Anticancer Activities of Chemical Constituents from Leaves and Twigs of *Mitrephora winitii*

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**Abstract:** The genus *Mitrephora* has been investigated, and its anti-inflammatory, antibacterial, and anti-parasitical activities were examined along with its potential as an anti-cancer cell line and inhibitor for platelet aggregation. In this work, air-dried leaves and twigs of *M. winitii* were grounded and extracted with n-hexane, ethyl acetate, and methanol, respectively. Chromatographic separations of these extracts led to the isolation of three known compounds and one new compound (compound 2). The chemical structures of these were identified using the spectroscopic investigation of 1D- and 2D-NMR, and the resulting data confirmed these as stigmasterol (1), (3,4-dimethoxyphenyl)(5-(3,4-dimethoxyphenyl)-4-(hydroxymethyl)tetrahydrofuran-3-yl)methanol (2), diayangambin (3), and methyl-L-inositol (4). The chemical constituents were reported for the first time in *M. winitii*. Compound 2 showed anti-cancer cell lines with ED₅₀ 13.07 µg/mL against KB cells and then was tested for cytotoxicity against MCF-7 cells with ED₅₀ 11.77 µg/mL.

**Keywords:** Mitrephora winitii; anticancer; extraction; extract

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**INTRODUCTION**

Within the pantropical family of shrubs, trees, and lianas, the *Annonaceae* family comprises an interesting group of medicinal plants. Consisting of roughly 130 genera and 2,500 species, the majority of these are found in Asia, Australia, and Pacific regions [1]. One of these is the genus *Mitrephora*, which is comprised of some 48 species found in Asia and Australia [2]. In Thailand, 12 species are found, and some plants in this genus have been used in the country for the production of folk medicine [3]. These genera have great potential for the treatment of cancers, bacterial infections, brain dysfunctions, and hypertension [1]. Phytochemical investigations have established that *Mitrephora* species contain diterpenoids, polycyctene carboxylic acids/esters, fatty acids, lignans, sesquiterpenes, alkaloids [4-9], the diterpenoids and alkaloids have shown significant anti-microbial, anti-malarial, anti-platelet aggregation, and cytotoxic potential [10-12]. Interestingly, the *M. winitii* was the new source and reported on the antitumor and potent cytotoxic activities of the genus showed high activity anticancer agents.

The *M. winitii* [13] extracts with solvents (n-hexane, ethyl acetate, and methanol) and isolated by column chromatography was found to contain four compounds. They were identified using IR, NMR, and ESI-MS spectrometry to yield stigmasterol (1), (3,4-dimethoxyphenyl)(5-(3,4-dimethoxyphenyl)-4-(hydroxy
methyl)tetrahydrofuran-3-yl)methanol (2), diayangambin (3), and methyl-L-inositol (4). In this paper, we investigated the cytotoxicity of the \( n \)-hexane extract of this plant tested against a panel of two mammalian cancer cell lines. We also report on the isolation and characterization of one new, and three known compounds were found in *M. winitii*.

**EXPERIMENTALSECTION**

**Materials**

*M. winitii* leaves and twigs were collected from Lampang Province in Thailand in January 2011. *M. winitii* was confirmed by the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand, where a voucher specimen (BKF16447) has been deposited. Silica gel (Merck 7734, Mesh 70–230) and TLC 60 PF254 sheets were purchased from Merck. All organic solvents used for extraction and chromatographic separation (CC) were distilled at their boiling point ranges of \( n \)-hexane, ethyl acetate, and methanol (laboratory grade), whereas AR grade solvents were used for crystallization were from Merck.

