-hexane, ethyl acetate, buthanol, and methanol.

The instrumentation used in this study included an extractor, a rotary evaporator (Buchi R214-Switzerland), micropipettes (Humapette, Germany), ovens, and a UV-Vis spectrometer (Hitachi U-2000, Series No. 0372-026).

2.1 Extraction and isolation

H. macrothyrsa leaves were sorted, washed with distilled water, and dried using a blower oven at 50°C. The dried leaves were processed in a grinder to obtain dried and powdered material. Subsequently, 650 g of powder were macerated in 5 L of methanol and evaporated using a rotary evaporator (Buchi R214-Switzerland) to yield 19.217% of methanol crude extract (HMM). The corresponding extract (100 g) was partitioned with n-hexane (HMH) 13.110 g, ethyl acetate (HME) 20.831 g, and *n*-butanol (HMB) 18.901 g. Potential fractions were obtained by eluting 15 g of the selected HME fraction using a gradient solvent system of n-hexane: HME, followed by column chromatography on a silica gel stationary phase (Riyadi et al., 2024). Thirteen fractions were generated when the column data were combined.

2.2 Antibacterial activity by disc diffusion method

The disc diffusion method was implemented with minor modifications, following the protocol described by Nafis et al. (2021) in modification (Megawati et al., 2023). Sterile nutrient agar (NA) plates were prepared and inoculated with bacterial suspension (10^8 CFU/mL). Ten microliters of samples (4 mg/mL diluted in 1% DMSO) were applied to sterile discs (6 mm) and allowed to dry. After preparing the NA medium, the sample containing discs were placed flat on a surface and incubated for 24 at 37°C. The inhibitions zone diameter around the tested discs was measured using a calliper to determine antibacterial activity. Tetracycline (4 mg/mL) served as a positive control, while 1% DMSO was used as a negative control.

2.2.1 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The NCCLS-described and modified Megawati et al. (2023) approach was used to determine MIC and MBC. To create the suspension, an overnight culture of the tested bacteria (0.5 McFarland standard, equivalent to 10^8 CFU/mL) was applied. A 96-well microliter plate was then inoculated with Mueller Hinton broth (MHB) to achieve a final volume of 200 µL and a final bacterial concentration of 10^6 CFU/mL. The extracts were subsequently serially diluted (in 1% DMSO) alongside positive controls (tetracycline and streptomycin) at concentrations ranging from 0.007 to 1 mg/ mL. The plates were incubated for 24 hours at 37°C. The minimum extract concentration (MIC) at which bacteria demonstrated inhibition was recorded.

2.3 Antidiabetic activity

The previously published method (Dewi et al., 2018) assessed the α-glucosidase inhibitory activity. Five concentrations ranging from 3.125 to 100 µg/mL were used in triplicate for the antidiabetic test. Mixtures containing 250 µL α-glucosidase (0.124 unit/mL), 495 µL of 0.1 M phosphate buffer (pH 7.0), and 5 µL of various sample concentrations in DMSO (50–200 µg/mL) were preincubated at 37°C for 5 min. The reactions were initiated by adding 250 µL of 5 mM p-NPG (Wako, Japan). The reaction mixtures were incubated at 37°C for 15 min and determined by adding 1 mL of 0.1 M Na_2CO_3. P-nitrophenol release was measured spectrophotometrically

at 400 nm. The enzyme inhibition percentage was calculated using Equation 1:

$$\% \text{ Inhibition} = ((A_{control} - A_{sample})/ A_{control}) \times 100 \quad (1)$$

2.4 Mass spectroscopy analysis

Mass spectrometry was performed using an LC-MS/MS Xevo, G2-XS QTof (Waters MS Technologies) with ESI ionization. The scan range was 100 to 1200 m/z. The capillary and cone voltages were set at 0.8 kV and 30 kV, respectively. Using positive electron spray mode. The desolvation gas was set to flow at 1,000 L/h at 500°C, while the cone gas flow rate was set to 50 L/h, with a source temperature of 120°C. UPLC analysis employed a Waters Acquity Ultra Performance LC system. Chromatographic separation utilized an ACQUITY UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 µm) at 40°C. The mobile phase comprised solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile), with a gradient polarity from 95:0.5 (A:B) to 0.5:95 (A:B). The flow rate was set at 0.3 mL/min. The column and autosampler were maintained at 40°C and 20°C, respectively. The injection volume was 1 µL. Data acquisition and processing used UNIFI, with the retention time (RT) ranging from 1–16 min.

