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Original Research Article

Sterculia foetida Leaf Fraction Against Matrix Metalloproteinase-9 Protein and 4T1 Breast Cancer Cells: *In-Vitro* and *In-Silico* Studies

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ABSTRACT

Sterculia foetida leaf extract has been shown to have cytotoxic activity. Matrix metalloproteinase-9 (MMP-9) has an important role in pathophysiological functions. Inhibition of MMP9 is an important therapeutic approach for combating cancer. This study was conducted to determine the most active fraction of *S. foetida* as anti-breast cancer agent with hemopexin-like domain of MMP-9 (PEX9) as the selective protein target and 4T1 cells line as metastatic breast cancer cell. The leaves *S. foetida* was extracted using 80% methanol and was fractionated into fractions of n-hexane, chloroform, ethyl acetate, n-butanol, and insoluble n-butanol with liquid-liquid partition. *In vitro* screening against MMP-9 was performed using FRET-based assay and cytotoxic tests were performed using the MTT assay. Identification of compounds in the most active fraction using GC-MS. The docking to PEX9 was run using AutoDock Vina embedded in PyRx program. The n-hexane fraction was the most active fraction to inhibit MMP-9 with an IC_{50} of 19.67 µg/mL and inhibit the growth of 4T1 cells with an IC_{50} of 34.65 µg/mL. NNGH was used as positive control for the *in-vitro* and *in-silico* studies. The GC-MS results of the n-hexane fraction showed that there were 23 compounds, and they had binding affinity score of -8.9 to -4.9 kcal/mol towards PEX9. It can be concluded that *S. foetida* leaf has the potential to be developed for therapeutic use, especially for breast cancer therapy.

***Keywords*:** *Sterculia foetida*, Fraction, Cytotoxic, MMP-9, 4T1, PEX9.

Introduction

Breast cancer is one of the most common cancers in women and is the leading cause of cancer deaths worldwide.[1] The incidence of breast cancer increases significantly from year to year, accompanied by the tendency to be diagnosed with cancer at a young age.[2] The genetic heterogeneity of breast cancer in different countries is shown to be significantly different.[3] The incidence of breast cancer in developed countries is higher than in developing countries.[4] Research has shown that breast cancer's predisposing factors is known to be associated with oral contraceptive use, age, time of menopause, and ethnicity.[5] Besides, single nucleotide polymorphism (SNP) is a risk factor for breast cancer in individuals, although the exact mechanism of breast cancer tumorigenesis is not fully understood.

Matrix metalloproteinases (MMPs) are a family of intracellularly present and zinc (Zn^{2+}) dependent endopeptidases that can regulate other proteases, chemokines, growth factors, cytokines, and cell receptor activity.[6] Also, MMPs can degrade extracellular matrix components.[7] MMPs participate in angiogenesis, cell proliferation, immune surveillance, and apoptosis and thus play an important role in tumor initiation and development.[8] Specifically, Matrix metalloproteinase-9 (MMP-9) is an important member of the MMP family that plays an important role in cancer cell metastasis. The expression of MMP-9 can change due to genetic variations that affect the effectiveness of breast cancer treatment.[9]

There has been many studies proving that chemical compounds in plants have cytotoxic activity. This is due to variations in the structures of the chemical compounds and the mechanisms for causing different cytotoxic effects.[10] This has led many researchers to conduct research to explore plants and chemical compounds that have anti-cancer effects.[11] *S. foetida* is a medicinal plant that has many pharmacological activities.[12] Mujumdar *et al.*[13] reported that the leaves of *S. foetida* contain luteolin, α-sitosterol, scutellarein, taraxerol, n-octacosanol, and procyanidin, which have analgesic and anti-inflammatory activity. Rajasekharreddy and Rani[14] reported finding cyclopropene fatty acid compounds, such as (2n-octylcycloprop-1-enyl)-octanoic acid (I) from *S. foetida* seeds which have antibacterial, antiviral, and cytotoxic activities.

In this study, we explored *S. foetida* leaves fractions of n-hexane, chloroform, ethyl acetate n-butanol, and insoluble n-butanol as a possible source of compounds that have cytotoxic activities. The in vitro inhibitory activities of these fractions against MMP-9 protein and 4T1 cancer cells were further studied. The compounds contained in the most active fraction were identified using Gas Chromatography-Mass Spectroscopy (GC-MS). The interaction of the compounds in the most active fraction against a protein target, MMP-9 involved in cancer pathogenesis was analyzed through molecular docking techniques.

Materials and Methods

Software and hardware

The 3D structure of PEX9 (PDBID: 1ITV), the hemopexin domain of MMP9, was downloaded from the Protein Data Bank (PDB, www.rcsb.org). and the structures of known PEX9 inhibitors (external

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validation data) that have arylamide structure linked to the planar pyrimidine ring by flexible ethylene chain were taken from published literatures (15). Lenovo laptop with Core i5 processor, Windows 10 operating system with 8 GB RAM and 1 TB Hard Disk was the hardware.

Chemicals

Methanol, n-butanol, ethyl acetate, chloroform, n-hexane were purchased from Merck (Darmstadt, Germany). 4T1 (ATTC^R CRL-2539) and Vero (ATTC^R CCL-81) cells were cultured in DMEM supplemented with 10% FBS (Fetal bovine serum) and 1% Pen-Strip at 37 ℃ and 98% humidity containing 5% CO_2 in humidified incubator. Cispaltin and 3-(4,5-dimethylthiazol-zyl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, USA). The MMP-9 enzyme kit was purchased from BioVision (Milpitas, USA), FRET-based MMP-9 substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg), MMP-9 assay buffer, and NNGH inhibitor (N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid) as its positive control.