**Instrumentation**

The melting points of the extracted compounds were measured on a digital electrothermal melting apparatus, and the uncorrected results were recorded in degrees Celsius (°C). IR spectra were recorded on KBr disks using a Shimadzu 8900 FTIR spectrophotometer, whereas major bands (\( \nu_{\text{max}} \)) were recorded at wavenumber (cm\(^{-1}\)) unit, \(^1\)H (400 MHz), and \(^{13}\)C (100 MHz). NMR spectra were determined using either CDCl\(_3\) or D\(_2\)O solution. Chemical shifts were recorded in \( \delta \) values, which were referenced to TMS as the internal standard at \( \delta 0.00 \) ppm. The signal of chloroform at \( \delta 7.26 \) was used as a reference in the case of \(^1\)H-NMR spectra and at \( \delta 77.00 \) in the case of \(^{13}\)C-NMR spectra. The instrument was achieved using a DPX on a Bruker AV 400 spectrometer for 1D and 2D determinations. Low resolution mass spectra were recorded on a Thermo Finnegan Polaris Q mass spectrometer at 70 eV (probe) for the EIMS. High-resolution mass spectra (made using the electrospray ionization mode, ESI-MS) were measured on a micro massQ-TOF-2\(^{\text{TM}}\) (Waters) spectrometer. Column chromatography was conducted on silica gel 60 (Merck 7734, 70–230 mesh). TLC was performed on aluminum backed pre-coated silica gel 60 PF\(_{254}\) sheets, and detections were made using a UV detector at 254 and 365 nm.

**Procedure**

**Extraction and isolation**

Dried and powdered leaves and twigs of *M. winitii* (2.0 kg) were treated at room temperature with \( n \)-hexane, ethyl acetate, and methanol successively. The \( n \)-hexane extract (25.0 g) was subjected to silica gel (Merck 7734, Mesh 70–230) and TLC 60 PF\(_{254}\) sheets were purchased from Merck. All organic solvents used for extraction and chromatographic separation (CC) were distilled at their boiling point ranges of \( n \)-hexane, ethyl acetate, and methanol (laboratory grade), whereas AR grade solvents were used for crystallization were from Merck.

**Evaluation of cytotoxic activity**

The cytotoxic activities of the compounds extracted from *M. winitii* were tested using the *in-vitro* sulforhodamine B (SRB) method. Ellipticine was used as a reference.
a positive control. Test samples were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 3 mg/mL, and these were tested in triplicate with a final concentration of DMSO at 0.5%. The cancer cell lines were grown in a 96-well plate in the following media: P-388, in RPMI-1640 with 5% fetal bovine serum (FBS). The P-388, KB, HT29, MCF-7, A549, ASK, and HEK293 cell lines were cultured in MEM (minimum essential medium with Earle’s salt and l-glutamine) with 10% FBS, while Lu-1 was grown in MEM with 5% FBS. After drug exposure was at 37 °C for 72 h (48h for P-388) with 5% CO2 in air and 100% relative humidity, cells were then fixed with a final concentration of 10% trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. The bound and dried stain was solubilized with 10 mM Trizma base after removing the unbound dye by washing. Absorbance was read on a Fluostar optima BMG plate reader at a wavelength of 570 nm. The cytotoxic activity is expressed as a 50% effective dose (ED50) [14].

The ED50 value was determined by:

\[
\text{% Survival} = \frac{\text{OD (test sample)} - \text{OD (Day 0)}}{\text{OD (0.5% DMSO control)} - \text{OD (Day 0)}}
\]

Criteria of activity: Extracts having an ED50 < 20 µg/mL and pure compounds having an ED50 < 4 µg/mL = Active; No Response = ED50 > 20 µg/mL

