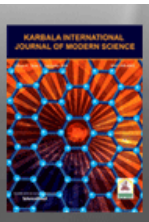

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Discovery of Horsfieldia macrothyrsa Bioactives with Cytotoxic Effects on Breast Cancer Cells Through In Vitro and Docking Analyses

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Abstract

This study investigated the phytochemical constituents and biological activities of *Horsfieldia macrothyrsa* leaves to identify their bioactive compounds. Methanol extracts were fractionated using *n*-hexane and ethyl acetate, followed by silica gel column chromatography with a stepwise polarity gradient. From 100 g of powdered leaves, three major compounds were successfully isolated from the ethyl acetate fraction: 1-(2,4,6-trihydroxyphenyl)dodecan-1-one (**1**), sesamin (**2**), and β-sitosterol (**3**). Their chemical structures were confirmed using UV, IR, LC–MS/MS, and NMR spectroscopy. Biological activities of the fractions and isolates were evaluated through DPPH antioxidant assays, α-glucosidase inhibition for antidiabetic activity, MTT assays on MCF-7 and T47D breast cancer cell lines for cytotoxicity, and antibacterial tests (MIC and MBC) against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The ethyl acetate fraction showed the strongest DPPH and α-glucosidase inhibitory activities, with IC_{50} values of 12.72 ± 0.12 and 6.69 ± 0.44 µg/mL, respectively. Isolate **1** exhibited the highest antioxidant potential and mild antibacterial effects against *B. subtilis*, *P. aeruginosa*, and *E. coli*. β-sitosterol (**3**) demonstrated significant cytotoxicity toward MCF-7 (IC_{50} = 20.71 µg/mL) and T47D (IC_{50} = 18.19 µg/mL), while sesamin (**2**) showed moderate cytotoxicity on MCF-7 cells. Molecular docking against EGFR (PDB ID: 4HJO) indicated strong binding affinities for all three isolate: -7.41 kcal/mol (**1**), -9.99 kcal/mol (**2**), and -9.15 kcal/mol (**3**). These results suggest that sesamin and β-sitosterol form stable interactions with EGFR. Overall, the methanol and ethyl acetate fractions of *H. macrothyrsa* represent promising natural sources of antioxidants, while isolate **1**-**3** exhibit potential antibacterial and anticancer activities.

Keywords

Cytotoxic evaluation, Horsfieldia macrothyrsa, 1-(2, 4, 6-trihydroxyphenyl)dodecan-1-one, Sesamin, β-sitosterol

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RESEARCH PAPER

Discovery of *Horsfieldia macrothyrsa* Bioactives With Cytotoxic Effects on Breast Cancer Cells Through In Vitro and Docking Analyses

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Abstract

This study investigated the phytochemical constituents and biological activities of *Horsfieldia macrothyrsa* leaves to identify their bioactive compounds. Methanol extracts were fractionated using *n*-hexane and ethyl acetate, followed by silica gel column chromatography with a stepwise polarity gradient. From 100 g of powdered leaves, three major compounds were successfully isolated from the ethyl acetate fraction: 1-(2,4,6-trihydroxyphenyl)dodecan-1-one (1), sesamin (2), and β-sitosterol (3). Their chemical structures were confirmed using UV, IR, LC−MS/MS, and NMR spectroscopy. Biological activities of the fractions and isolates were evaluated through DPPH antioxidant assays, α-glucosidase inhibition for antidiabetic activity, MTT assays on MCF-7 and T47D breast cancer cell lines for cytotoxicity, and antibacterial tests (MIC and MBC) against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The ethyl acetate fraction showed the strongest DPPH and α-glucosidase inhibitory activities, with IC_{50} values of 12.72 ± 0.12 and 6.69 ± 0.44 μg/mL, respectively. Isolate 1 exhibited the highest antioxidant potential and mild antibacterial effects against *B. subtilis*, *P. aeruginosa*, and *E. coli*. β-sitosterol (3) demonstrated significant cytotoxicity toward MCF-7 (IC_{50} = 20.71 μg/mL) and T47D (IC_{50} = 18.19 μg/mL), while sesamin (2) showed moderate cytotoxicity on MCF-7 cells. Molecular docking against EGFR (PDB ID: 4HJO) indicated strong binding affinities for all three isolate: −7.41 kcal/mol (1), −9.99 kcal/mol (2), and −9.15 kcal/mol (3). These results suggest that sesamin and β-sitosterol form stable interactions with EGFR. Overall, the methanol and ethyl acetate fractions of *H. macrothyrsa* represent promising natural sources of antioxidants, while isolate 1−3 exhibit potential antibacterial and anticancer activities.

