Characterization and Investigation of Stigmasterol Isolated from Rodent Tuber Mutant Plant (Typhonium flagelliforme), Its Molecular Docking as Anticancer on MF-7 Cells

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Abstract: Typhonium flagelliforme is an Indonesian herbal plant used and applied traditionally to treat cancer diseases. Gamma rays have irradiated rodent tuber mutant plant at six doses gray to increase the chemical compounds of anticancer activity. The effect of isolated compounds from rodent tuber mutant plants has never been studied and published yet. Our study unveiled the potential of stigmasterol as a remarkable cytotoxic agent and the significant contribution of NMR spectroscopy, IR, Mass spectra, QTOF MS towards the isolation and identification of this anticancer agent. Stigmasterol was isolated from T. flagelliforme mutant plant. Stigmasterol was more effective against MCF-7 cells with an IC50 value of 0.1623 µM than Cisplatin with IC50 value 13.2 µM. It is the most potential and active fraction in the human breast cancer cell line. The molecular docking study analyzed the chemical profile of stigmasterol to confirm the receptor in agonist binding sites. The prediction of the toxicity of stigmasterol compounds using in silico and analysis of its interaction with the receptor can act as a competitive regulator with a high-affinity binding site on FXR. Stigmasterol has potential as a candidate for an anticancer drug that promoting further clinical action.

Keywords: Typhonium flagelliforme, MCF-7 cell line, stigmasterol, agonistic, mutant plant

1. Introduction

Rodent tuber with latin name Typhonium flagelliforme, known as a local name in Indonesia, belongs to the Araceae family. T. flagelliforme is a herbal plant from Indonesia that plays an active role in anticancer activity in various cancer cell lines, including lung and breast cancer cells [1], T4-lymphoblastoid leukemia [2], T47D breast [3], MCF-7 breast [4]. Gamma rays successfully irradiated T. flagelliforme plants through combined mutagenesis of somaclonal variations in vitro to increase the number of bioactive compounds contained therein. T. flagelliforme mutant plants have been detected to undergo genetic changes in the first generation (MV1) [5]. The second-generation (MV2) and third-generation (MV3) T. flagelliforme mutants have shown genetic changes through molecular RAPD by comparing wild-type [6]. T. flagelliforme mutant plant received Plant Variety Protection by the Indonesian Ministry of Agriculture in 2020. T. flagelliforme mutant plant has been shown to have
bioactive compounds, and its potential as an anticancer agent has increased [6-8]. The ethyl acetate fraction of the superior *T. flagelliforme* mutant has potential as an anticancer with an IC	extsubscript{50} value of 1.09 µg/mL. In comparison, the ethanol extract has an IC	extsubscript{50} value of 1.60 µg/mL [9]. This indicated that the cytotoxic activity of *T. flagelliforme* mutant extract was three times more effective than wild-type extract, with an IC	extsubscript{50} value of 19.11 µg/mL [8].

Based on the GC-MS analysis of the chemical components of the KB 6-3-3-6 *T. flagelliforme* mutant plant extract, it was found that hexadecenoic acid, octadecadienoic acid, stigmasterol, and beta-sitosterol compounds were more significant in quantity than wild type [10]. The stigmasterol compound can be an antioxidant and reduces Ehrich Ascite Carcinoma (EAC) by reducing lipid peroxidation and increasing catalase in the liver of EAC rats [11]. Stigmasterol is the main phytoster in various herbal plants with anti-inflammatory properties and anticancer agents [12]. Stigmasterol can inhibit the growth of uterine cancer cells ES2 and OV90 by 50% at a treatment concentration of 20 µg/mL [13]. However, the molecular mechanism of stigmasterol compounds isolated from *T. flagelliforme* mutant plants has not yet been revealed on breast cancer cell death (MCF-7 cell line).