### RESULTS AND DISCUSSION

#### Structure Elucidation

Compound 1 was obtained as a white powder; mp 163–164 °C. From the EIMS spectrum [M+H2O+H]+ at m/z 395.37, this could be assigned the molecular formula C29H48O. The IR spectrum showed the broad absorption bands of a hydroxyl group at 3460 cm–1. Absorption bands appearing at 2955 and 2870 cm –1 were due to C–H stretching. C–H bending showed weak absorption bands at 1465 cm–1 and 1377 cm–1, and C=C stretching appeared as a weak absorption band at 1650 cm–1. The medium absorption band at 1050 cm –1 was assigned to C–O stretching. The structure was further elucidated by examination with NMR techniques. The 1H-NMR spectra of compound 1 showed the presence of six methyl signals, which appeared at δ 0.72 (3H, s, H-28), 0.81 (3H, d, J=5.0 Hz, H-27) 0.85 (3H, d, J=5.0 Hz, H-26), 0.87 (3H, t, J=5.0 Hz, H-24), 0.94 (3H, d, J=10.0 Hz, H-19), and 1.06 (3H, s, H-29). The spectra also showed protons at δ 5.03, 5.16, and 5.62 ppm, suggesting the presence of protons corresponding to a tri-substituted and a di-substituted olefinic bond. A comparison of the 1H and 13C-NMR spectral data to data in the literature, together with the melting point of the sample, points to the molecular structure of stigmasterol (1) [9,15].

Compound 2 was isolated as a colorless needle crystal; mp 126-127 °C. Its ESIMS gave a molecular ion peak [M+2H+] at m/z 406, consistent with the molecular formula C22H28O7 (cal. for C22H28O7, 404). In addition, the mass spectrum of the compound found m/z 406 [M+2H+]. The key fragmentation ions in the mass spectrum were at 359, 324, 323, 249, and 151, which was useful in obtaining the structure of the compound (Fig. 2) [16]. The IR spectrum showed absorption bands attributable to hydroxyl at 3400 cm–1 and aromatics at 1617, 1589, and 1519 cm–1. The absorption bands appearing at 2945 and 2850 cm–1 were due to C–H stretching. The C–H bending appeared as weak absorption bands at 1464 and 1375 cm–1. In addition, the methoxy groups showed typical C–O stretching absorptions, which appeared at 1269 and in the range from 1234 to 1160 cm–1. The 1H-NMR signals at δ 6.85–6.93 ppm (6H) represented a tri-substituted phenyl moiety. Two oxygen bearing methylene protons were

![Fig 1. Structure of compounds from M. winitii](image-url)
suggested at δ 3.36 ppm (2H, m, H-3a) and two nonequivalent methylene protons at δ 3.85 (1H, dd, J=5.0, 10.0 Hz, H-5s) and 4.13 (1H, d, J=10.0 Hz, H-5a) ppm. Four methine protons were indicated at δ 2.92 (1H, dd, J=5.0, 10.0 Hz, H-4), 3.32 (1H, m, H-3), 4.45 (1H, d, J=10.0 Hz, H-4a), and 4.88 (1H, d, J=5.0 Hz, H-2) ppm. Four methoxy groups were also indicated at δ 3.89, 3.87, 3.88, and 3.91 (each 3H, s) ppm in the 1H-NMR spectrum. Further spectral evidence was required to confirm the structure of 2. The 1H-1H COSY showed coupling correlations through the sequence of H-2 to H-3, H-3 to H-3a, H-4 to H-5, and H-4 to H-4a for the connectivity of the protons to the structure. The connectivity of the aromatic carbon skeleton (e.g., C-5', C-6', C-5'', and C-6'') was also confirmed by the COSY correlations (Fig. 3). The HMBC spectrum showed crossed peaks between the aromatic signals (H-2', H-5', and H-6') and C-1', C-2', C-3', and C-4' and between H-6', H-2' and C-2, which indicated the aromatic ring was connected to C-2. Further, the aromatic signals (H-2'', H-5'', and H-6'') and C-1'', C-2'', C-3'', and C-4'' and between H-6'', H-2'' and C-4a indicated the aromatic ring was connected to C-4a (Fig. 3). The 1H-13C spectrum revealed signals from 22 atoms, and DEPT experiments also showed 16 protonated carbon signals, thereby revealing the presence of six quaternary carbons in the molecule. The presence of a trisubstituted phenyl ring was evident from the signals at δ 109.11, 111.14, 117.76, 131.02, 148.09 and 148.92 (a ring connected C-2) and signals at δ 109.26, 111.14, 118.47, 133.75, 148.78 and 149.29 (a ring connected C-4a). Additionally, oxymethine carbon signals were found at δ 82.07, 87.63, and methoxy carbons at δ 55.93, 55.96, and 55.97. A literature search revealed that cis- and trans- orientation of substituents at C-2 and C-3 give a signal of H-2 at δ 4.76 and 4.91 (J=4, 4.8 Hz), respectively. The H-2 signal of compound 2 (δ 4.88 and J=5.0 Hz) thus agreed well with the assignment of a cis-configuration. The relationship between the torsion angle and vicinal coupling constant $J$ is given theoretically by the Karplus equation: 