Keywords: Cytotoxic evaluation, *Horsfieldia macrothyrsa*, 1-(2,4,6-trihydroxyphenyl)dodecan-1-one, Sesamin, β-sitosterol

1. Introduction

Species of the genus *Horsfieldia* (family Myristicaceae) were discovered in the virgin forests of Indonesia. These tropical plants, locally known as *pala*, are characterized by the crimson resin found in their bark. Many *Horsfieldia* species in Southeast Asia have been traditionally used to treat various ailments due to their cytotoxic [1,2], antioxidant [3−5], and antibacterial [6,7] properties. A review of the literature reveals that numerous phytochemical investigations on plants belonging to this genus have been conducted, which identified a wide range of secondary metabolites, including lignans [2,8,9], terpenoids [1,8,10], flavonoids [1,2,4,9,12], polyketides [1,3,5,10,12−15], as well as chalcones, quinones, and alkaloids [1,11,12].

H. macrothyrsa is a species endemic to Sumatra, Indonesia, and is recognized as a valuable essential oil producing plant. However, compared with other

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species in this genus, scientific information on *H. macrothyrsa* remains very limited. No detailed studies have reported the isolation of pure compounds from its twig extracts [16], nor have its biological potential, including cytotoxic effects against breast cancer cell lines been investigated. Additionally, the α-glucosidase inhibitory activity of this species has not been documented, leaving a gap in understanding its potential therapeutic applications in the treatment of metabolic disorders.

Therefore, this study provides the first report on the isolation and structural elucidation of secondary metabolites from the ethyl acetate fraction of *H. macrothyrsa* twigs, along with an evaluation of their *in vitro* biological properties. Specifically, this work investigates their cytotoxicity on MCF-7 as well as T47D breast cancer cell lines, antioxidant activity using the DPPH assay, and α-glucosidase inhibitory potential. These findings are expected to broaden the phytochemical knowledge of *H. macrothyrsa* and support its prospective development as a promising natural resource for anticancer and antidiabetic therapeutics.

2. Materials and methods

2.1. Materials

In March 2023, *H. macrothyrsa* leaves were collected from the Cibinong Botanical Garden in West Java, Indonesia. The specimen was identified and assigned as 04-SHU-12-2023 in the Pharmaceutical Ingredients Standardization Laboratory, BRIN, Tawangmangu, Central Java.

2.2. General experimental setup

The instruments used in this investigation include ovens, a micropipette (Humapette, Germany), an extractor, a rotary evaporator (Buchi R214, Switzerland), and glassware. Among other methods, the purity of the separated isolate crystals was evaluated by measuring the melting point later in the isolate separation process using a Fisher-Johns melting point device. Isolate **1**–**3** were isolated and identified using various analytical techniques. Agilent Technologies' Cary 60 UV–Vis spectrophotometer was used to determine the maximum absorbance of isolates that have been diluted with methanol. The ^{1}H, ^{13}C, HMQC and HMBC spectra were using a Bruker AV 700 MHz and a JEOL JMN-A 500 MHz NMR. Shimadzu Prestige 21 was utilized to measure the FTIR spectrum. Molecular weight was determined using the ESI source technique (Mariner Biospectrometry) in an LC-MS/MS Xevo, G2-XS QTOF (Waters MS Technologies). For the antibacterial test, several bacterial strains were acquired from the Food and Nutrition Study Program's culture collection at Gadjah Mada University in Indonesia. Among these strains were the bacteria *E. coli* (FNCC-0195), *P. aeruginosa* (ATCC 9027), *B. subtilis* (FNCC-0059), and *S. aureus* (FNCC-0047).

2.3. Extraction and isolation

The dry powder of *H. macrothyrsa* (650 g) was macerated with methanol (3 × 2 L). The mixture was then filtered, and the filtrate was evaporated under decreased pressure to obtain a crude methanolic extract. The methanolic extract was fractionated with Vacuum Liquid Chromatography (VLC) employing *n*-hexane (3 × 2 L) and ethyl acetate (EtOAc) (3 × 2 L) as solvents, respectively. Each extract was subjected to evaporation at 45 °C under decreased pressure. Ethyl acetate fraction (20 g) was subjected to silica gel column chromatography and eluted using gradients of *n*-hexane and ethyl acetate, yielding a total of 13 fractions (F1–F13). Isolate **1** (15 mg) was acquired by purifying fraction F4 through two successive Sephadex LH-20 column chromatographies, utilizing chloromethane-methanol (1:1) as the eluent. Recrystallization of fraction F2 with an extra solvent yielded isolate **2** (12 mg), while recrystallization of fraction F3 resulted in isolate **3** (13 mg).

2.3.1. Characterization of isolate 1, 2, and 3

The structural identities of the three isolates were established using UV, IR, MS, and both 1D and 2D NMR analyses.

a. **Isolate 1: 1-(2,4,6-trihydroxyphenyl) dodecan-1-one**

Isolate **1** occurred as a white solid.