*In silico* methods, such as molecular docking, have been used to predict the molecular mechanism of bioactive compounds at the atomic level [14-16]. Therefore, in this work, we also explored the anti-breast cancer activity of the isolated compound using the molecular docking method. Molecular docking is critical in the planning and layout of new drugs. The aim of having a compulsory experimental mode and small molecule affinity within the binding site of the desired target receptor. A successful splicing methodology must correctly predict the native ligand to which the receptor binds and the associated physicochemical molecular interactions [17]. In this study aims, stigmasterol compound was selected to characterize the anticancer activity and examine the pathway expression mechanisms in vitro through an *in silico* evaluation using molecular docking studies to explore the binding mode and its interaction with the MCF-7 breast cancer cell line.

### 2. Materials and Methods

#### 2.1. Plant Material

*T. flagelliforme* mutant plant KB 6-3-3-6 was collected from Sianipar & Purnamaningsih’s collection. It has been irradiated by gamma-ray with a dose of 6 Gy to produce *in vitro* mutagenesis and obtained as mutant plants. The mutant plant was acclimatized and maintained at Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Ministry of Agriculture, Bogor, Indonesia.

#### 2.2. Extraction and Isolation

The tubers of *T. flagelliforme* mutant KB 6-3-3-6 were harvested and dried into a powder. *T. flagelliforme* mutant plant powder was submerged in ethanol 96% and repeatedly macerated seven times until the extract indicated colorless. The solvents were evaporated using a rotary evaporator (Rotavapor® R-300, Buchi) at 50°C. The highly concentrated ethanol extract was obtained. Extracting ethyl acetate separation was chosen using the KCV method to use vacuum assistance to make it faster and the separation more efficient. Separation with KCV is based on the principle of adsorption with the adsorbent as the stationary phase used is silica gel G60 (70-230 mesh) with *n*-hexane-ethyl acetate-methanol solvent system about 10% gradient. The results of KCV of ethyl acetate extract obtained 21 fractions, and each fraction was analyzed by TLC on a GF	extsubscript{254} silica plate eluted with *n*-hexane:ethyl acetate (1:1) to determine the staining pattern of the compounds contained in each fraction of the separation results. Sub-fraction was obtained and separated by column chromatography on silica gel (70-230 mesh) with a gradient *n*-hexane-methylene chloride-ethyl acetate about 5% solvent system. The combined sub-fraction was collected.
and separated by column chromatography on silica gel (230-400 mesh) with a solvent system of *n*-hexane-methylene chloride-isocratic ethyl acetate (6:2.5:1.5), then obtained compound 1 as yellow solid (4.8 mg).

2.3. Characterization of Chemical Structures from the Mutant Plant

The IR spectra and mass spectra were recorded on a SHIMADZU IR Prestige-21 in KBr and Waters Xevo QTOF MS using an NMR JEOL ECZ-500 and Variant Unity INOVA-500 Spectrometer (Agilent Technologies, Santa Carla, United States). The NMR data were recorded at 500 MHz for *¹H* and 125 MHz for *¹³C*, using TMS as internal standard. Column chromatography was conducted on the silica gel 60 (70–230 and 230–400 mesh, Merck), after which TLC analysis was carried out on 60 GF₂₅₄ (Merck, 0.25 mm) using various solvent systems to detect spots by irradiating under ultraviolet-visible light (257 and 364 nm) and heating of silica gel plates, sprayed with H₂SO₄ in EtOH (10%).

2.4. Cell Viability Evaluation by PrestoBlue Assay

Cell viability of MCF-7 breast cancer cells was performed by the PrestoBlue assay method. MCF-7 cells were purchased from American Type Cell Cancer (Manassas, VA, US) that cultured in Eagle’s Minimum Essential Medium (EMEM) medium (Sigma Aldrich; Merck KGaA, Darmstadt, German) contained 10% Fetal Bovine Serum (FBS) (Sigma Aldrich; Merck KGaA) and 1% of 100 µg/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich; Merck KGaA). Cisplatin (Sigma Aldrich; Merck KGaA) was used as a positive control. Each well of 96 well plates contained the cells to be tested with cultured medium replacement. 100 µL of medium containing stigmasterol from *T. flagellforme* superior mutant with serial concentration obtained 2423.05, 1211.52, 605.76, 302.88, 151.440, 75.72, 37.86, 18.92 µM of test solution. The amount of 100 µl of PrestoBlue solution (Invitrogen; Thermo Fisher Scientific, Waltham, MA, US) (10% in medium without FBS) was added to each well plate incubated at 37°C and 5% CO₂ gas for 24 h. The change in the fluorescence of the test reagent (resazurin to resorufin) was measured using an Infinite M200 PRO microplate reader (Tecan, Männedorf, Switzerland) with the excitation wavelengths set at 570/600 nm. The conversion value is proportional to the number of metabolically active cells and can be measured quantitatively. The percentage of cell viability equals (absorbance of treated cells/absorbance of untreated cells) × 100%. The morphological features were captured using by microscope EVOS XL Core Imaging System (Invitrogen; Thermo Fisher Scientific).