Plant materials and extractions

Fresh leaves of *S. foetida* were collected from Kupang City, East Nusa Tenggara, Indonesia (March 2020). Washed thoroughly with water, dried, and homogenized to a fine powder. A voucher specimen (MCH/003/X) was identified by Dr. Budi Sumarta as Taxonomist and deposited at Laboratory of Pharmacognosy, Ma Chung University Malang, Indonesia. 2 Kg of *S. foetida* leaves powder was soaked in 6 liters of 80% methanol by maceration method. The solvents were then removed under pressure to obtain sticky residues. Fractionation was carried out by mixing 70 g of the extract with 600 mL of 80% methanol and then mixing it until it was homogeneous. After that, separated by n-butanol, ethyl acetate, chloroform, and n-hexane, respectively to 600 mL using a separating funnel by liquid-liquid fractionation. All fractions were used for in vitro MMP-9 Inhibition assay and cytotoxic assay.

In vitro MMP-9 inhibition assay

The MMP-9 assay was adopted from Hariono *et al.*[16]. The lyophilized enzyme was re-constituted using 110 µL of glycerol 30% in deionized water. The reconstituted enzyme was diluted into 550 µL of buffer and ready to be used in assay. The crude fraction sample was prepared by dissolving it in DMSO to yield a final concentration of 1 mg/mL in the 96-microwell plate. The final concentration of DMSO in the well plate did not exceed 2%. Firstly, the samples were properly mixed with the buffer before adding the enzyme. The mixture was then incubated at 37^{o}C for 30 minutes. The substrate (40 µM) was added to the mixture and then incubated at 37^{o}C for 60 minutes. The fluorescence was read using Jasco F08500 Microplate Reader at 325/393 nm. The NNGH inhibitor was prepared using a similar process except for the final concentration which was 0.02 µM. When the least % inhibition attained 50%, the compounds were proceeded for IC_{50} determination by preparing a series of concentrations. The data calculation and the drug-dose dependent curve were prepared by Microsoft excel.

In vitro cytotoxicity assay

Cytotoxic assay on 4T1 cells using the MTT colorimetric test 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide based on Rollando[17] with modification. Cells (2.5×10^4/well) were seeded 96-well flat-bottomed plates and incubated with% CO_2 at 37 °C for 24 h. After that, the treatments were performed with 50 µg/mL Cisplatin, eight concentrations of test solutions (3.125, 6.25, 12.5,25, 50, 100, 250, and 500 µg/mL), negative control was DMSO 1%. The cytotoxic assay was carried out for 24 h. Results were expressed as percentage of cell viability with respect to untreated control cells (as 100%).

GC-MS analysis

GC-MS was used to identify structures in the most active fraction. The sample was dissolved in methanol and injected in an Agilent 7890A GC system coupled with an MS (Agilent Technologies) with an injection volume of 2 µL. The column used was a 35 × 950 mm glass column, using a flame ionization detector (ionization: 70eV), and helium carrier gas with a flow rate of 1 mL/min. Operating conditions on GC-MS: initial temperature of 160°C held for 2 min, then increased to 300°C at 5°C/min, and the final temperature of 300 °C. The total running time of the GC was 30 min. The structures of the identified compounds were compared with the data base from the NIST14.L library (2018).

Molecular docking

The first step was carried out by internal validation. The crystal structure of PDB ID 1ITV consisting of PEX9 with sulfate ion was downloaded from Protein Data Bank.[18] The sulfate ion was separated from PEX9 using Discovery Studio 3.5 and stored in a PDB format.[16] Granted Gasteiger Charge using AutoDockTools1.5.6. (This is not a complete sentence) [15] Polar hydrogen and a Kollman charge are applied to the PEX9 structure created by the same program. Docking was performed with AutoDock Vina embedded in the PyRx program[19] with a grid (completeness = 8; sizes 25, 25, 25 and center x = -42.05, y = -30.82, z = -7.26). If the RMSD value is given to be less than 2Å, the docking is declared valid.[20] The second step is external validation, using compounds that have been proven to be inhibitors for PEX9.[16] Inhibitor structures with inhibitory activity against PEX9 in the range of 0.30-4.89 M were sketched and optimized (MM+ force field) using the ChemDraw Ultra 12.0 Program.[21] The docking of the inhibitor compound was carried out with the same parameters as the internal validation. Free energy of binding and True Positive Rate (TPR = sensitivity) were observed. The last step was carried out in silico prediction of the compounds identified by GC-MS in the most active fraction. Ligands were sketched and geometrically minimized using ChemDraw Ultra 12.0 2D and 3D. The same protocol carried out in docking on internal validation. The output data is collected as a CSV file, and the compounds were tabulated according to the binding free energy. The selected docking pose was visualized using Discovery Studio 3.5.

Statistical analysis

Statistical analysis was performed using SPSS 20 software (SPSS, Chicago, IL, USA). Data were analysed using a one-way analysis of variance followed by post hoc Tukey's multiple comparisons test in SPSS 20 software. Values of $P < 0.05$ were considered to be statistically significant.