^{1}H-NMR (500 MHz, CD_3OD): δ_H 5.80 (1H, *s,* H-3 and H-5), δ_H 1.25–1.37 (22H, H-2′– H-11′), δ_H 0.89 (3H, *t*, H-12′).

^{13}C-NMR (125 MHz, CD_3OD): δ_C 207.6 (C-1′), δ_C 105.4 (C-1), 165.9 (C-2 and C-4), 166.1 (C-6) and 95.8 (C-3 and C-5), δ_C 44.9–23.8 (C-2′– C-11′), δ_C 14.5 (C-12′). LC-MS/MS: *m/z* 309.20668.

UV $_{\lambda max}$: 285 nm.

IR: O–H absorption at 3392 and 3262 cm^{-1}, C=O at 1714 cm^{-1}.

b. **Isolate 2: Sesamin**

Isolate **2** formed white crystalline material.

^{1}H-NMR (700 MHz, $CDCl_3$), spectrum showed characteristic aromatic proton signals at δ_H

6.78–6.88 ppm, indicating a substituted aromatic system. Two methylenedioxy groups were observed as singlets at δ_H 5.95 ppm. Oxygenated methylene protons appeared in the region δ_H 3.24–4.10 ppm, while several oxygenated methine protons resonated between δ_H 3.40 and 4.41 ppm, confirming the presence of a highly oxygenated lignan framework.

^{13}C-NMR (175 MHz, CDCL3). revealed signals corresponding to oxygenated aliphatic carbons at δ_C 51.3–89.3 ppm and aromatic carbons at δ_C 102.4–149.5 ppm. The presence of methylenedioxy carbons was supported by resonances around δ_C 102.4 ppm, while multiple oxygenated aromatic carbons appeared above δ_C 140 ppm. Overall, the NMR data are consistent with a polyphenolic lignan structure.GC-MS/MS *m/z* 354.0.

Melting point: 119–122 °C.

UV λ_{max}: 207, 236, 287 nm.

IR bands: C–H at 2898 cm^{-1}, C=C at 1605 cm^{-1}, C–O at 1071 cm^{-1}.

c. Isolate 3: β-sitosterol

Isolate 3 was obtained as a white powder.

^{1}H-NMR (700 MHz, Acetone), spectrum exhibited several upfield methyl resonances between δ_H 0.73–1.02 ppm, corresponding to six methyl groups typical of a sterol skeleton. An olefinic proton was observed at δ_H 5.31 ppm, indicating the presence of a double bond, while an oxygenated methine proton resonated at δ_H 3.62 ppm, confirming a hydroxyl-substituted carbon. The remaining aliphatic methylene and methine protons appeared as a broad multiplet in the range of δ_H 0.98–2.26 ppm.

^{13}C-NMR (175 MHz, acetone) showing 29 signals. displayed 29 carbon signals, consistent with a phytosterol framework. Key resonances included an oxygenated carbon at δ_C 71.9 ppm, olefinic carbons at δ_C 121.6 and 142.4 ppm, and multiple aliphatic carbons distributed between δ_C 12.3 and 57.7 ppm. These spectral features collectively support the presence of a β-sitosterol-type structure.

GC-MS/MS: *m/z* 414.0.

Melting point: 134–135 °C.

IR: broad absorption at 3287 cm^{-1} and a C=C signal at 1675 cm^{-1}.

2.4. Bioactivities

a. Antioxidant Assay

The antioxidant assays were conducted by following the methodology of Minarti (2024) [17], with minor changes. A sample solution of 1000 μg/mL was prepared in methanol. Five concentration series (10–100 μg/mL) were subsequently prepared and mixed with an equal volume of 1 mM DPPH in methanol. The mixtures were incubated in the dark for 30 min to ensure the reaction was completed. The absorbance was measured at 517 nm. The following formula was used to calculate the inhibition percentage:

$$\text{Inhibisi}\,(\%) = \frac{(A_{control} - A_{sample})}{(A_{control})} = 100$$

$A_{control}$ represents the absorbance without samples

A_{sample} = the absorbance of the samples

The IC_{50} values were obtained using linear regression analysis of concentration versus inhibition percentage. Quercetin was used as a positive control at five different concentrations (1–20 μg/mL), while methanol and DPPH served as negative controls.