2.5. In Silico Bioprospection of Stigmasterol

The crystal structure of natural ligand and agonist ligand binding with Farsenoid X Receptor (FXR) was downloaded from the PDB library (PDB ID: 7D42 and 1OT7) [18],[19]. The ligands structure was separated from the receptor using Discovery Studio 2021, the water molecules and heteroatoms were also removed. Chem3D modeled the 3D structure of stigmasterol. Docking simulation with a rigid receptor structure was performed with AutoDock 4.2.6 [20]. Furthermore, the 3D structure of stigmasterol was converted into the PDBQT format by employing the Gasteiger charges and adding the atom types using AutoDock Tools 1.5.6. The size of the grid box of the receptor was 40 x 40 x 50 points with a grid spacing of 0.375 Å, centered in the binding site of the ligand. The Lamarckian Genetic algorithm was used with the following parameters: trials of 100 runs, energy evaluations of 2,500,000, and population size of 150. The energy breakdown analysis and ligand efficiency (total binding energy divided by a number of atoms) were also calculated. The docking result was visualized using Biovia Discovery Studio Visualizer and sorted based on
the free energy of binding on the most populated cluster and their intermolecular interactions.

3. Results and Discussion

3.1. Chemical Structure Characterization

The fraction ethyl acetate from the dry powder of tubers *T. flagelliforme* was chromatographed over a vacuum-liquid chromatographed (VLC) column, packed with silica gel 60 by gradient elution. The VLC fractions were repeatedly subjected to standard and reversed-phase column chromatography and preparative TLC on silica gel GF254 to accommodate compounds 1.

Stigmasterol (1) was observed as a colorless amorphous solid, with its molecular composition established as C29H49O2, based on HR-TOFMS. This showed a [M+H]+ ion peak at m/z 429.3654 (calc. C29H49O2: m/z 429.3650), requiring to six degrees of unsaturation. The IR spectrum showed bands which were ascribed to a hydroxyl (νmax 3495 cm⁻¹).

Furthermore, the 1H-NMR spectrum (Table 1) showed six primary methyls at δH 0.68 (3H, s, H-18), 0.85 (3H, t, H-29), 0.81 (3H, d, H-27), 0.84 (3H, d, H-26), 0.92 (3H, d, H-21) and 1.00 (3H, s, H-19) which indicates the characteristics of the compounds within the phytosterol group steroid framework [21]. Nine methylene protons sp³ at δH 1.51 (2H, H-11), 1.27 (2H, H-28), 1.13; 1.61 (2H, H-15), 1.35; 1.86 (2H, H-16), 1.85; 1.56 (2H, H-2), 1.53 (2H, H-7), 1.15; 1.89 (2H, H-1), 1.19; 2.03 (2H, H-12) and 2.28 (2H, H-4) and there are seven methine protons sp³ at δH 1.71 (1H, H-25), 1.91 (1H, H-8), 1.37 (1H, H-20), 0.97 (1H, H-24), 0.98 (1H, H-9), 1.10 (1H, H-17) and 1.00 (1H, H-14), one oxygenated methine proton at δH 3.53 (1H, m, H-3) and three methine protons sp² at δH 5.36 (1H, dd, 2.5; 7.2 H-6), 4.97 (1H, dd, 5.5; 14.2, H-22) dan 5.11 (1H, dd, 4.5; 14.2, H-23) indicated olefinic binding to C-22 and C-23 is trans.