Results and Discussion

In-vitro MMP-9 assay

The five fractions obtained from the fractionation were tested on MMP-9 using in-vitro FRET-based assay. The test results showed that the five fractions had an inhibitory activity of more than 50% against MMP-9. The positive NNGH control had an inhibition of 95.13%. The highest inhibition was shown by the fraction of n-hexane (89.12%). This was followed by ethyl acetate (74.72%), insoluble n-butanol (74.72%), n-butanol (65.83%), and chloroform (63.82%) (Figure 1).

Following the IC_{50} determination (Table 1), the most potent fraction is n-hexane (19.67 µg/mL) followed by n-butanol fraction (78.82 µg/mL). In addition, there were 3 fractions that had IC_{50} values above 100 µg/mL which are chloroform fraction (162.72 µg/mL), the insoluble n-butanol fraction (172.13 µg/mL), and the ethyl acetate fraction (348.53 µg/mL). The n-hexane fraction showed a high percentage of inhibition and IC_{50} because the compounds in the n-hexane fraction can bind stronger with amino acids in the active site on MMP-9.

In-vitro cytotoxic assay

In-vitro tests on 4T1 cells were performed to support the MMP-9 test. 4T1 cells are metastatic cancer cells known to express MMP-9. The five fractions tested against 4T1 showed a trend that suggests a concentration-dependent inhibition of 4T1 cell growth (Figure 2). The cytotoxic test results on 4T1 cells showed that Cisplatin as a positive control had an IC_{50} of 6.98 µg/mL. The n-hexane fraction was the most active fraction against 4T1 cells with an IC_{50} of 34.65 µg/mL,

followed by the chloroform fraction (55.34 µg/mL), ethyl acetate fraction (76.89 µg/mL), n-butanol fraction (79.55 µg/mL), and the insoluble n-butanol fraction (283.42 µg/mL). The IC_{50} fractions of n-hexane and cisplatin were not statistically different. This indicates that n-hexane and cisplatin fractions were equivalent in cytotoxic activity against 4T1 cells. The fractions of n-hexane, chloroform, and n-butanol were classified as active (10-100 µg/mL). Meanwhile, the insoluble n-butanol fraction was classified as moderate (100-500 µg/mL).

In addition to the testing of 4T1 cells, the fraction was also tested against Vero cells to evaluate the toxicity to normal cells. The evaluation parameter used was the selectivity index as a reference that the cells were less toxic to normal cells but toxic to cancer cells with a selectivity index value >2.[17] n-hexane fraction, which was the most active fraction, had a selectivity index value of 3.39, followed by chloroform (3.67), ethyl acetate (2.45), and n-butanol (4.80) (Table 2).

The treatment of the n-hexane fraction caused morphological changes in 4T1 cells (Figure 3). The nucleus appeared shrunken, rounded, and some cells experienced membrane blebbing. In contrast, the untreated cells showed normal morphology. The treatment of the n-hexane fraction also caused morphological changes in Vero cells; the cells were shrunken, rounded and no longer attached to the bottom of the well. However, higher concentration was required to produce the same effect on 4T1 cells. The untreated vero cells showed normal morphology. Further investigation is needed to confirm that these changes that caused cell death are due to the process of either apoptosis or cell cycle inhibition.

Compounds identification of the most active fraction

Identification of the structure of chemical compounds in the n-hexane fraction using GC-MS. The identification results showed six compounds with different retention times (Rt) (Figure 4). These compounds were identified based on their molecular weight and fragmentation profiles according to the database. Six compounds with molecular weight (m/z) 104 (3.11%), 222 (21.91%), 414 (45.38%), 426 (12.78%), 440 (2.18%), and 468 (3.23%) were identified as 3, 4 Furanthiol (4; Rt 8,874 minutes), 2,4-cyclohexadiene-1-one, 3,5 bis (1,1-dimethyl ethyl) -4-hydroxy (5; Rt 21,910 minutes), gamma sitosterol (12; Rt 16,636 minutes), Lupeol (14; Rt 20,101 minutes), 9,19-cyclo-9β-lanostan-3β-ol, 24 methylene (23; 24,086 minutes), and Lupeol acetate (23; Rt 24,086 minutes) (Table 3), respectively. Not all compounds detected using GC-MS can determine their structure; this is because their fragmentation patterns do not match the database. The compounds identified are hydroxy, phenolic and steroid compounds that meet the Lipinski Rule of Five so that the analysis of ligand-receptor interactions with molecular docking can be carried out.

Molecular docking

Docking control is carried out to ensure that the parameters in the docking simulation are correct. The control docking test results show the native ligand position (sulphate ion) and the docked position is 1.80 Å. The RMSD control docking value is included in the required RMSD, which is <2.[21] After that, an external validation was carried out to increase confidence that the simulation protocol is correct. The external validation used 13 compounds which were proven to have PEX9 inhibitory activity experimentally. Figure 5 shows the initial pose of the sulphate ion as a native ligand and the overlapped docked poses of 13 ligands used in the external validation. The published binding affinity of the 13 compounds[22] used in the external validation is shown in Table S1 (Supporting Information).