b. Antidiabetic Assay

The α-glucosidase inhibitory assay was carried out according to a previously published protocol, with a few minor adjustments [18]. Briefly, 5 μL of the test sample was dissolved in DMSO to create a series of final concentrations (6.25–100 μg/mL), which was then subsequently mixed with 250 μL of p-NPG (3 mM) and 495 μL of 100 mM phosphate buffer (pH 7.0). After incubating at 37 °C for 5 min, 250 μL of α-glucosidase (0.065 units/mL) was added, and the mixture was incubated for an additional 15 min. The reaction was stopped by adding one milliliter of 0.2 M Na_2CO_3. The α-glucosidase inhibitory effect was measured by observing the absorbance of *p*-nitrophenol product at 400 nm. To account for background absorbance, a blank was made in the same way, but without the test sample. Similar to the DPPH assay, linear regression analysis was used to determine an IC_{50} value. All of the reagents and DMSO were used as negative control, and quercetin, an effective α-glucosidase inhibitor, was used as the positive control at five different concentrations (1.5625–25 μg/mL).

c. Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Antibacterial Activity using the Disc Diffusion Approach

The disc diffusion method was carried out as previously described [19] with a few minor adjustments. Bacterial cultures were assessed for MIC and MBC values using tetracycline (T) and streptomycin (S) as positive controls. Briefly, an

inoculum suspension equal to 0.5 McFarland standard (roughly 10^8 CFU/mL) was made using an overnight culture of each test bacterium. The test extracts (prepared in 1 % aqueous DMSO) and the positive control (tetracycline) were dispensed to 96-well microtiter plates and subsequently inoculated with the bacterial suspension prepared in Mueller–Hinton Broth (MHB). The final volume in each well was adjusted to 200 μL, resulting in a final bacterial concentration of approximately 10^6 CFU/mL. The plates were incubated at 30 °C for 24 h. The MIC was defined as the lowest extract concentration that completely inhibited visible bacterial growth. Aliquots from wells exhibiting no discernible growth were subcultured into nutrient agar (NA) plates and incubated for 24 h at 37 °C to calculate the minimum bactericidal concentration (MBC). The MBC was defined as the lowest concentration of the extract that achieved 100 % bacterial kill.

d. **Cytotoxicity Assay**

Methanolic extract of *H. macrothyrsa* leaves was preparated at a concentration 10,000 μg/mL. The cytotoxicity of *H. macrothyrsa* was evaluated by an MTT-based cytotoxicity assay on MCF-7 (ATCC) [20] and T47D [21] cell lines (ATCC). Samples were administered to cells that were grown in growth medium (DMEM supplemented with 10 % FBS and 1 × antimycotic-antibiotic solution) for 48 h. Doxorubicin was used as a positive control. After 48 h, the medium was removed and replaced with 0.5 mg/mL MTT solution in DMEM, and the cells were incubated for another 3 h. The absorbance of the formazan crystal product was measured at 570 nm. The following formula was used to calculate cell viability (%):

$$\text{Viability}\,(\%) = \frac{(A_{sample} - A_{blanko})}{(A_{control} - A_{blanko})} \times 100$$

The IC_{50} value was determined from the linear regression of concentration versus viability percentage. According to the NCI (National Cancer Institute) standard, crude extracts and fractions with IC_{50} values below 30 μg/mL are considered active [22].

2.5. Molecular docking

Compounds isolated from the EF of *H. macrothyrsa* were selected for further investigation due to their potential anticancer activity. These isolates were molecularly docked to the active site of EGFR (PDB ID: 4HJO) [23], retrieved in PDB format from the RCSB Protein Data Bank. Protein–ligand complex preparation was performed using UCSF Chimera. All water molecules were removed from both the protein and ligand structures to prevent interference during docking. Structural optimization was then carried out in UCSF Chimera by the addition of hydrogen atoms, assignment of appropriate partial charges, and reconstruction of missing side chains. The optimized protein and ligand structures were saved in PDB format for subsequent docking simulations [24]. Redocking analyses were performed using AutoDock4. The EGFR protein was docked within a 40 × 40 × 40 $Å^3$ grid box along the x, y, and z axes, and the Lamarckian Genetic Algorithm (LGA) was executed for 50 iterations. The docking interactions were displayed graphically with Discovery Studio Visualizer (DSV) 2019 [25].

3. Result and discussion

The *H. macrothyrsa* leaf extracts were evaluated for their antioxidant and antidiabetic activities (Table 1). The ethyl acetate fraction (EF) demonstrated the IC_{50} values for the antidiabetic and antioxidant activities of 6.69 ± 0.44 μg/mL and 12.72 ± 0.12 μg/mL, respectively (Table 1). These data suggest that the ethyl acetate fraction has the strongest DPPH radical scavenging and α-glucosidase inhibitory activities in comparison with *n*-hexane (HF) and methanolic (ME) extract.

The antioxidant activity of plants is mainly dependent on the content of their bioactive compounds. One such compounds, flavonoids, is a secondary metabolite with recognized strong antioxidant potential and is considered a natural phenolic compound [26]. Besides flavonoids, polyphenols also take a primary role as powerful antioxidants. Polyphenolic compounds are gaining popularity due to their multifunctional properties as they may act as decomposition agents, hydrogen donors, and free radical scavengers [27].