$$J(HH) = P_1 \cos^2 \phi + P_2 \cos^2 \phi + P_3 \Delta \chi \{P_1 + P_2 \cos^2 (\xi \phi + P_3 \Delta \chi)\}$$

[17]. So, the relative configuration at H-4 and H-4a could be determined by the $J_{4,4a}$, H–C–C–H (10 Hz) coupling constant, which would indicate that the two protons were located on opposite sides with a dihedral angle of 180°. Furthermore, the H-4a showed a signal at δ 4.45 ppm

**Fig 2. The mass spectral fragmentation of compound 2**

**Fig 3. 1H-1H COSY correlations and the selected HMBC correlations of compound 2**
Table 1. $^{13}$C and $^1$H-NMR data of compound 2 and $^1$H-$^1$H, $^1$H-$^1$C correlations exhibited in the 2D NMR spectra in CDCl3

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_c$(ppm)</th>
<th>$\delta_d$(ppm)</th>
<th>COSY</th>
<th>HMBC</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>2</td>
<td>82.07</td>
<td>4.88, d(5.0)</td>
<td>H-3</td>
<td>3, 3a, 1', 2', 6'</td>
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<tr>
<td>3</td>
<td>50.18</td>
<td>3.32, m</td>
<td>H-2, H-3a, H-4</td>
<td>2, 3a, 4, 4a</td>
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<tr>
<td>3a</td>
<td>69.73</td>
<td>3.36, m</td>
<td>H-3</td>
<td>2, 3, 4</td>
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<tr>
<td>4</td>
<td>54.50</td>
<td>2.92, d(5.0,10.0)</td>
<td>H-3, H-4a, H-5</td>
<td>2, 4a, 1''</td>
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<tr>
<td>4a</td>
<td>87.63</td>
<td>4.45, d(10.0)</td>
<td>H-4</td>
<td>3, 4, 5, 1'', 2'', 6''</td>
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<tr>
<td>5</td>
<td>71.04</td>
<td>4.13, d(10.0)</td>
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<tr>
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<td>6.93, s</td>
<td>2, 1', 3', 4', 6'</td>
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<tr>
<td>3'</td>
<td>148.92</td>
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<td>4'</td>
<td>148.09</td>
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<tr>
<td>5'</td>
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<td>2, 1', 2', 3', 4', 6'</td>
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<tr>
<td>6'</td>
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<td>H-5'</td>
<td>2, 1', 2', 3', 4'</td>
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<td>2''</td>
<td>109.26</td>
<td>6.92, s</td>
<td>4a, 1'', 3', 4', 6''</td>
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<tr>
<td>3''</td>
<td>149.29</td>
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<tr>
<td>4''</td>
<td>148.78</td>
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<tr>
<td>5''</td>
<td>11.14</td>
<td>6.85, m</td>
<td>H-6''</td>
<td>1'', 2'', 3', 4', 6''</td>
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<tr>
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<td>4a, 1'', 2''</td>
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<tr>
<td>4''-OMe</td>
<td>55.96</td>
<td>3.91, s</td>
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</table>