The results of the external validation show that the 13 compounds that have been docked to PEX9 show bond energies of -8.80 to -5.20 (kcal/mol) (Table S1). All of these compounds have high interaction and lower energy than the sulphate ion as a native ligand (-3.5 kcal/mol), and this shows that the 13 compounds are more active than the sulphate ion. The activity of a compound can be categorized as active (Kd <1.00 µM) or inactive (Kd> 1.00 µM).[23] Based on this, there are nine compounds classified as active and four compounds classified as inactive. Correlation of Kd values and docking affinity can be used to classify into true positive (TP) for active compounds that are predicted to be active, false positives (FP) for active compounds that are predicted to be active, true negative (TN) for the inactive, and false negatives (FN).[24] The test results showed that there were five compounds in the true positive (TP) category, three compounds in each of the true negative and false positive categories, and two compounds in the false-negative category (Table S2). The validity of the docking control parameters was evaluated by TPR (True Positive Rate), FPR (False Positive Rate), and TNR (True Negative Rate). The results showed that the TPR and TNR approached 1.00 and the FPR approached 0.00 indicated that the protocol docking could validate the activity of published compounds. Based on docking control and external validation, the docking parameters were used for further virtual screening.

The docking protocol was used for screening interactions of compounds identified by GC-MS. Six compounds are docking with PEX9 with a binding affinity of -4.5 to -8.9 kcal/mol (Table 4). The test results showed that Gamma-sitosterol had the highest interaction with PEX9, followed by lupeol, lupeol acetate, 2,4-cyclohexadiene-1-one, 3,5 bis (1,1-dimethyl ethyl) -4-hydroxy, 9,19 -cyclo-9β-lanostan-3β-ol, 24 methylene, and 3,4 furanthiol. The six compounds had a higher interaction than the native ligand (sulphate ion), furthermore, 3,5 bis (1,1-dimethyl ethyl)-4-hydroxy, 9,19-cyclo-9β-lanostan-3β-ol, 24methylene, and 3,4 furanthiol had a higher interaction than positive control (NNGH). This indicates that the three compounds have great potential as MMP-9 inhibitors. The binding affinity was found to be contributed by the molecular interactions such as steric interaction, hydrophobic interaction, and hydrogen bonding (Figure 6 and 7). Six possible binding residues such as SER172, LEU113, ARG106, ALA159, LEU113, and SER107 were found to be involved in an interaction with lead inhibitors. Moreover, the docking studies provide useful insight into the mechanism of reference inhibitor binding to the active site. Protein-ligand interaction plays a significant role in structure-based drug designing.

Sterculia foetida is empirically used to treat infectious diseases, hypertension, and anti hyperlipidemia.[14] However, the use of *S. foetida* for cytotoxic assay have not been widely studied. Therefore, we conducted this research to increase knowledge and the use of *S. foetida* as raw material for cancer drugs. The results of the in-vitro MMP-9 assay showed that the n-hexane fraction had an inhibitory activity and an IC_{50} which was almost equivalent to a positive control for N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH) (P> 0.05). These results indicate that the compounds in the n-hexane fraction can bind to the catalytic site in MMP-9. The catalytic site of the MMP-9 enzyme consists of the amino acid residue GLU402 and zinc ion, which is coordinated by the histidine triad (401, 405 and 411).[25]

The results of the molecular docking analysis showed that the lead compound did not interact with the essential amino acids in the receptor. This may be due to leads are working on other sites with allosteric activity. However, compounds in the n-hexane fraction are hypothesized to be able to form chelates with zinc ions and GLU402 to attract water closer to the scissile amide inhibitor bonds, thereby preventing proteolysis of the peptide substrate by enzymes.

The n-hexane fraction showed the highest inhibitory activity against the MMP-9 enzyme and 4T1 cells. Thus, there is a positive correlation between the MMP-9 assay and the cytotoxic assay on 4T1 cells. 4T1 cells are breast cancer cells that have undergone metastasis and can express MMP-9 for proliferation and angiogenesis.[26] MMP-9 causes degradation of gelatin and collagen types IV, V, XI and XVI, which are essential for metastasis.[6] MMP-9 promotes metastasis through the degradation of the ECM physical barrier resulting in cell migration. The cells penetrate the blood vessels and lymphatic vessels so that the cells attach to the endothelium. Finally, this leads to secondary tumour cell growth in other organs.[7] Hypothetically, if the activity of the MMP-9 protein is inhibited, the proliferation and metastasis processes of 4T1 cells will be inhibited. However, further investigation is still required to confirm the activity of the n-hexane fraction on the cell migration assay.

The chemical compounds in the n-hexane fraction are responsible for the inhibition of the enzyme MMP-9 and 4T1 cell lines. The group of compounds mostly identified in that fraction are steroid compounds.

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Steroid compounds have been widely studied to have cytotoxic activity[27-29]. Gamma-sitosterol is a steroid compound that is mostly found in the n-hexane fraction. Major compounds have significant contribution in enhancing pharmacological effects.[30] Khan dand Mlungwana[31] reported that gamma sitosterol isolated from the *Markhamia zanzibarica* and *Kigelia africana* plants had an activity to inhibit the growth of MCF-7 and T47D breast cancer cells. Also, it has been reported that gamma sitosterol from the leaves of *Acacia nilotica* L. inhibits the growth of Human MCF-7 and A549 cell lines with apoptotic mechanisms and inhibits the cell cycle at the G2/M phase and decreases the c-Myc expression.[32]

On the other hand, lupeol and lupeol acetate are steroid compounds found in the n-hexane fraction. Bednarczyk-Cwynar *et al.*[33] in their research reported that the lupeol found in *Mangifera indica* leaves inhibited the growth of HeLa, KB, MCF-7, and A-549 cell lines in the active category. Previous study reported that triterpene lupeol was obtained from the leaves of *Dendropanax cf. querceti* which has cytotoxic activity against Hep-G2, A-431, and H-4IIE tumour cell lines.[34] Nguyen *et al.*[35] isolated lupeol acetate from *Plumbago zeylanica*, which resisted the growth of MCF-7 cells by apoptotic mechanism and inhibited the cell cycle. Ogunlaja *et al.*[36] reported that lupeol acetate compound from bark *Ficus burtt-davyi* was found to inhibit MCF-7 and colorectal adenocarcinoma (Caco-2) cancer cell lines in the active category.