The first part of the present work has pointed out that the methanolic extract (ME) and ethyl acetate frac (EF) displayed inhibiting activities with IC_{50}

Table 1. In vitro assays (IC_{50}) of fractions from H. macrothyrsa.

Sample	In Vitro Assays IC_{50} (μg/mL)	
	α-Glu	DPPH
MeOH ext (ME)	8.47 ± 0.27	15.97 ± 0.24
n-hexane Fr (HF)	59.73 ± 0.79	54.50 ± 1.33
Ethyl acetate Fr (EF)	6.69 ± 0.44	12.72 ± 0.12
Quercetin	11.83 ± 1.96	7.08 ± 0.05

Results presented as mean ± SE (n = 3).

values inferior to 20 μg/mL and, thus, can be considered as strong and promising agents in the fight against oxidative stress and diabetes. The *n*-hexane frac (HF), on the other hand, was only moderately active as its IC_{50} values were above 50 μg/mL. The authors suggest that the extract could serve as a protector against mitochondrial oxidative stress and an antioxidant source [28].

3.1. Isolation and characterization

The ethyl acetate fraction of *H. macrothyrsa* leaves was purified by gradient solvent methods. Vacuum liquid chromatography (VLC) was used for the purification of the Ethyl acetate frac (EF) with silica gel as the stationary phase and *n*-hexane: EtOAc and EtOAc: methanol as the mobile phase. Three pure isolates were obtained and their chemical structures were determined with UV-spetcrophotometry, Infrared spectroscopy (IR), LC-MS/MS, and NMR.

Using the Sephadex LH-20 column and CH_2Cl_2: MeOH (1:1) as the eluent, isolate **1** was developed as a white amorphous solid after being purified from fraction 4. Its chemical formula was $C_{18}H_{28}O_4$ and a peak at *m/z* 309.20668 $[M+H]^+$ according to LC-MS/MS. ^{1}H-NMR, ^{13}C-NMR, HSQC, and HMBC were carried out to better characterise isolate **1**. The first isolate was a solid white. The maximum wavelength of the UV spectrum was 285 nm. A carbonyl group (1714 cm^{-1}) and hydroxyl groups (3392, 3262 cm^{-1}) were visible in the infrared spectrum. An aromatic signal was detected at δ_H 5.80 (1H, *s*, H-3 and H-5) in the ^{1}H-NMR spectrum. Aliphatic groups were indicated by the proton signals at δ_H 1.25–1.37 and a methyl signal at δ_H 0.89 (3H, *t*, H-12′). Carbonyl group (δ_C 207.6, C-1′), six aromatic carbons (δ_C 105.4, 165.9, 166.1, 95.8), ten methylene groups (δ_C 44.9–23.8 ppm), and a methyl group at δ_C 14.5 were all confirmed by the ^{13}C-NMR spectrum. Isolate **1** was determined to be 1-(2,4, 6-trihydroxyphenyl) dodecan -1-one based on spectroscopic data and data comparison with the relevant references [29].

GC-MS/MS revealed that isolate **2**, which was purified by recrystallisation from fraction 3, had white crystals with the molecular formula $C_{20}H_{18}O_6$, with a peak at *m/z* 354.0$[M]^+$. Mp 119–122 °C. The examination of HSQC, HMBC, ^{1}H and ^{13}C-NMR spectra were carried out to better characterise isolate **2**, contained a six aromatic proton group: δ_H 6.88 (1H, *dd*, J = 1.4 and 7.7, H-2′ and H-2″) correlations δ_C 89.3 (C-2), δ_C 83.4 (C-7), δ_C 149.5 (C-5′), δ_C 120.0 (C-2″); δ_H 6.78 (1H, d, J = 1.4 Hz, H-3′ and H-3″) correlations δ_C 136.5 (C-1″), δ_C 149.5 (C-5′); δ_H 6.84 (1H, *d*, J = 8.4 Hz, H-6′) correlations C-2 (δ_C 89.3), C-6 (δ_C 83.4), C-5′ (δ_C 149.5), C-2″ (δ_C 120.0). Four germinal protons of an oxygenated methylene group as δ_H 3.22 (1H, t, J = 8.4 Hz, H-4a) and 3.78 (1H, *t*, J = 9.1 Hz, H-4b) correlations C-7 (δ_C 83.4), C-3 (δ_C 55.8), and C-2 (δ_C 89.3); δ_H 4.10 (1H, *d*, J = 9.1 Hz, H-8a) and δ_H 3.83 (1H, *dd*, J = 6.32 Hz, H-8b) correlations C-7 (δ_C 51.3), C-4 (δ_C 70.7), C-6 (δ_C 83.4), and C-2 (δ_C 89.3). H-7′ δ_H 5.93 (2H, *s*) and H-7″ δ_H 5.92 (2H, *s*) are two methylenedioxy groups that correlate with C-4′/4″ (δ_C 148.1) and C-5′/5″ (δ_C 149.5). Consequently, isolate **2** was determined to be 1,4-di(benzo[1,3]dioxol -5-yl)hexahydrofuro[3,4-c]furan, known as sesamin [30].