2.5 Statistical analysis

Data were presented as mean ± standard deviation from at least triplicate determinations. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range test. A $p<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Antidiabetic activity

The assessment of α-glucosidase impact on *H. macrothyrsa* extracts indicates that the HMM and HME extracts demonstrate potential antidiabetic properties through their α-glucosidase inhibitory activities (Table 1). The IC_{50} values were 7.318 ± 0.57 and 6.694 ± 0.44 µg/mL, respectively. The IC_{50} value of methanol and ethyl acetate extracts were comparable to the quercetin standard (2.670 ± 0.2 µg/mL).

This enzyme catalyzes the final stage of glucose breakdown in intestinal cells. This strategic approach, which limits carbohydrate absorption from meals and reduces postprandial hyperglycemia, may be an effective method for developing antidiabetic drugs. The study evaluated the antidiabetic potential of different *H. macrothyrsa* fractions using the glucosidase inhibition assay. This enzyme is known to catalyze the final stage of glucose digestion in intestinal cells. This targeted action, which limits carbohydrate absorption from meals and reduces postprandial hyper-glycemia, may be viable approach to developing antidiabetic drugs (Omari et al., 2019).

The ethyl acetate fraction was selected for purification using gravity column chromatography based on the antidiabetic test results. Since the ethyl acetate fraction compounds are components of the methanol extract, they are likely to be predominantly semi-polar.

Table 1. Antidiabetic activity of extract and fractions of *H. macrothyrsa* leaves

H. macrothyrsa	**IC $_{50}$ values (µg/mL)**
Methanolic extract (HMM)	7.318 ± 0.57
Hexane extract (HMH)	50.303 ± 2.32
Ethyl acetate extract (HME)	6.694 ± 0.44
Butanol extract (HMB)	64.016 ± 1.02

Note: Data is mean ± SD of three determinations, values bearing different letters are significantly different by multiple Duncan ($p\leq0.05$)

Furthermore, we found that F11 and F12 exhibited noteworthy IC_{50} values of 19.270 ± 0.3 and 17.380 ± 0.4 µg/mL after producing 13-column chromatography fractions (Table 2). The IC_{50} value of quercetin, the positive standard, is 2.670 ± 0.2 µg/mL. These findings, suggest that F11 and F12 contain promising compounds that could be developed into antidiabetic medications.

Table 2. Antidiabetic activities of column chromatography ethyl acetate fraction of *H. macrothyrsa* leaves

Samples & fractions	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**
IC_{50} (µg/mL)	>100	>100	>100	>100	54.387 ± 4.3	35.249 ± 1.1	43.874 ± 3.0	87.003 ± 0.4	49.230 ± 2.2	44.860 ± 2.1	19.270 ± 0.3	17.380 ± 0.4	>100

Note: Data is mean ± SD of three determinations, values bearing different letters are significantly different by multiple Duncan ($p\leq0.05$)

The inhibitory effect of the F11 and F12 fractions on the α-glucosidase enzyme indicates that they can reduce elevated blood glucose levels. Our findings align with previous of similar *Horsfieldia* species, which documented α-glucosidase inhibitory activities in *myristinin* D (53.8 µM) and *myristinin* E (67.0 µM) from from *H. motleyi* fruits extracts (Ramadhan et al., 2018), and 43 µg/mL ethyl acetate from *H. spicata* leaves extracts (Megawati et al., 2023). Horsfieldones A (22.61 µM) and maingayone D (5.65 µM) were found stem bark extracts from *H. macrobotrys* (Ramadhan & Phuwapraisirisan, 2015). Variations in the plant species's enzymatic inhibition capabilities can be attributed to the differences in reported inhibiton percentages. Through ongoing research efforts to conducts a comprehensive study, we have identified dominant compounds that characterize F11 and F12, representing promising research area.

3.2 Antibacterial activity

The antibacterial activity of HMM and its fractions was evaluated using disk diffusion and microwell dilution techniques against four common bacteria. It was discovered that none of the fractions exhibited an inhibitory zone against *E. coli*, *B. subtilis*, *S. aureus*, or *P. aeruginosa*, with a clear zone diameter of 7 mm (Table 3).

