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Triterpenoids from the Stems of *Nauclea orientalis* (L.) L. (Rubiaceae)

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ABSTRACT

Nauclea orientalis (L.) L. is a well-known source of varied secondary metabolites that underlie its traditional therapeutic uses and pharmacological activity. Studies on this plant in Vietnam have identified various compounds, but an investigation into the triterpenoids from its Vietnamese species and their specific biological activities has not been thoroughly conducted. This study focuses on the isolation, structural elucidation, and bioactivity evaluation of triterpenoids from the chloroform-soluble fraction of *Nauclea orientalis* stems. Through combined chromatographic methods, four triterpenoids (**1**–**4**) were successfully isolated. These compounds were identified as 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid (**1**), rotundic acid 3,23-acetonide (**2**), oleanolic acid (**3**), and ursolic acid (**4**), based on spectroscopic analysis and comparison with literature data. Notably, a complete comprehensive NMR dataset, including ^{1}H, ^{13}C, HSQC, HMBC, and NOESY correlations, was provided for compound **1**. This analysis confirmed its relative stereochemistry, resolving ambiguities present in previous literature reports. All isolated compounds were evaluated for cytotoxicity against the human breast cancer MCF-7 cell line using an MTT assay. Compound **1** exhibited the most promising moderate cytotoxicity with an IC_{50} value of 34.17 μM (48 h incubation), while compound **3** showed an IC_{50} of 87.63 μM (24 h incubation). *In silico* target prediction and molecular docking studies identified compound **1** as a promising hit, binding to several breast cancer-related targets (e.g., PI3K, PDGFRα). Pharmacophore modeling further suggested that the C-23 hydroxy group is a prime target for structural modification to enhance potency. These findings highlight compound **1** as a valuable scaffold for designing new cytotoxic derivatives.

Keywords: Triterpenoid, *Nauclea orientalis*, Cytotoxicity, *In Silico*, Ursanes, Oleananes.

Introduction

Nauclea orientalis (L.) L. (Rubiaceae) was first recorded in regions from Sri Lanka and China to New Guinea and Australia. In Vietnam, *N. orientalis* is widely distributed in the Southwest Region, where it thrives in alluvial soils near riverbanks, stream edges, and coastal areas. The vernacular name "*Gao Vang*" in Vietnam refers to the yellow hue of the wood when cut. The leaves and bark of *N. orientalis* are widely utilized in traditional medicine to alleviate abdominal pains and pains from animal bites or wounds.[1,2] *Nauclea orientalis* is a rich source of diverse secondary metabolites responsible for its traditional medicinal uses and documented pharmacological effects. Studies on the chemical constituents of *N. orientalis* have revealed the presence of alkaloids, triterpenoids, steroids, and saponins.[3-6] Indole-type alkaloids, including nauclorienine, antirhine, *iso*-antirhine, alangine, naucline, neonaucline, angustidine, subditine, naucleaoral A, naucleaoral B, angustine, 18,19-dihydroangustine, nauclefine, angustoline, 10-hydroxyangustine, 3,14-dihydroangustine, 3,14,18,19-tetrahydroangustine, 3,14-dihydroangustoline, isolated from different parts of *N. orientalis*, have shown potent cytotoxicity against several human cancer cell lines, including HL-60 (blood), SMMC-7721 (liver), A-549 (lung), MCF-7 (breast), SW480 (colon), HeLa (cervix), KB (mouth), and T-24 (bladder).[4,6,7]

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An aqueous bark extract demonstrated significant cardioprotective activity against doxorubicin-induced cardiotoxicity in rats by mitigating oxidative stress, inflammation, apoptosis, and DNA fragmentation.[8] The leaf ethanolic extract and its fractions showed potent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.[9]

Building upon previous studies on the chemical constituents of *Nauclea orientalis* cultivated in Vietnam, it is evident that while various compounds have been identified,[10] a comprehensive investigation of triterpenoids from the stems of the Vietnamese species, along with their specific biological activities, remains less explored. For compound **1** (3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid), its structural elucidation in previous reports often lacked complete spectral data, especially NOESY correlations, leading to ambiguities in its stereochemistry. Detailed cytotoxic evaluations and *in silico* analyses to explore the therapeutic potential of such triterpenoids, specifically from the stems of Vietnamese *N. orientalis*, are also not extensively documented.