The 13C NMR (Table 1) together with the DEPT spectra revealed twenty-nine carbons consisting of an six methyl carbon signals on δc 11.9 (C-18), 11.9 (C-29), 18.9 (C-27), 19.0 (C-26), 19.5 (C-21), and 19.7 (C-19), nine methylene carbon sp³ at δc 21.2 (C-11), 23.2 (C-28), 24.4 (C-15), 28.4 (C-16), 31.7 (C-2), 31.9 (C-7), 37.3 (C-1), 39.9 (C-12) and 42.5 (C-4) and there are seven metine carbons sp³ at δc 29.2 (C-25), 31.9 (C-8), 36.4 (C-20), 45.9 (C-24), 50.2 (C-9), 56.0 (C-17), 56.8 (C-14), one oxygenated metine carbon at δc 71.9 (C-3), three metine carbons sp² at δc 121.9 (C-6), 129.4 (C-22) dan 138.5 (C-23), two quaternary carbon sp³ at δc 36.6 (C-10), 42.4 (C-13) and one quaternary carbon sp² at δc 140.9 (C-5).

![Figure 1. Structures of stigmasterol (1).](image)
### Table 1. NMR data compound 1 (500 MHz for $^1$H dan 125 MHz for $^{13}$C).

| Position | $^{13}$C-NMR δc/ppm | $^1$H-NMR δH (∑H, Mult, J/Hz) |
|----------|----------------------|-------------------------------|
| 1        | 37,3                 | 1,15; 1,89 (2H, m)            |
| 2        | 31,7                 | 1,85; 1,56 (2H, m)            |
| 3        | 71,9                 | 3,53 (1H, m)                  |
| 4        | 42,5                 | 2,28 (2H, m)                  |
| 5        | 140,9                | -                             |
| 6        | 121,9                | 5,36 (1H, dd, 2,5; 7,2)       |
| 7        | 31,9                 | 1,53 (2H, m)                  |
| 8        | 31,9                 | 1,93 (1H, m)                  |
| 9        | 50,2                 | 0,98 (1H, m)                  |
| 10       | 36,6                 | -                             |
| 11       | 21,2                 | 1,51 (2H, m)                  |
| 12       | 39,9                 | 1,19; 2,03 (2H, m)            |
| 13       | 42,4                 | -                             |
| 14       | 56,8                 | 1,00 (1H, m)                  |
| 15       | 24,4                 | 1,13; 1,61 (2H, m)            |
| 16       | 28,4                 | 1,35; 1,86 (2H, m)            |
| 17       | 56,0                 | 1,10 (1H, m)                  |
| 18       | 11,9                 | 0,68 (3H, s)                  |
| 19       | 19,7                 | 1,00 (3H, s)                  |
| 20       | 36,4                 | 1,37 (1H, m)                  |
| 21       | 19,5                 | 0,92 (3H, d, 7)               |
| 22       | 129,4                | 4,97 (1H, dd, 5,5; 14,2)      |
| 23       | 138,5                | 5,11 (1H, dd, 2,5; 14,2)      |
| 24       | 45,9                 | 0,97 (1H, m)                  |
| 25       | 29,2                 | 1,70 (1H, m)                  |
| 26       | 19,0                 | 0,84 (3H, d, 7)               |
| 27       | 18,9                 | 0,82 (3H, d, 7)               |
| 28       | 23,2                 | 1,27 (2H, m)                  |
| 29       | 11,9                 | 0,85 (3H, t, 7)               |