Isolate **3**, a white powder with the chemical formula $C_{29}H_{50}O$, a peak at 414.4, and infrared spectra of 3750, 3440, 1675, 1505, and 1455 cm^{-1}, was refined by recrystallisation from fraction 2. δ_H 1.02 (3H, *s*, H-18); δ_H 0.73 (3H, *s*, H-19); δ_H 0.98 (3H, *d*, J = 8.4 Hz, H-21); δ_H 0.85 (3H, *d*, J = 7.0 Hz, H-26); δ_H 0.81 (3H, *d*, J = 6.1 Hz, H-27); and δ_H 0.84 (3H, *t*, J = 7.7 Hz, H-29) were detected by the ^{1}H-NMR spectrum. Signals for methylene and methine at δ_H 0.98–2.26 ppm, oxygenated methine δ_H 3.62 (1H, *m*, 4.2 Hz, H-6), and triplet δ_H 5.31 (1H, *t*, J = 5.6 Hz, H-6). The 29 carbons were confirmed by the ^{13}C-NMR spectrum. Important carbons were δ_C 71.9 (C-3), δ_C 121.6 (C-6), and δ_C 142.4 (C-5). These validated the double bond and hydroxyl group. β-sitosterol was identified as the third isolate [31].

These isolates were discovered in this genus for the first time. The structures of the three known isolates were determined to be 1-(2,4,6-trihydroxyphenyl) dodecan-1-one (isolate **1**), sesamin (isolate **2**), and β-sitosterol (isolate **3**) by comparing their spectroscopic data with values reported in the literature (Fig. 1).

3.2. Bioactivities of isolates

Isolate **1–3** have been previously identified in several genera of the Myristicaceae family, including *Horsfieldia*, *Knema*, and *Myristica*. However, this study focused on evaluating their

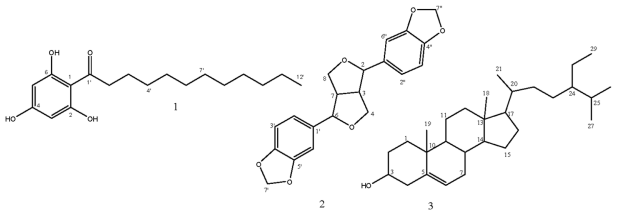

*Fig. 1. The structure of isolate **1–3**.*

antioxidant activity, cytotoxicity against breast cancer cell lines, and inhibitory effects on α-glucosidase.

According to Table 2, isolate **1** showed moderate antioxidant effectiveness, yielding an IC_{50} of 31.06 ± 1.5 μg/mL in comparison with quercetin, which served as the positive control. In contrast, isolates **2** and **3** demonstrated negligible antioxidant activity, as their IC_{50} values exceeded 100 μg/mL. Similarly, none of the isolates exhibited meaningful α-glucosidase inhibitory activity. β-sitosterol **3**, a naturally occurring plant sterol, is known to possess mild to moderate antioxidant properties and has been shown *in vitro* to reduce reactive oxygen species levels [32,33]. Prior investigations reported an antioxidant IC_{50} value of 150 μg/mL for *β*-sitosterol [34]. There have been no previous reports describing the antioxidant or antidiabetic activities of isolate **1** and **2**.

Isolate **2**, sesamin, is a polyphenolic lignan that exhibits potent activity against gallbladder cancer [35]. In contrast, isolate **3**, *β*-sitosterol, inhibited MCF-7 cell proliferation with an IC_{50} value of 20.71 μg/mL, while T47D cells were inhibited up to 50 μg/mL *β*-sitosterol also acts as a chemosensitizer for cancer cells. Structural derivatives of *β*-sitosterol have been reported to possess anticancer properties [36] and *β*-sitosterol itself exhibits a wide range of biological activities [37]. When tested against breast cancer (BC), epidermoid carcinoma, and small-cell lung cancer cell lines, isolate **1**, 1-(2,4,6-trihydroxyphenyl) dodecan-1-one, demonstrated mild to moderate cytotoxicity [38]. Conversely, our study revealed substantial efficacy of *β*-sitosterol **3** against T47D cells, with an IC_{50} value of 18.19 μg/mL.