Therefore, this study aimed to address these gaps by conducting a detailed phytochemical investigation of the stems of *Nauclea orientalis* (L.) L. collected in Vietnam. The primary objectives were to isolate and unequivocally characterize triterpenoid compounds, with a special focus on providing a complete and revised NMR dataset for 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid (**1**) to resolve previous structural uncertainties. In addition, this study sought to evaluate the cytotoxicity of the isolated triterpenoids against the human breast cancer MCF-7 cell line and to utilize *in silico* approaches, including target prediction, molecular docking, and pharmacophore-guided structural modifications, to highlight the potential of compound **1** as a novel scaffold for designing new cytotoxic derivatives targeting breast cancer.

Materials and Methods

General experimental procedures

NMR spectra were obtained on the Bruker Advance III 500 and 600 MHz spectrometers (Bruker BioSpin AG, Bangkok, Thailand) with deuterated solvents, and chemical shifts are expressed in δ values. Silica

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gel 60 (40–63 μm) for column chromatography was obtained from Scharlau (Scharlau, Barcelona, Spain). Silica gel 60 F_{254} plates (0.25- or 0.5-mm thickness) for TLC were purchased from Merck (Merck KGaA, Darmstadt, Germany).

Plant material

The stems of *Nauclea orientalis* L. were collected at Phu Quoc National Park, An Giang Province, in June 2024. Ms. Le Minh Dung, Phu Quoc National Park, An Giang Province, Vietnam, identified the plant [voucher specimen (DOC-2024-029)].

Extraction and isolation

The powdered dry stems of *N. orientalis* (10 kg) were extracted with 70% MeOH–H_2O (75 L, reflux, 3 h × 3) to obtain an aqueous MeOH extract (473.5 g). This extract was suspended in H_2O (6 × 3 L) and successively partitioned with *n*-hexane (6 × 3 L), $CHCl_3$ (6 × 3 L), EtOAc (6 × 3 L) to yield an *n*-hexane-soluble (13.1 g), $CHCl_3$-soluble (17.4 g), EtOAc-soluble (13.2 g), and residual aqueous (403.7 g) fractions, respectively. The $CHCl_3$-soluble fraction was subjected to a silica gel column chromatography (diameter × length, 10 × 100 cm) and eluted with Me_2CO–*n*-hexane mixtures (*v/v*, 0:100 → 100:0), to obtain four fractions (C1–C4). Fraction C1 (6.6 g) was re-chromatographed on a silica gel column with EtOAc–*n*-hexane mixtures (*v/v*, 0:100 → 100:0) to obtain 4 fractions (C1.1–C1.4). Fraction C.1.2 (0.3 g) was subjected to a silica gel column and eluted with EtOAc–*n*-hexane (*v/v*, 0:100 → 100:0) and MeOH–$CHCl_3$ (*v/v*, 2:98 → 30:70) mixtures, and the resulting fractions were purified by preparative TLC using MeOH–$CHCl_3$–*n*-hexane (*v/v/v*, 2:28:70) to yield compound **2** (3.5 mg, R_f = 0.3). Fraction C2 (1.0 g) was also subjected to a silica gel column and eluted with Me_2CO–*n*-hexane mixtures (*v/v*, 0:100 → 100:0), to give four fractions (C2.1–C2.4). Fraction C2.3 (0.5 g) was chromatographed on a silica gel column using $CHCl_3$–*n*-hexane mixtures (*v/v*, 50:50 → 100:0), and then purified by preparative TLC with MeOH–$CHCl_3$ (*v/v*, 2:98) to obtain compounds **1** (3.0 mg, R_f = 0.4), **3** (3.4 mg, R_f = 0.3), and **4** (3.8 mg, R_f = 0.3).