Currently, the n-hexane fraction activity test provides a new added value to the use of *S. foetida* as herbal medicine, especially in inhibiting the enzymes MMP-9 and 4T1 cancer cell lines. This study provides important information on the n-hexane fraction, which can inhibit 4T1 cancer cell lines with high selectivity to normal cells (SI = 3.59). GC-MS determination showed that the n-hexane fraction contained steroid-derived compounds that have never been reported to inhibit the MMP-9 enzyme and selectively against 4T1 cells. Therefore, the n-hexane fraction from *S. foetida* is considered to have great potential to be developed as herbal medicine that can inhibit the growth of metastatic cancer cells.

Table 1: Percentage inhibition against MMP-9 *in vitro* and IC_{50}

Crude extract	% Inhibition	IC_{50} (µg/mL)
n-hexane	89.12 ± 3.62	19.67 ± 4.84
Chloroform	63.82 ± 5.82	162.72 ± 23.98*
Ethyl acetate	76.61 ± 1.12	348.53 ± 13.43*
n-butanol	65.83 ± 3.13	78.82 ± 9.53*
Insoluble n-butanol	74.72 ± 2.39	172.13 ± 2.31*
NNGH	95.13 ± 1.22	5.18 ± 0.23

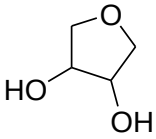
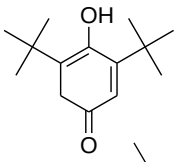
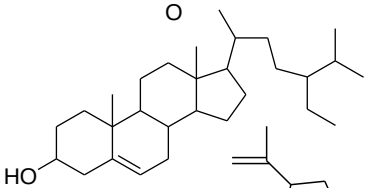
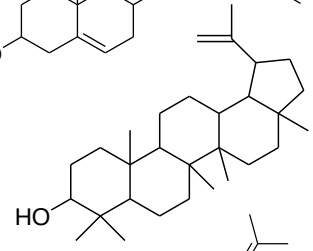
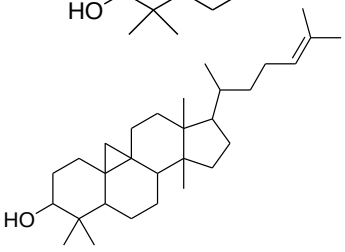
Values area means ± SD, n = 3 Replicates, *p value < 0.05 then indicate statistically significant differences in comparison to NNGH.

Table 2: IC_{50} and selectivity index on 4T1 and Vero cells

No	Sample	IC_{50} (µg/mL ± SD)		Selectivity Index
		4T1	Vero	
1	n-hexane	34.65 ± 4.11	124.55 ± 7.31	3.59
2	Chloroform	55.34 ± 3.87*	203.13 ± 8.95*	3.67
3	Ethylacetate	76.89 ± 5.56*	188.73 ± 3.83*	2.45
4	n-butanol	79.55 ± 1.54*	381.84 ± 89.74*	4.80
5	Insoluble n-butanol	283.42 ± 65.97*	231.87 ± 45.24*	0.82
6	Cisplatin	6.98 ± 0.85	93.34 ± 2.74	13.37

Values area means ± SD, n = 5 Replicates, *p value < 0.05 then indicate statistically significant differences in comparison to cisplatin.

Table 3: Compounds have been identified from n-hexane fraction of the *S. foetida* leaf

Codes	R_t (Min)	Percent Area (%)	Molecular Weight	Chemical Names	Structure
4	8.874	3.11	104	3,4 Furanthiol	
5	10.185	21.91	222	2,4-cyclohexadiene-1-one,3,5 bis (1,1-dimethyethyl)-4-hydroxy	
12	16.636	45.38	414	Gamma-sitosterol	
14	20.101	12.78	426	Lupeol	
18	22.513	2.18	440	9,19-cyclo-9β-lanostan-3β-ol, 24 methylene	

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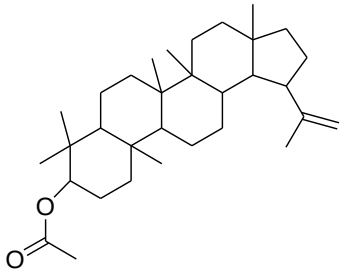
23	24.086	3.23	468	Lupeol acetate	

Table 4: The binding affinity of the positive control (NNGH), top six ligands from n-hexane fraction, and amino acid residues

Ligand	Binding Affinity (kcal/mol)	Amino Acid Residues
NNGH	-5.7	Phe142, Leu113, Ala159, Gln154 Glu157, Arg106
3,4 Furanthiol	-4.5	Ser172
2,4-cyclohexadien-1-one,3,5 bis (1,1-dimethyethyl)-4-hydroxy	-5.5	Leu113
Gamma-sitosterol	-8.9	Arg106, Ala159, Leu113
Lupeol	-7.9	Arg106
9,19-cyclo-9β-lanostan-3β-ol, 24 methylene	-5.4	Ser107
Lupeol acetate	-7.3	Leu113, Ser107