3.2. Cytotoxicity of Stigmasterol on MCF-7 Cell Line

Effects of the *T. flagelliforme* mutant plant isolate known as stigmasterol have inhibition activity against breast cancer MCF-7 cell line at 24 h. The minimum concentration of the extract giving a 50% inhibition (IC$_{50}$) was about 0.1623 µM (Fig. 4). Stigmasterol showed to be the most active and potential, comparing favorably with Cisplatin about 13.2 µM [22]. Based on the IC$_{50}$ value of stigmasterol showed the most robust cytotoxic activity on MCF-7 cell lines. The morphological cytotoxic activity was expressed as a percentage of cell viability in MCF-7 cell lines (Fig. 5). Observation of MCF-7 cells after treatment with stigmasterol for 24 hours showed slow cell proliferation. Furthermore, decreasing cells revealed that treatment at 605.76 M compared to control (DMSO) decreased the ability to adhere to MCF-7 breast cancer cell lines. The significant
anticancer effect of the *T. flagelliforme* mutant plant is maybe due to the presence of the isolated bioactive compound of stigmasterol from the extract.

Al-Fatlawi [23] reported that stigmasterol exerts anti-proliferative activities against the human cancerous breast MCF-7 cancer line with the IC$_{50}$ value of about 27.38 µM. The cytotoxicity concentration of stigmasterol evaluated against MCF-7 cell line with IC$_{50}$ value was 14.5 µg/mL from *Kopsia singapuraensis* Ridl. [24]. In another study, cytotoxicity stigmasterol isolated from *Polygonum hydropiper* L. more effective against MCF-7 by killing 87.50% cancerous cells at 1 mg/mL concentration [25]. Previous studies above consistent with this study that stigmasterol has cytotoxic activity in MCF-7 cells, although the IC$_{50}$ value stigmasterol that isolated from *T. flagelliforme* mutant plant much lower than the stigmasterol obtained in other plants. The plants extract with IC$_{50} \leq$ 4 µg/mL as the most effective crude extracts and pure compounds as cytotoxic agents against cancerous cells [26]. The IC$_{50}$ value of the stigmasterol isolated from the *T. flagelliforme* mutant plant is 0.1623 µM. This value is much lower than the extract of 1.60 µg/mL, and the ethyl acetate fraction of 1.09 µg/mL of the *T. flagelliforme* mutant plant is rational [9].

Our previous studied demonstrated that triterpenoids (pentandrucines A to K) isolated from the *n*-hexane extract of *Chisocheton pentandrus* is more robust cytotoxic activity against MCF-7 breast cancer cells in vitro with an IC$_{50}$ value of 16.84 µM than the crude extract [22].

This study indicates that a stigmasterol isolated compound from *T. flagelliforme* mutant compared to the crude and fraction extract is the most potent to inhibit cell proliferation on MCF-7 cell lines. Li et al. [27] reported that stigmasterol inhibits the proliferation and induced apoptotic cells in human gastric cancer cell line SNU-1 through JAK/STAT signaling pathway with an IC$_{50}$ value of 15 µM. Stigmasterol has been clarified
can induce apoptosis and autophagy in gastric cancer cells by blocking the Akt/mTOR signaling pathway [28]. Stigmasterol isolation from an n-hexane extract of Cheilanthes tenuifolia reported has LC\textsubscript{50} value of about 205.984 µg/mL on shrimp nauplii. The effect of stigmasterol was isolated from Bacopa monnieri showed that the inhibition of tumor cells of EAC-bearing mice with 60.11% at 10 mg/kg [11].

Stigmasterol has been investigated for its pharmacological prospects such as antiosteoarthritic, anti-hypercholesterolemic, cytotoxicity, antitumor, hypoglycaemic, antimutagenic, antioxidant, anti-inflammatory, and CNS effects [29]. The anticancer potential of stigmasterol on various cancer has been revealed in recent times. In lung cancer, it reduces the expression of Bcl-2 as an antiapoptotic protein and enhances the BAX as a proapoptotic protein [30]. The development of skin cancer also has been suppressed by the VEGFR-2 and TNF-alpha downregulation [12], the lipid peroxide enhancement, and also by causing DNA damage [31]. The stigmasterol also induces the expression of p53 protein while inhibits the expression of p21 and p27 protein to represses prostate cancer development [33]. Furthermore, in breast cancer, stigmasterol induced growth inhibition and apoptosis \textit{in vitro} in a time- and dose-dependent manner [32].