We decided to uncover the antibacterial potentials of three isolates obtained from the ethyl acetate fraction of *H. macrothyrsa*. These isolates were subjected to tests against four common pathogenic bacteria. None of them produced a clear inhibition zone of more than 6 mm in the agar diffusion assay, indicating that the activity of these isolates via this method was very limited. As it turns out, an entirely different outcome was recorded in the microdilution assay. Among the three isolates, isolate **1** exhibited the most pronounced antibacterial activity, showing the most significant influence, in particular, the case of *B.subtilis, E.coli*, and *P. aeruginosa*, with MIC/MBC values of 15.625/>15.625 μg/mL (Table 3). The activity against *S. aureus* was very low as evidenced by MIC/MBC values of 1000/>1000 μg/mL which were comparable to those observed for the other fractions.

Reports on other *Horsfieldia* species show similar trends. For example, the seed extract of *H. glabra* was previously found to inhibit *S. aureus* at an MIC of 15.62 mg/mL [39]. Another species, *H. helwigii*, also demonstrated antibacterial activity, producing inhibition zones of 22 mm against *B. coagulans* and 18 mm against *P. aeruginosa* [6].

Isolate **1** from *H. macrothyrsa* still demonstrates meaningful activity against *B.subtilis*, *P.aeruginosa* and *E.coli*, although the absence of a larger inhibition zone, particularly against *P. aeruginosa*, indicates that its performance does not yet surpass that of *H. helwigii* extracts. Molecular docking studies of isolate **1–3** were conducted against the epidermal growth factor receptor (EGFR). EGFR, a member of the tyrosine kinase receptor family, is

*Table 3. The MIC and MBC values of Isolate **1–3** of* H. macrothyrsa *Leaves on Tested Bacteria.*

Bacteria	Sample	MIC (μg/mL)	MBC (μg/mL)
B.subtilis	**1**	15.625	≥15.625
	2	1000	>1000
	3	1000	>1000
	T	62.5	>15.625
	S	31.25	≥31.25
P.aeruginosa	**1**	15.625	≥15.625
	2	1000	>1000
	3	1000	>1000
	T	62.5	>0.015
	S	62.5	62.5
E.coli	**1**	15.625	≥15.625
	2	1000	>1000
	3	1000	>1000
	T	62.5	0.021
	S	15.625	≥15.625
S,aureus	**1**	1000	>1000
	2	1000	>1000
	3	1000	>1000
	T	62.5	31.25
	S	31.25	62.5

Results presented as mean ± SE (n = 3).

Table 2. In vitro assays of isolates from H. macrothyrsa.

Isolate	% Viability Vero cells	In Vitro Assays (IC_{50}, μg/mL)			
		MCF-7 cells	T47D cells	α-Glu	DPPH
1	100.06	36.34	50.12	>100	31.06 ± 1.5
2	79.89	21.97	>100	>100	>100
3	99.79	20.71	18.19	>100	>100

Results presented as mean ± SE (n = 3).

expressed on the surface of epithelial cells and becomes activated upon binding to its endogenous ligand, epidermal growth factor (EGF).

Activated EGFR triggers various intracellular signaling pathways that regulate normal cellular functions. However, EGFR overexpression can lead to tumor formation and progression through mechanisms such as angiogenesis, tissue invasion, and metastasis by activating a number of signaling pathways, including Ras/Raf/MAPK, PI3K/AKT, PLC/PKC, and STAT. Consequently, EGFR is a vital molecular target in anticancer drug discovery and development [40,41]. Redocking of erlotinib (native ligand) and doxorubicin as a positive control with the EGFR receptor gave a result an RMSD value of 0.93 Å, which is regarded as valid (RMSD <2 Å) [42].

The binding energies of isolate **1**, **2**, and **3** were −7.41, −9.99, and −9.15 kcal/mol, respectively (Table 4 and Fig. 2), where erlotinib and doxorubicin exhibited binding energies of −8.27 and −8.46 kcal/mol, respectively. Among these, isolate **2** and **3** showed lower binding energies than erlotinib, indicating stronger and more stable interactions with the EGFR protein, and it also implies that these isolates may act as potential EGFR inhibitors. Analysis of formed hydrogen bonds isolate **1** with Met769 and Gln767, isolate **2** to Thr830, and isolate **3** with Met769. It is worth mentioning that Met769 was a hydrogen bond partner with both the reference ligands, and therefore, it played a critical role in stabilizing ligand binding inside the EGFR active site. The similar interactions and good binding affinities of

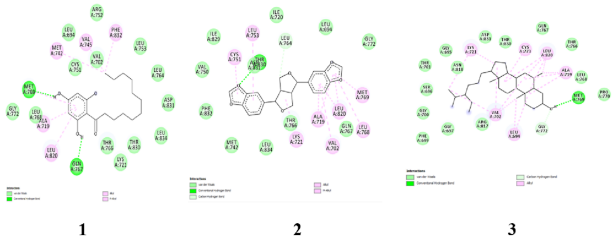

Fig. 2. Two-dimensional representation of molecular docking interaction between EGFR and tested isolates.

isolate **2** and **3** suggest that these isolates might be potential anticancer agents targeting EGFR.