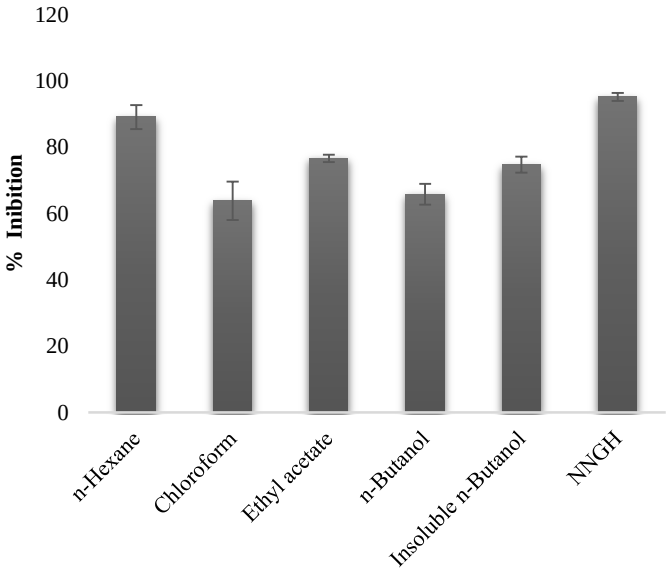


Figure 1: The histogram of inhibition percentage from 5 partitions from methanol extract of *S. foetida* leaf against MMP-9 activity in vitro.

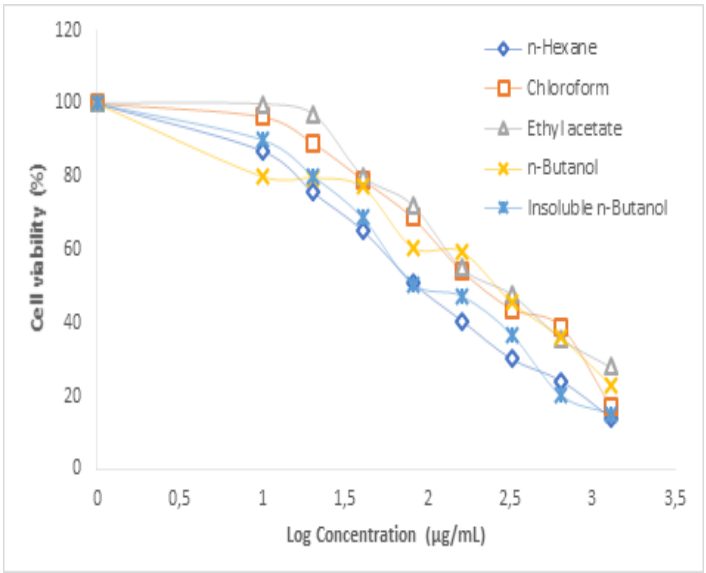


Figure 2: The drug dose-dependent curves of five fractions against 4T1 cells.

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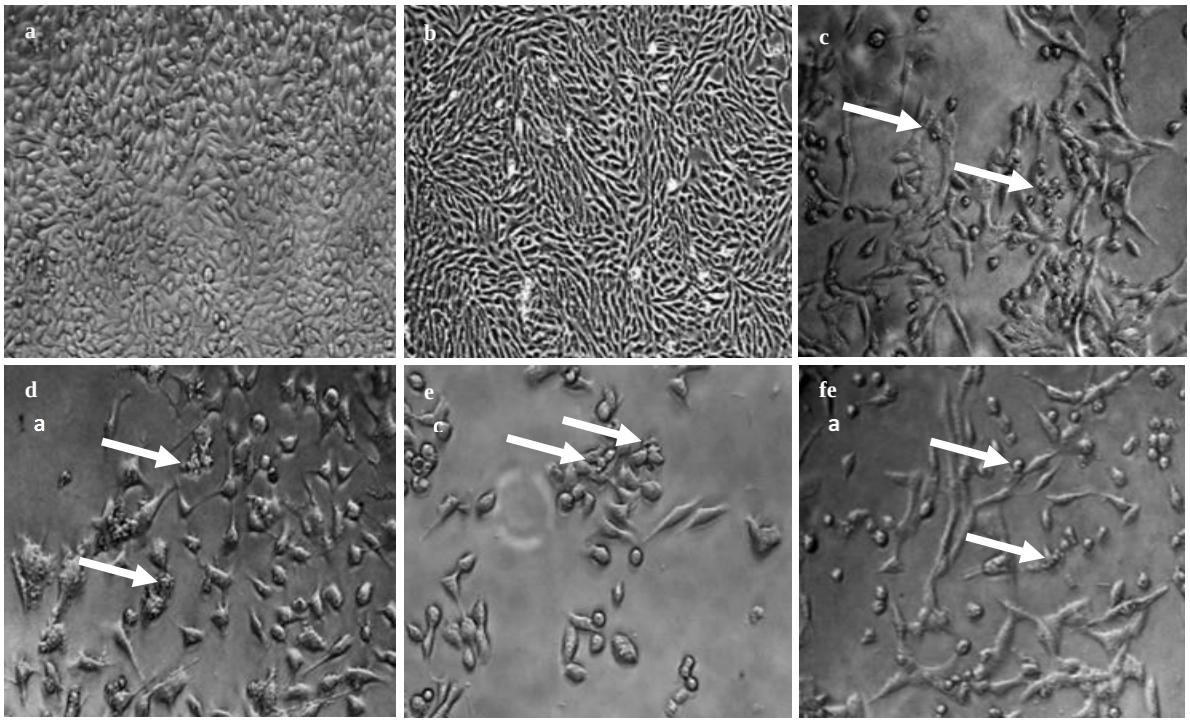