Table 3. Diameter of inhibition zone, MICs and MBCs values of extract and fractions of *H. macrothyrsa* leaves against bacterial tested

Samples	Diameter of inhibition zone (mm)				MIC/MBC (mg/mL)			
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***B. subtilis***	***S. aureus***	***E. coli***	***P. aeruginosa***
HMM	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00	1/>1	1/>1	1/>1	1/>1
HMH	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00	1/>1	1/>1	0.31/>0.31	1/>1
HME	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00	1/>1	0.63/>0.63	0.31/>0.31	1/>1
HMB	7.00±0.00	7.00±0.00	7.00±0.00	7.00±0.00	1/>1	1/>1	0.5/>0.5	1/>1
Tetracycline	13.00±0.82	18.00±0.00	13.00±0.82	10.33±0.47	1/>1	0.08/>0.08	0.08/>0.15	0.16/>0.16
Streptomycin	20.33±0.94	22.33±0.47	15.00±0.00	18.67±0.47	0.31/>0.31	0.31/>0.62	0.15/>0.15	0.78/>0.78

Note: Values are mean ± SD of 3 replicates; (-) MIC or MBC values was higher than 1 mg/mL

The microdilution assay demonstrated that the ethyl acetate fraction (HME) exhibited the strongest antibacterial activity against *S. aureus* and *E. coli*, with MIC/MBC values of 0.63/>0.63 mg/mL and 0.31/>0.31 mg/mL, respectively. In contrast, the n-hexane (HMH) and n-butanol fractions showed comparatively lower antibacterial activity against *E. coli*, with MIC/MBC values of 0.31/>0.31 mg/mL and 0.50/>0.50 mg/mL, respectively.

Overall, all extracts and fractions displayed weaker antibacterial effects than the positive controls (tetracycline and streptomycin), as evidenced by Tables 3 and 4 inhibition zone diameters (which were larger than the sample) and MIC/MBC concentrations (which were lower than the sample).

Interestingly, after column chromatography, it was discovered that fractions F2 (8.76±0.47 mm) against *S. aureus*, F3 (9.00±0.00 mm) and F7 (8.33±0.47 mm) against *B. subtilis* demonstrated greater clear diameter inhibition zone diameters than the previous ones (Table 4). These active fractions demonstrated improved antibacterial potency, with MIC/MBC value rates of 0.15/>0.15 mg/mL.

The antibacterial activities of HMM and its fractions against four common bacteria were determined using disk-diffusion and microwell- dilution assays methods. None of the fractions established an inhibitory zone against *E. coli*, *B. subtilis*, *S. aureus*, or *P. aeruginosa* with an apparent diameter of 7 mm (Table 3). However, combined analysis of disc diffusion and microdilution results indicated that fractions F2, F3, and F7 exhibited relatively higher antibacterial activity compared to the other fractions, suggesting their potential activity against multiple bacterial targets (Figure 1).

Table 4. Diameter of inhibition zone, MIC and MBC values of column chromatography ethyl acetate fraction of *H. macrothyrsa* leaves on tested bacteria test

Ethyl acetate extract	Diameter of inhibition zone (mm)				MIC/MBC (mg/mL)			
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***B. subtilis***	***S. aureus***	***E. coli***	***P. aeruginosa***
1	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	1/>1	1/>1	1/>1	1/>1
2	6.00±0.00	8,67±0.47	6.00±0.00	6.00±0.00	1/>1	0.15>0.15	1/>1	1/>1
3	9.00±0.00	7,33±0.47	6.00±0.00	8.00±0.00	0.15>0.15	1/>1	1/>1	0.15/>0.15
4	6.00±0.00	6.00±0.00	6.00±0.00	6.67±0.82	1/>1	1/>1	1/>1	1/>1
5	6.00±0.00	6.00±0.00	7.33±0.47	6.00±0.00	1/>1	1/>1	1/>1	1/>1
6	6.33±0.47	7.00±0.00	6.00±0.00	7.00±0.00	1/>1	1/>1	1/>1	1/>1
7	8,33±0.47	7.67±0.94	7.00±0.00	7.33±0.47	0.15>0.15	1/>1	1/>1	1/>1
8	6.00±0.00	6.00±0.00	9.00±0.47	7.00±0.00	1/>1	1/>1	1/>1	1/>1
9	6.00±0.00	6.33±0.47	6.00±0.00	7.33±0.00	1/>1	1/>1	1/>1	1/>1
10	8.00±0.82	6.00±0.00	6.00±0.00	6.67±0.94	1/>1	1/>1	1/>1	1/>1
11	6.00±0.00	6.00±0.00	6.00±0.00	6.67±0.94	1/>1	1/>1	1/>1	1/>1
12	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	1/>1	1/>1	1/>1	1/>1
13	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	1/>1	1/>1	1/>1	1/>1
Tetracycline	13.00±0.82	18.00±0.00	13.00±0.82	10.33±0.47	1/>1	0.50/>0.50	0.13/>0.13	0.13/>0.13
Streptomycin	20.33±0.94	22.33±0.47	15.00±0.00	18.67±0.47	0.13/>0.13	0.31/>0.62	0.15/>0.15	0.78/>0.78