3.3. FXR- Stigmasterol Docking

The binding mode of stigmasterol in the FXR was investigated from its crystal structure (PDB ID: 7D42). It showed that the interactions between stigmasterol and FXR are favorable and tend to be stable. The ligand-binding site was in the center of FXR-LBD, which is around the helix-3, helix-5, helix-6, and helix-10 (Fig. 4a). It is also far from the NcoA co-regulator peptide binding site. Hence it can be assumed that there will be no alteration in the whole structure conformation of FXR-LBD. The lowest binding energy of stigmasterol-FXR is -12.01 kcal/mol, while the binding with 3-deoxy CDCA as its natural ligand results in -11.17 kcal/mol. The average RMSD value of stigmasterol conformations is lower than 2 Å (Fig. 4b) which is also proved the stability of its binding with FXR.

It is shown that the hydroxyl groups in C2 of stigmasterol formed hydrogen bonds with Thr288. The steroidal core of stigmasterol formed CH-pi interaction with Trp454, Leu287, and Ala291, while the rest of the interactions between stigmasterol and FXR was hydrophobic (Fig. 5a-b). It can be assumed that the hydrophobic interactions play a significant role in binding stability.
As a member of the nuclear receptor superfamily, the function of FXR is to regulate bile acid homeostasis. A high level of FXR expression was detected in epithelial cells of normal and tumor breast and the invasive ductal carcinoma cells. This FXR was also present in the human breast cancer cells (MCF-7) and carcinoma cells (MDA-MB-468) [33],[34]. Moreover, in the MCF-7 cell line, FXR activation has been assumed to down-regulate breast cancer target genes such as the MRP-1, MDR3, SLC7A5, and aromatase with the inhibition of cell proliferation [34],[36]. Previous studies have also shown that the stigmasterol emerged as a potent FXR agonist in breast cancer cases [36-39]. In this work, the results from PrestoBlue Assay showed that stigmasterol could inhibit the activities of the MCF-7 cell line, which is consistent with the above findings. As displayed in Fig. 4 and 5, stigmasterol could be docked to FXR and result in high binding affinity by hydrophobic interactions. It is indicated that the steroid moiety in the structure of stigmasterol might be crucial in the binding affinity. The residues involved in stigmasterol-FXR interaction were in the ligand-binding domain [39]. This indicated that stigmasterol might act as a competitive activator with a single high-affinity binding site on FXR. These findings showed that the proper drug-like characteristics and potency cytotoxicity of stigmasterol from \textit{T. flagelliforme} mutant plant were found.

4. Conclusions

In summary, stigmasterol isolated compound from \textit{T. flagelliforme} mutant plant is more robust against MCF-7 breast cancer cell line \textit{in vitro} with an IC$_{50}$ value of 0.1623 $\mu$M. Stigmasterol can induce oxidative stress in MCF-7 cells that have led to apoptosis. This is facilitated by a competitive activator with a single high-affinity binding site on FXR with hydrophobic interactions. The molecular docking study showed that the interactions between stigmasterol and FXR are favorable, whereas its binding energy is lower than its natural ligand. It is postulated that the cytotoxic effect of stigmasterol towards MCF-7 cells may be due to the receptor’s agonist binding sites.

![Figure 5 The ligand-receptor interactions between stigmasterol and FXR. (a) The stigmasterol as steroid ligand. (b) The 2D diagram interactions of stigmasterol-FXR was visualized using discovery studio.](image-url)
**Authors Contributions:** Conceptualization N.F.S., Y.E.H., K.A., and M.Y.; methodology, Y.E.H., K.A., S.S., W.D.; formal analysis, Y.E.H., K.A., S.S., W.D.; writing-review and supervision, N.F.S., Y.E.H., and M.Y.; writing-original draft preparation, K.A., and W.D.; editing, K.A.; resources the mutant plant, N.F.S., and R.P.; funding acquisition, I.G.S. All authors have read and agreed to the published version of the manuscript.

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Farnesoid X receptor inhibits tamoxifen-resistant MCF-7 breast cancer cell growth through downregulation of HER2

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