Cytotoxicity assay

The cytotoxic efficacy was assessed using the MTT assay. Human MCF-7 breast cancer cells (ATCC, Manassas, USA) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, US) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, US) and 1% antibiotics (penicillin, streptomycin, and neomycin) (Gibco™, Thermo Fisher Scientific, Geel, Belgium). The cells were maintained in a humidified incubator at 37 °C with 5% CO_2. For the assay, MCF-7 cells were seeded in 96-well plates at a density of 10^4 cells/well in 90 μL of culture medium and allowed to attach overnight. Subsequently, cells were treated for 24 or 48 hours with varying concentrations of test compounds, each dissolved in 10% *v/v* DMSO. Following the treatment, MTT solution (Roche Diagnostics, Tokyo, Japan) was added to each well and incubated for 4 hours. The resulting formazan crystals were then solubilized by adding DMSO. The absorbance at 540 nm was measured using a Varioskan™ microplate reader (Thermo Fisher Scientific, Massachusetts, United States). Cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$, where $A_{control}$, A_{sample}, and A_{blank} represent the absorbance of the control, the samples, and the blank, respectively.

All experiments were conducted in triplicate, and the data are presented as the mean ± standard deviation of three independent samples. Doxorubicin (Sigma-Aldrich, St. Louis, USA) served as a positive control. IC_{50} values were determined by fitting the dose-response curves using Prism 10.

Molecular Docking

Docking studies were performed with the Molecular Operating Environment 2024 (MOE 2024.06) suite (Chemical Computing Group ULC, Montreal, Canada). The Builder module was employed to construct the structures of these compounds. All structures were minimized up to gradients of 0.0001 using the Amber10:EHT force field. The crystal structures of these target proteins (8ESV for ADAM10, 3B68 for AR, 4JPS for PI3K p110-α/p85-α, 6JOL for PDGFRα, 5UGW for TERT) were taken from the RCSB Protein Data Bank. These structures were prepared using the QuickPrep module. The binding site was identified by referencing the positions of standard ligands in the complex. Molecular docking was conducted using the Dock module, employing Triangle Matcher placement, Induced Fit refinement, and London dG and GBVI/WSA dG scoring methods. The top five poses were selected based on the negative binding free energy values (*S* values). The best pose was selected as the model to perform the Add Group to Ligand function. This function used the default linker database in MOE and applied descriptor filters (MW < 550 Da and 40 Å^2 < TPSA < 140 Å^2). Pharmacophore features and ligand R-vectors were analyzed to identify appropriate positions for structural modification to enhance binding affinity. The results showed the linkers with their respective new docking scores (*S* values).

Results and Discussion

Isolation and characterization of compounds

Four triterpenoids (**1**–**4**) were isolated from the $CHCl_3$-soluble fraction of *Nauclea orientalis* stems. These were identified as 3β,6β,23-trihydroxyolean-12-en-28-oic acid (**1**), rotundic acid 3,23-acetonide (**2**),[11] oleanolic acid (**3**),[12] and ursolic acid (**4**).[13] (Figure 1).