Figure 3: Morphological changes after 24 hours
(a) Untreated 4T1 cells, (b) Untreated Vero cells, **(c)** IC_{50} treatment of Cisplatin on 4T1 cells, **(d)** IC_{50} treatment of Cisplatin on Vero cells, **(e)** IC_{50} treatment of n-hexane fraction on 4T1 cells, **(f)** IC_{50} treatment of n-hexane fraction on Vero cells.

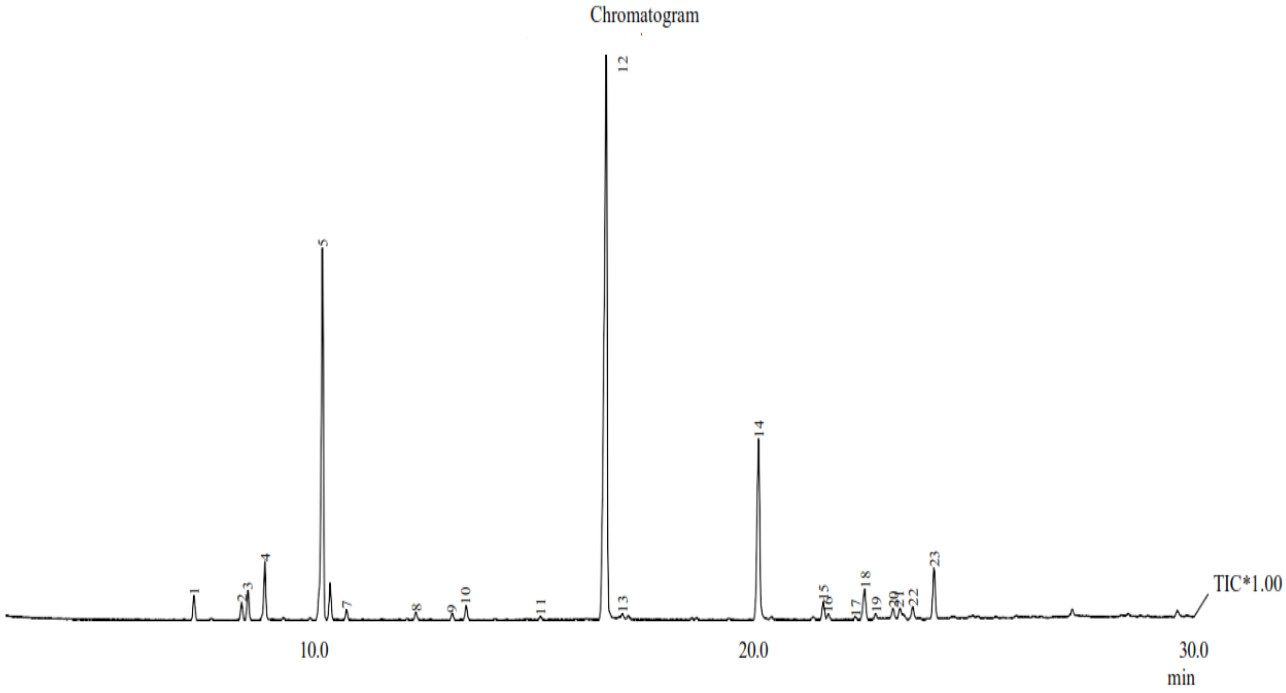

Figure 4: GC chromatogram of n-hexane fraction of *S. foetida* leaf

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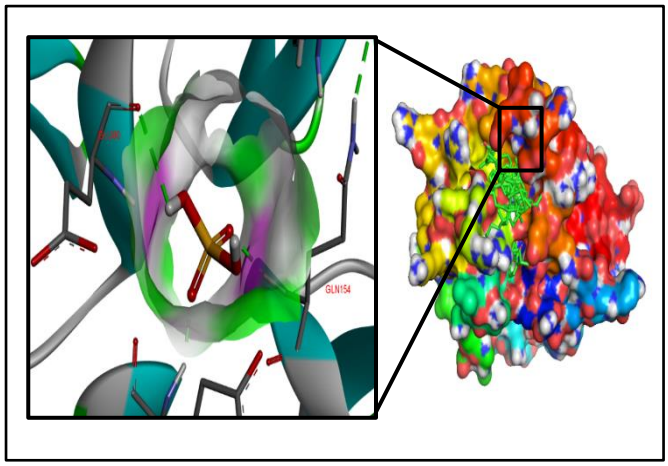

Figure 5: Superimposition of 13 published PEX9 inhibitor six ligand scores docked into PEX9 with deep pocket in the left side and shallow pocket in the right side.
Inset is the control docking of sulfate ion with red and yellow stick interacts with amino acids GLU60 and GLN154. The external ligands are colored in green stick.