and $J$=10.0 Hz, giving a spectra data assignment to the trans-configuration. On the basis of the above data [18-19], the structure of 2 was determined to be (3,4-dimethoxyphenyl)(5-(3,4-dimethoxyphenyl)-4-(hydroxylmethyl)tetrahydrofuran-3-yl)methanol, which has not been previously reported. The resonances for the protons of the tetrahydrofuran skeleton between two propargyl groups showed nonequivalent for H-3/H-4, H-2/H-4a, and H-5/H-3a; therefore, requiring an unsymmetrical substitution stereochemistry of the furan system; especially the chemical shifts of the benzylic oxymethine protons H-2 (4.88, 5.0 Hz, d) and H-4a (4.45, 10 Hz, d) confirmed that two the propanyl groups substituent are asymmetrical in compound 2 [18-19]. When compared, the protons for the furan system of compound 3 showed the equivalents of H-7/H-7’ and H-8/H-8’, therefore, requiring a symmetrical substitution stereochemistry for the aryl substituents and furan ring. Moreover, the few coupling constants and the chemical shifts of the benzylic oxymethine protons of H-7 and H-7’, showed $J=5.0$ Hz, $\delta$ 4.92, and for bridge carbons, C-8/C-8’ (49.43/49.43) confirmed that two aryl substituents are symmetry in compound 3 [20]. Support the absolute configuration of compound 2 was studied by electronic circular dichroism (ECD) spectroscopy. The optimized structure of compound was performed by the density functional theory (DFT) calculation at the B3LYP/6-31G (d,p) level of theory (Fig. 4). ECD spectra were carried out by using TD-DFT method at the CAM-B3LYP/6-311G++(d,p) including PCM model (MeOH) (Fig. 5). The rotary strengths of 80 excited states were calculated. All calculations were performed using Gaussian09 program package. Gaussian bandshape with a bandwidth of 0.25 eV was used to simulate ECD spectra.
The ECD curve was generated by SpecDis 1.64 (University of Wurzburg, Wurzburg, Germany) softwares. The proposed biosynthesis pathway correctly showed to reasonably assure the specific structure for the unknown compound 2 (Fig. 6) [21].

Medicinal plants are important sources of bioactive compounds in cancer suppression and treatment [1]. Plants of the *Mitrephora* genera were used to treat sickness in folk medicines [3]. The *in-vitro* sulforhodamine B (SRB) method assay to study the inhibition of cell viability on seven cancer cell lines P-388, KB, HT29, MCF-7, A549, ASK, and HEK293 by treatment of seven naturally occurring pure compounds, compared with an ellipticine drug. Compound 2 showed moderated anti-proliferative activity against KB and MCF-7 cell lines, with ED_{50} values of 13.07, and 11.77 µg/mL, respectively, which is reported here for the first time.