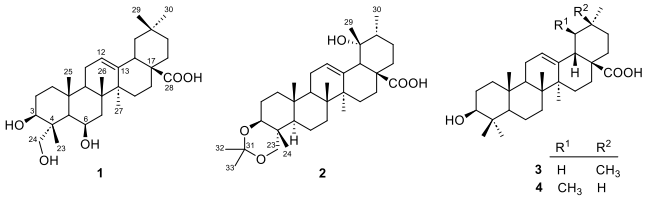

Figure 1: Chemical structures of isolated compounds **1**–**4**

*Structural elucidation of compound **1***

In the ^{1}H NMR spectrum, compound **1** showed signals of an olefinic methine proton [δ_H 5.55 (t, *J* = 3.7 Hz, H-12)], two oxygenated methine protons [δ_H 4.21 (dd, *J* = 11.7 and 4.5 Hz, H-3), 5.02 (brs, linewidth (W½) = 8.5 Hz, H-6)], one oxygenated methylene proton [δ_H 4.33 (d, *J* = 10.6 Hz, H-24b), 4.01 (d, *J* = 10.6 Hz, H-24a)], six methyl groups [δ_H 1.66 (s, H-23), 1.61 (s, H-25), 1.57 (s, H-26), 1.23 (s, H-27), 0.97 (s, H-29), 0.90 (s, H-30)], and other methine and methylene protons at δ_H 1.10–3.29 ppm. These ^{1}H NMR data were reprocessed from the initial dataset using water suppression (HOD signal at δ_H ~5 ppm) and receiver gain optimization (increased from 57.8 to 101) to enhance signal visibility and improve the signal-to-noise ratio. The ^{13}C NMR spectrum of compound **1** displayed 30 carbon signals, including one carbonyl carbon (δ_C 180.5, C-28), two olefinic carbons [δ_C 144.3 (C-13), 123.1 (C-12)], two oxygenated methine carbons [δ_C 73.4 (C-3), 67.6 (C-6)], one oxygenated methylene group (δ_C 67.0, C-24), six methyl carbons [δ_C 33.4 (C-30), 26.4 (C-27), 23.8 (C-29), 18.8 (C-26), 17.6 (C-25), 14.8 (C-24)], three methine carbons [δ_C 49.4 (C-5), 48.8 (C-9), 42.2 (C-18), nine methylene carbons [δ_C 46.6 (C-19), 41.2 (C-1), 41.1 (C-7), 34.3 (C-21), 33.3 (C-22), 28.4 (C-15), 27.8 (C-2), 24.0 (C-16), 23.9 (C-11)], and six quaternary carbons [δ_C 46.9 (C-17), 44.1 (C-4), 42.9 (C-14), 31.1 (C-20), 39.3 (C-8), 37.1 (C-10)]. The ^{1}H and ^{13}C NMR signals of compound **1** indicated that it is an oleanane-type triterpenoid. The HMBC correlations, observed from H-24 to C-3, C-4, C-5, C-23, from H-25 to C-1, C-10, from H-26 to C-9, C-8, C-14, from H-27 to C-8, C-13, C-14, C-15, from H-29 and H-30 to C-19, C-20, C-21 (Figure 2), provided obvious evidence supporting this structural assignment. In addition, the HMBC correlations from H-3 to C-4, C-23, C-24, from H-6 to C-8 and C-10, and from H-23 to C-3, C-4, C-24, showed the presence of the three hydroxy groups at C-3, C-6, and C-23.

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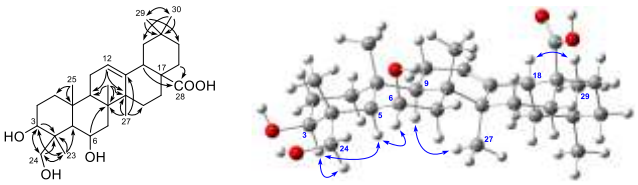

Figure 2: Key HMBC and NOESY correlations observed for compound **1**

Furthermore, the position of the carboxylic acid group was determined at C-17 based on the HMBC correlations from H-18 and H-22 to the COOH. The location of an olefinic group was confirmed at C-12 through the HMBC correlations from H-12 to C-9 and C-14. These NMR data closely resembled those of 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid, except for the larger W½ value.[14]

The structure of 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid was first isolated from *Timonius timon* leaves and published in 1993 based solely on its ^{1}H and ^{13}C NMR spectral data.[14] In particular, the 6*β*-OH group was deduced from the linewidth (full width at half height) (W½ = 5.0 Hz) of the unresolved equatorial H-6 proton signal. Since then, it has been reported from various plant species, including *Centella asiatica*,[15] *Kalopanax pictus*,[16] *Spermacoce latifolia*,[17] *Nauclea latifolia*,[18] *Ophiorrhiza baviensis*,[19] and *Mussaenda recurvata*.[20] However, most of these studies did not provide spectral data. None of these studies recorded NOESY spectra, leaving its stereochemistry ambiguous. In addition, uncargenin C has been partially synthesized from oleanolic acid.[21] However, in its ^{1}H NMR spectrum, the H-6 proton signal overlapped with the HOD signal. The structural assignment was based on a comparison of its ^{13}C NMR data with that of 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid.