In fact, molecular docking experiments confirmed the significant cytotoxic activity of isolate **2** and **3** in breast cancer cell lines. Among the tested compounds, isolate **3** was the most potent growth inhibitor of MCF-7 and T47D, with the IC_{50} values of 20.71 and 18.19 μg/mL, respectively. Isolate **2** demonstrated moderate activity against MCF-7 cells (21.97 μg/mL) but was showed negligible activity T47D cells (IC_{50} > 100 μg/mL). On the other hand, isolate **1**, which had the highest binding energy (−7.41 kcal/mol), showed relatively low cytotoxic effects. Molecular docking study showed the relationship between lower binding energies and lower IC_{50} values, and isolate **3** (*β*-sitosterol) is identified as the most viable EGFR -targeting anticancer candidate.

β-Sitosterol **3** was identified as the major active compound that strongly inhibited the growth of MCF-7 and T47D breast cancer cells (IC_{50} = 20.71 and 18.19 μg/mL, respectively) while demonstrating less toxic effects on normal Vero cells

Table 4. Molecular docking results obtained from bioactive isolates sourced from H. macrothyrsa.

Isolate	Binding Energy (kcal/mol)	Interacting Amino Acids in the Receptors
1 (1-(2,4,6-trihydroxyphenyl)dodecan-1-one)	−7.41	**Conventional H-bonds:** Met(A:769), Gln(A:767) **Van der Waals**: Gly(A:772), Leu(A:768, 834, 764, 753, 694), Thr(A:766, 830), Lys(A:721), Asp(A; 831), Val(702), Cys(A:751) **Pi-Alkyl:** Val residues, along with several π-alkyl interactions
2 (sesamin)	−9.99	**Conventional H-bonds:** Thr(A:830) **Van der Waals**: Exhibited the strongest binding affinity, stabilized mainly by hydrophobic interactions and π-alkyl contacts
3 (β-sitosterol)	−9.15	**Conventional H-bonds**: Met(A:769) extensive hydrophobic interactions distributed across the binding pocket, including multiple alkyl and aromatic residues.
Erlotinib	−8.27	**Conventional H-bonds**: Met(A:769), Cys(A:773), Lys(A:704) **Van der Waals**: Interacted through multiple hydrogen bonds and hydrophobic contacts, including π-related interactions with residues lining the active site.
Doxorubicin	−8.46	**Conventional H-bonds**: Arg(A:817), Lys(A:721), Asp(A:831), Thr(A:766), Met(A:769) **Van der Waals**: Established several hydrogen bonds accompanied by van der Waals and π-sigma interactions, indicating stable receptor binding

(IC_{50} = 100.06 μg/mL). Sesamin **2** was slightly active against MCF-7 cells and weakly inhibited T47D cells, whereas 1-(2,4,6-trihydroxyphenyl) dodecan-1-one **1** only showed weaker effects. The molecular docking results were supported the cytotoxicity trend, as isolate **2** and **3** showed lower (more negative) binding energies to EGFR (−9.99 and −9.15 kcal/mol) compared to isolate **1** (−7.41 kcal/mol). Overall, these findings indicate that β-sitosterol **3** is the most promising EGFR-targeting cytotoxic agent among the isolates, showing potent anticancer activity while maintaining relatively high normal-cell viability. Sesamin **2** also demonstrates potential, albeit with lower efficacy.

4. Conclusion

This research supports the potential of *H. macrothyrsa* leaves as an environmentally friendly source of pharmacologically active secondary metabolites. Three isolate, namely 1-(2,4,6-trihydroxyphenyl)dodecan-1- one (**1**), sesamin (**2**), and β-sitosterol (**3**) were successfully isolated from the ethyl acetate fraction. Among them, isolate **1** exhibited strong antioxidant and antibacterial activities, with an IC_{50} value of 31.06 μg/mL and MIC/MBC values of 15.625/>15.625 mg/mL, respectively. Molecular docking studies of isolate **1**–**3** with EGFR (PDB ID: 4HJO) were consistent with the *in vitro* cytotoxicity results, indicating that isolate **2** and **3** possess favorable binding affinities and cytotoxic effects against MCF-7 breast cancer cells. In addition, isolate **3** demonstrated a certain degree of selectivity toward T47D cells. Overall, the compounds isolated from *H. macrothyrsa* exhibit antioxidant, antidiabetic, and antibacterial potential, while also emerging as promising candidates for anticancer drug development. These findings provide a scientific basis for mechanism-based drug discovery in future studies.

Ethics information

This research did not have any ethical concerns because it involved neither human participants nor animals tests.

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Conflicts of interest

The authors declare that they have no competing interests.

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