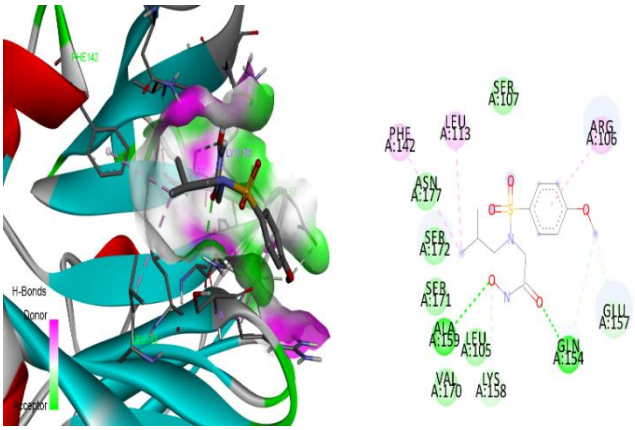

Figure 6: The docking poses of NNGH bindings. NNGH interact with amino acid residues GLN154 and GLU157. The H-bond interaction is visualized as green dashed line

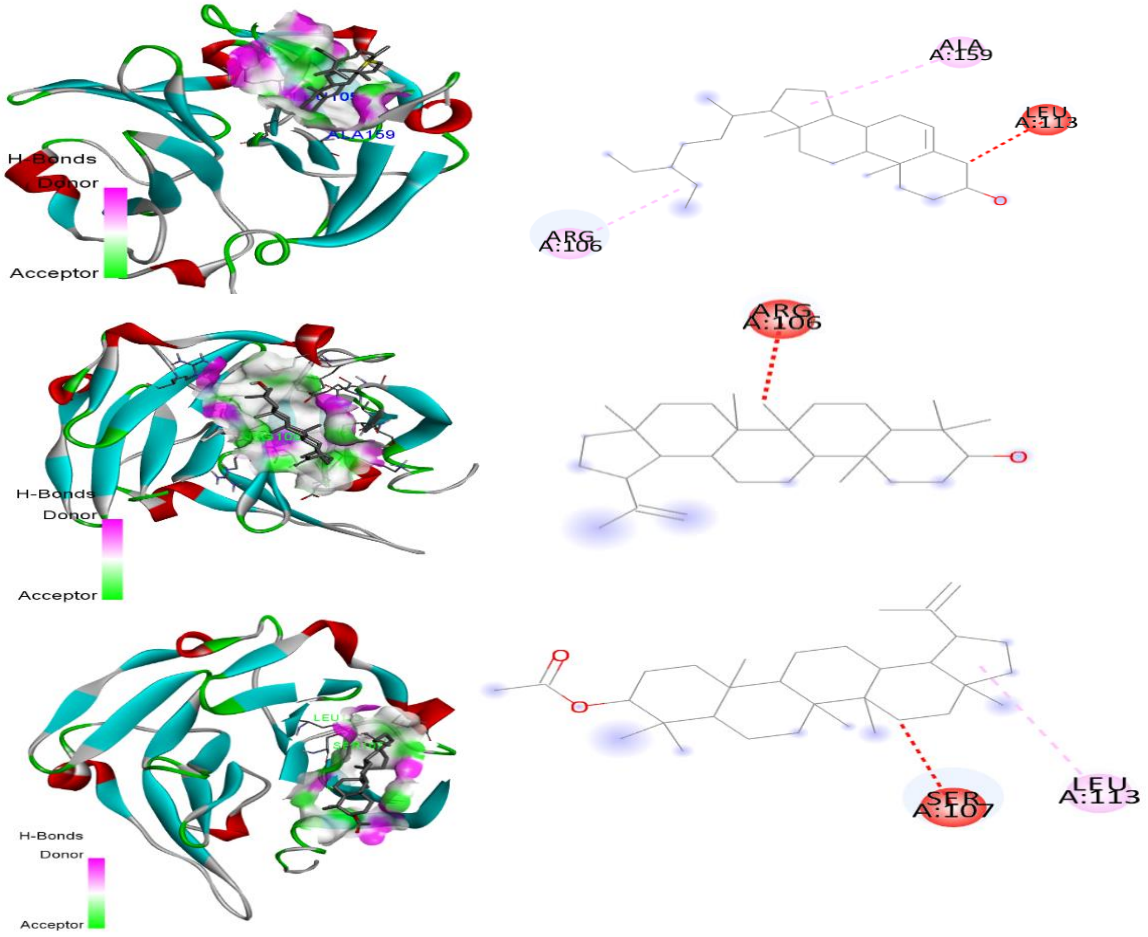

Figure 7: The docking poses of three top bindings (a) Gamma-sitosterol, **(b)** Lupeol and **(c)** Lupeol acetate at the binding site of PEX9 The H-bond interaction is visualized as black dashed line

Conclusion

Traditionally, the leaves of *S. foetida* have been shown to have pharmacological activities. The leaves of *S. foetida* were fractionated using five solvents. The n-hexane fraction was the most active fraction in inhibiting the MMP-9 enzyme with an IC_{50} value of 19.67 ± 4.84 µg/mL. The cytotoxic test results of the n-hexane fraction showed the highest inhibitory activity against 4T1 cells with an IC_{50} value of 34.65 ± 4.11 µg/mL. However, this fraction was inactive against normal Vero cells. The GCMS of the n-hexane fraction was determined and this detected 23 compounds of which only the structures of six compounds were identified. These six compounds include steroid and phenolic derivatives. The ligand-receptor interaction revealed that gamma-sitosterol showed the greatest inhibitory activity against PEX9 with the lowest binding energy of -8.9 kcal/mol.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

This research article is part of the doctoral thesis, and this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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