Therefore, determining the relative configuration of the 6-OH group based solely on linewidth can be challenging and prone to misidentification, especially if poor shimming leads to broadened or distorted NMR signals and lines or reduced signal-to-noise ratio or if an inappropriate NMR solvent causes signal overlap and sample inhomogeneity. Thus, this study presents a complete NMR spectral analysis of compound **1** to clarify its structure. The 3D structure of compound **1** was built and optimized using density functional theory (DFT) calculation at the B3LYP/6-31G(d) level with polarizable continuum model (PCM) solvation for pyridine (Gaussian 16, Gaussian, Inc., Wallingford, USA). This optimized structure was used to depict NOESY correlations. The NOESY correlations between H-3/H$_2$-24, H-3/H-5, H-5/H-6, H-9/H$_3$-27, and H-18/H$_3$-29 clearly established the relative configuration of the three hydroxy groups. Thus, the structure of compound **1** was confirmed as 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid. In this study, the revised W½ value of the H-6 proton signal was reported, providing a comprehensive NMR dataset of 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid. Compound **1** showed a positive Cotton effect at 204 nm ($\Delta\varepsilon$ = +24.42) and a negative n → π* Cotton effect at 221 nm ($\Delta\varepsilon$ = −11.11), showing trends similar to those of 3*β*-hydroxy-18*βH*-olean-12-en-28-oic acid.[22]

Evaluation of cytotoxicity

Four oleanane-type triterpenoids (**1**–**4**) were screened for their cytotoxicity against the human MCF-7 breast cancer cell line. First, MCF-7 cells were incubated with test samples for 24 hours to assess the initial response of cancer cells to treatment. Compounds **1**, **2**, and **4** were non-cytotoxic or weakly cytotoxic at a concentration of 100 μM, with cell viability remaining above 80%. Compound **3** showed moderate cytotoxicity, with cell viability of 47.1% and had an IC$_{50}$ value of 87.63 μM, compared to the positive control, doxorubicin (IC$_{50}$ = 74.15 μM). Compound **1**, 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid, was reported to exhibit strong cytotoxicity against MCF-7 cell line, with an IC$_{50}$ value of 11.8 μM, after six-day incubation.[16] This prolonged incubation period may have contributed to the significant difference in cytotoxic results. Many approved drugs remain relatively stable in cell culture medium but have variable half-lives in human circulation. The terminal half-life of doxorubicin was 20–48 hours.[23] A half-life of 12–48 hours is generally ideal for once-daily oral dosing. The too-long incubation time (i.e., 6 × 24 hours) may not be suitable for cytotoxic assay. Thus, the cytotoxicity against the MCF-7 cell line of compound **1** and doxorubicin was re-performed with a 48-hour incubation. Compound **1** showed moderate cytotoxicity, with an IC$_{50}$ value of 34.17 μM, compared to the positive control, doxorubicin (IC$_{50}$ = 2.23 μM).

In silico studies

In the structure of compound **1**, the presence of two hydroxy groups at C-6 and C-23, compared to oleanolic acid (**3**), facilitated structural modifications. Target prediction for compounds **1** and **3** was performed to apply the structure-based design approach using SuperPred 3.0.[24] The results indicated that compound **1** was associated with the following predicted breast cancer-related protein targets: phosphoinositide 3-kinase p110-*α*/p85-*α* (PI3K), disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), telomerase reverse transcriptase (TERT), platelet-derived growth factor receptor *α* (PDGFR*α*), and androgen receptor (AR), with a probability > 50% and model accuracy > 90%. Oleanolic acid (**3**) was predicted to bind with the same breast cancer-related protein targets, also with a probability > 50% and model accuracy > 90%.

Table 1: Cytotoxicity against MCF-7 cancer cell line of compounds **1**–**4**

Compounds	Cell viability (%)					IC$_{50}$ (μM)
	6.25 μM	**12.5 μM**	**25 μM**	**50 μM**	**100 μM**	
1	–	–	–	–	95.9 ± 4.1	>100[a]
2	–	–	–	–	94.7 ± 4.5	>100
3	100.0 ± 4.0	89.0 ± 3.2	74.8 ± 5.5	63.0 ± 3.8	47.1 ± 2.6	87.63
4	–	–	–	–	89.7 ± 6.0	>100
	7.4 μM	**36.8 μM**	**73.7 μM**	**110.5 μM**	**184.2 μM**	
Doxorubicin	89.6 ± 1.4	64.7 ± 2.5	58.4 ± 8.4	35.3 ± 7.1	27.0 ± 4.8	74.15[a]
	6.25 μM	**12.5 μM**	**25 μM**	**50 μM**	**100 μM**	
1	81.1 ± 0.8	72.7 ± 1.2	60.2 ± 2.4	42.5 ± 2.1	22.8 ± 3.1	34.17[b]
		0.5 μM	**1 μM**	**2 μM**	**4 μM**	
Doxorubicin		89.4 ± 3.3	60.4 ± 2.2	56.1 ± 3.2	35.3 ± 2.1	2.23[b]