Note: Values are mean ± SD of 3 replicates; (-) MIC or MBC values were higher than 1 mg/mL

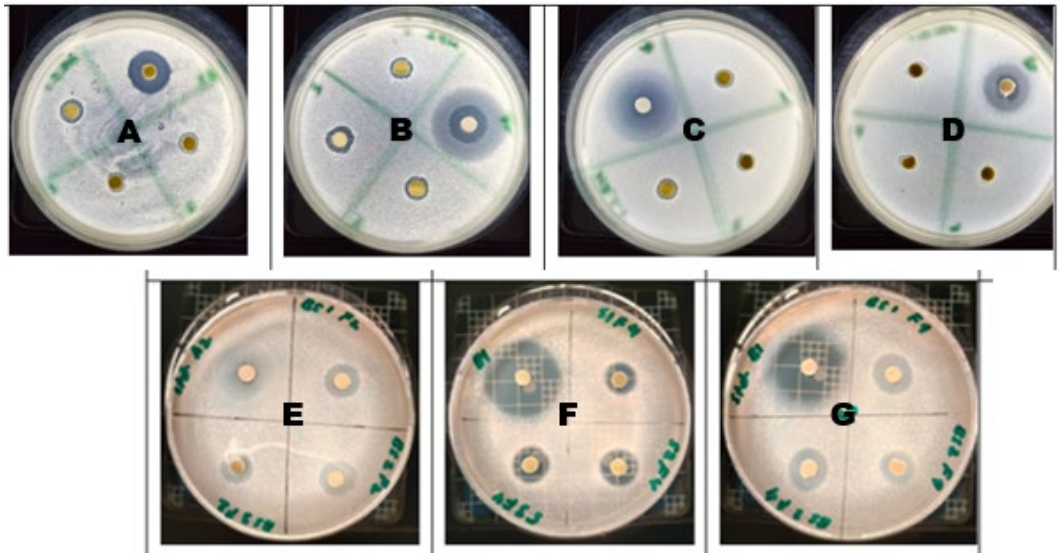

Figure 1. Antibacterial activity via disc diffusion method of (A) HMM, (B) HMH, (C) HMEA, and (D) HMB, against (E) *S. aureus* F2, (F) *B. subtilis* F3, and (G) F7
Note: all fractions were 4 mg/mL; (+) indicates positive control, tetracycline (4 mg/mL); assay was carried out in standard petri-discs (90×15 mm)

Other species from the *Horsfieldia* genus, such as *H. glabra* seed extract, have demonstrated antibacterial activity against *S. aureus* with a MIC value of 15.62 mg/mL (Chaicana, 2016). *H. helwigii* has also exhibited antibacterial activity against numerous bacteria, including *B. coagulans* and *P. aeruginosa*, with inhibition zone diameters of 22 and 18 mm, respectively (Khan et al., 2001).

Consequently, the selected fractions (F2, F3, and F7) exhibited MIC value against *S. aureus* and *B. subtilis* comparable to those reported for *H. glabra* extract. However, these fractions showed smaller inhibition zone diameter against *P. aeruginosa* than those observed for *H. helwigii* extract.

3.3 LC-MS/MS profile of selected fraction

We further identified the dominant compounds in the most potent HME fraction based on the results above. The LC-MS/MS analysis showed that this fraction contained five dominant compounds (Figure 2).