Compound 3 was obtained as a white needle crystal; mp 144–145 °C. Its EIMS gave a molecular ion peak [M]^+ at m/z 445, which was consistent with the molecular formula C_{24}H_{30}O_{8} (cal. for C_{24}H_{30}O_{8}^+, 445). The mass showed the fragmentation characteristics described for liriosinol-B dimethyl ether. The EIMS spectrums showed fragmentation ions in the mass spectrum at m/z 249, 219, 195, 181, 177, and 165. The IR spectrum of the compound showed medium absorption bands at 1634, 1614, 1589, and 1509 cm⁻¹, which were characterized as aromatic C=C stretching. The strong absorption bands at 2935 and 2840 cm⁻¹ were characterized as C–H stretching, while the corresponding bending vibrations appeared at 1422 and 1367 cm⁻¹. The
C–O stretching showed medium absorption bands at 1129 and 1004 cm⁻¹. The structure of compound 3 was further elucidated through 1D- and 2D-NMR experiments. The ¹H-NMR spectrum showed the presence of tetra-substituted aromatic protons at δ 6.62 (4H, s, H-2,6,2',6') ppm. A doublet of methine proton H-7,7' was observed at δ 4.92 (2H, d, J=5.0 Hz, H-7,7') ppm and the resonances at δ 3.90 (12H, s, 3,5,3',5'-OMe) and 3.87 (6H, s, 4,4'-OMe) ppm indicated a methoxy proton. A doublet of methylene protons (Hα,β-9) on a tetrahydrofuran ring was observed at δ 3.74 (2H, dd, J₁=1.8 Hz, J₂=9.6 Hz, Hα-9,9') and 3.58 (2H, dd, J₁=6.7 Hz, J₂=9.6 Hz, Hβ-9,9') ppm. The ¹³C-NMR spectrum exhibited the resonances of a quaternary aromatic carbon at δ 153.22 (C-3,5,3',5'), 137.02 (C-1,1'), 134.58 (C-4,4'), and four aromatic methine carbons at δ 103.17 (C-2,6,2',6') ppm. Methine carbons were found at δ 84.08 (C-7,7') and 49.43 (C-8,8') ppm, and methylene carbons at δ 68.89 (C-9,9') ppm. Deshieldedoxymethyl carbons were indicated at δ 68.89 (C-9,9'), 60.89 (4,4'-OMe), and 56.10 (3,5,3',5'-OMe) ppm. The HMBC correlation of H-2 to C-4, C-6, and C-7, and H-7 to C-1, and C-9 confirmed an aromatic ring connecting to the tetrahydrofuran ring. These data were in accordance with those of diayangambin (3) [20,22].

Compound 4 was obtained as a white crystal; mp 190–192 °C. The IR spectrum showed the broad absorption band of a hydroxyl group at 3420 cm⁻¹. The absorption bands at 2940 and 2840 cm⁻¹ were due to C–H stretching. In addition, the hydroxyl and methoxy groups showed typical C–O stretching absorptions, which appeared at 1146 cm⁻¹ and in the range from 1119 to 1066 cm⁻¹. The structure of the compound was further elucidated by 1D- and 2D-NMR experiments. The ¹H-NMR displayed signals for protons of oxygenated carbons at δ 3.46 (1H, m, H-2), 3.48 (1H, m, H-3), 3.58 (1H, m, H-4), 3.92 (1H, m, H-5), and 4.13 (1H, t, J=5 Hz, H-6) ppm, while a methoxy group was indicated at δ 3.31 (3H, s, -OMe) ppm. One proton at δ 3.26 (1H, m, H-1) ppm was assigned to a methine proton adjacent to the oxygen of an ether group. The ¹³C-NMR spectrum showed a resonance signal at δ 80.06 ppm, which was assigned to a carbon of ether, while the methoxy carbon showed at δ 56.79 ppm. The signals at δ 72.74, 71.83, 70.27, 71.28, and 67.03 ppm also indicated oxymethylene carbons. The COSY spectrum showed correlations between H-1 and H-2, H-2 and H-3, H-3 and H-4, H-4 and H-5, H-5 and H-6, and H-6 and H-1. The HMBC spectrum demonstrated the correlation of H-OMe to C-1, indicating the methoxy was connected at C-1, and this confirmed the position of the methoxy group. These data were in accordance with those recorded for methyl-L-inositol (4) [23-25].

**CONCLUSION**

The investigation focused on the phytochemical of medicinal plant together with biochemical evaluation.
The results presented four compounds derivative from \textit{M. winitii} were carried out from crude extract of \textit{M. winitii} found the compounds; stigmasterol (1), (3,4-dimethoxyphenyl)(5-(3,4-dimethoxyphenyl)-4-(hydroxy methyl)tetrahydrofuran-3-yl)methanol (2), diayangambin (3), and methyl-L-inositol (4). Chemical constituents were the first report isolated from \textit{M. winitii}, in addition, compound 3,4-dimethoxyphenyl)(5-(3,4-dimethoxyphenyl)-4-(hydroxymethyl)tetrahydrofuran-3-yl)methanol (2) was new the structure. Other than the compound 2 can effectively inhibit the growth of the KB and MCF-7 cancer cell lines; when compared with an ellipticine as the positive control.

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