[a]24 h incubation; [b]48 h incubation

Table 2: Breast cancer-related target proteins and docking scores of **1** and **3**

Target Name (PDB ID)	3*β*,6*β*,23-Trihydroxyolean-12-en-28-oic acid (1)			Oleanolic acid (3)		
	Probability (%)	**Model accuracy (%)**	**Docking score**	**Probability (%)**	**Model accuracy (%)**	**Docking score**
PI3K (4JPS)	75.40	94.33	−6.73	73.27	94.33	−6.70
ADAM10 (8ESV)	74.27	97.50	−6.11	77.95	97.50	−5.96
TERT (5UGW)	69.28	90.00	−6.11	63.30	90.00	−5.74
PDGFR*α* (6JOL)	68.96	91.07	−7.47	64.55	91.07	−7.08
AR (3B68)	59.12	96.43	−5.59	62.49	96.43	−6.66

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Molecular docking studies were performed for compounds **1** and **3** with the corresponding specific targets (Table 2). Pharmacophore analysis of the best-docked pose was performed, and the ligand structure was modified (by adding or modifying groups) to affect its binding affinity, selectivity, and overall activity toward a specific target. This approach led to the rational design of new structures with enhanced interactions with these breast cancer-related protein targets. In contrast, oleanolic acid exhibited limited feasibility for structural modifications.

The resulting best-docked poses indicated that compound **1** fit within the binding pockets of ADAM10, AR, and TERT. This presented no apparent ligand R-vectors suitable for ligand modification. However, results involving PI3K and PDGFRα demonstrated a different situation, wherein ligand R-vectors were observed at the C-23 hydroxy group. In addition, pharmacophore feature analysis suggested the possible features to introduce new groups, such as H-bond donor (purple sphere) in PI3K and H-bond acceptor (cyan sphere) in PDGFRα (Figure 3). Various groups were suggested for addition to the hydroxy group at C-23 to interact with the Ser854 residue of PI3K and the Asp836 residue of PDGFRα to increase the binding affinity. This hydroxy group was attached to a primary carbon, making it reactive and susceptible to functionalization, such as esterification and etherification. Thus, these results suggested that compound **1** could be a versatile scaffold for further structural modifications to generate new compounds with more potent cytotoxicity against breast cancer cells.

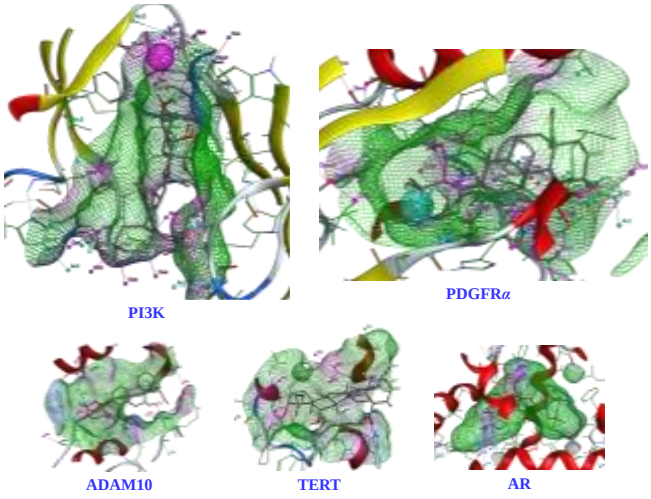

Figure 3: Pharmacophore model of the best-docked pose of **1** with breast cancer-related target proteins

Conclusion

In conclusion, four triterpenoids (**1**–**4**) were isolated from the $CHCl_3$-soluble fraction of *Nauclea orientalis* (L.) L. stems. Compound **1** was identified as 3*β*,6*β*,23-trihydroxyolean-12-en-28-oic acid. These compounds were evaluated for cytotoxicity against the MCF-7 breast cancer cell line. Compound **1** showed moderate cytotoxicity with an IC_{50} = 34.17 μM after 48 h incubation, while compound **3** exhibited an IC_{50} = 87.63 μM after 24 h incubation. These findings highlight compound **1** as a promising scaffold for the design new cytotoxic compounds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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