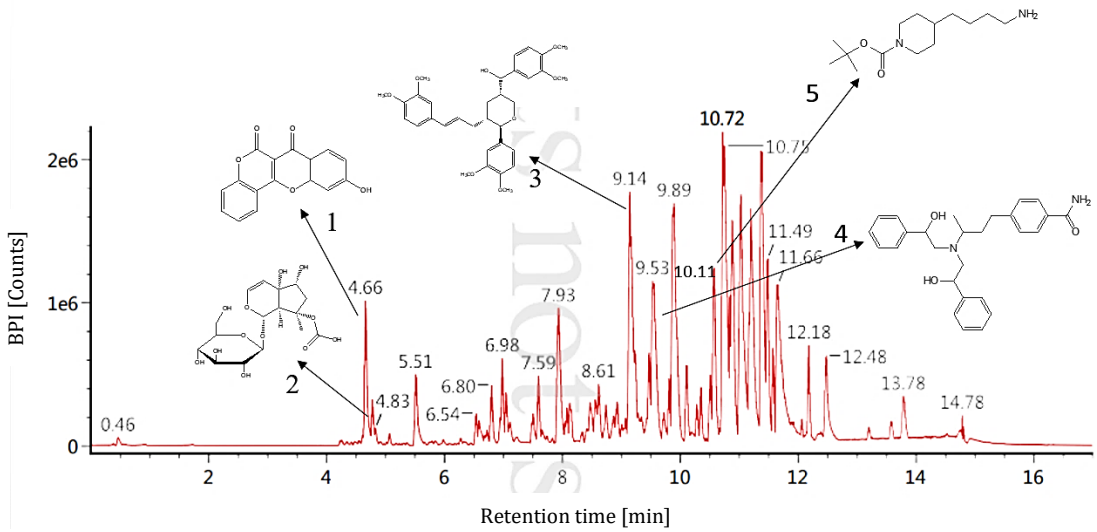

Figure 2. Chromatogram of *H. macrothyrsa* by LCMS-MS HME (*H. macrothyrsa* Ethyl acetate extract)

The compound morinol ($C_{33}H_{40}O_8$), a flavonoid derivative, can be isolated from *Morina Chinensis* and exhibits anti-microbial activity (Akiyama et al., 2009). 4-[(3R)-3[Bis((2R)-2-hydroxy-2-phenylethyl)amino]butyl]benzamide ($C_{27}H_{32}N_2O_3$) and 1,1-dimethylethyl 4 (4-aminobutyl)-1piperidinecarboxylate ($C_{14}H_{28}N_2O_2$) are alkoxide groups with anticancer activities. 8-acetyl harpagide ($C_{16}H_{24}O_{12}$) can be isolated from *Stachys* species and exhibits anticancer activity (Háznagy-Radnai et al., 2008). 7-Hydroxy-3-methoxyflavone-2'-carboxylic acid ($C_{16}H_8O_5$) can be isolated from *Distemonanthus* species (King et al., 1954), and belongs to the glucoside lignan group, which has several bioactivities, including antimicrobial (Khan et al., 2001; Minarti et al., 2022), and antidiabetic effects (Ramadhan & Phuwapraisirisan, 2015; Ramadhan et al., 2018) (Table 5).

Table 5. Compound name from LCMS-MS ethyl acetate extract from *H. macrothyrsa* leaves

No.	Component name	Chemical formula	MS	Retention time (min)
1	7-Hydroxy-3-methoxyflavone-2'-carboxylic acid	$C_{16}H_8O_5$	280.04	4.66
2	8-Acetyl harpagide	$C_{16}H_{24}O_{12}$	408.13	4.83
3	Morinol	$C_{33}H_{40}O_8$	564.27	9.14
4	4-[(3R)-3-[Bis((2R)-2-hydroxy-2-phenylethyl)amino]]benzamide	$C_{27}H_{32}N_2O_3$	432.24	9.53
5	1,1-Dimethylethyl 4-(4-aminobutyl)-1-piperidinecarboxylate	$C_{14}H_{28}N_2O_2$	256.22	10.11

The HME fraction's predominant constituents have demonstrated strong antibacterial and anticancer activities, while the presence of numerous other bioactive compounds, including lignans and flavonoids, may also have contributed to those effects.

4. CONCLUSION

We successfully extracted HME, a potent bioactive fraction, which exhibited antibacterial and antidiabetic properties, from *H. macrothyrsa* metabolites. As an inaugural investigation reporting the putative pharmacological activities of *H. macrothyrsa*, these findings suggest that the predominant compounds in this bioactive fraction, specifically the lignans and alkaloids groups, are responsible for the plant fraction's therapeutics effects. The results should provide a novel insight for studying and characterizing phyto-chemicals isolated from *H. macrothyrsa*. However, this promising discovery necessitates comprehensive investigation to elucidate its fundamental mechanisms.

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