Antiproliferative Effects of *Nerium oleander* Stem and Mitotic Arrest Induced by Cardenolide Odoroside B on NCI-H460 Cancer Cells

Kehkashan Arshad Qamar*a, Ahsana Dar Farooqab, Bina S. Siddiquia, Nurul Kabirb, Naseema Khatoonb, Shakil Ahmedc, Shaista Erumdb and Sabira Begumaa

*aH.E.J. Research Institute of Chemistry, bDr. Panjwani Center for Molecular Medicine and Drug Research, cIndustrial Analytical Center, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

**Abstract**

Background: *Nerium oleander* extract preparations have been used in the Arab folk-medicine for the treatment of solid tumors.

Objective: In the current investigation, bioassay-guided fractionation of *N. oleander* stem methanol extract was performed to identify the active compound(s) responsible for its antiproliferative activity and the mechanism of action of the active compounds was explored.

Methods: The methanolic extract, fractions and sub-fractions were screened against four human cancer cell lines: HT-144, MCF-7, NCI-H460 and SF-268 using sulforhodamine B assay. The effects of the active compounds on the cytoskeleton and nuclei of NCI-H460 cells were studied using immunofluorescence microscopy.

Results: The most active petroleum ether insoluble sub-fraction led to the isolation of five pure compounds viz adynergenin, adynerin, hemidesmin-2, odoroside A and odoroside B. Odoroside A was the most potent compound with GI50: 0.04 and LC50: 0.74 μM against NCI-H460 cell line, while odoroside B demonstrated moderate growth inhibition and cytotoxicity (GI50: 6.7; LC50: 54 μM). After 24 hours’ treatment with odoroside B (50 μM) abnormal mitotic spindles were observed, while > 90% mitotic cells were arrested in the prophase stage.

Conclusion: *N. oleander* stem possesses significant antiproliferative effects against the aforementioned cell lines and the cardenolide odoroside B induces mitotic arrest of NCI-H460 cells in the prophase stage.

Keywords: *Nerium oleander*, antiproliferative, odoroside B, cytoskeleton, immunofluorescence, mitotic arrest.

1. INTRODUCTION

*Nerium oleander* (Apocynaceae family) is an evergreen tree distributed in Indo-Pakistan subcontinent, tropical and subtropical regions, and the Mediterranean. It has been used in the folklore medicine for the treatment of solid tumors and skin diseases like corns, eczema, herpes, psoriasis, ringworm infections, scabies, sores and warts [1-3]. Ingestion of parts of this plant causes toxicity due to the presence of cardiac glycosides such as oleandrin and oleandrigenin [4]. Many cardiac glycosides and pregnanes e.g. neriidione A and neriidione B have been isolated from the stem and twigs of *N. oleander* [5, 6]. Several of these compounds have demonstrated anticancer effects, e.g. neriidione A exhibited IC50 of 0.68 and 2.5 μg/ml against human malignant fibroblast and liver tumor cell lines, respectively [5]; the compound 3β-O-(β-D-digitosyl)-14-hydroxy-5β,14β-card-20(22)-enolide also demonstrated significant antiproliferative effects (IC50: 0.18 and 1.5 μM) against the aforementioned human cancer cell lines [7]. It has been recently demonstrated that the cardenolides from the *Apocynaceae* family including the genus *Nerium* exhibit the capability to regulate cancer cell survival and death through multiple signaling pathways [8].

The proteins involved in cell division such as microtubules are important targets for anticancer drugs [9]. Microtubules form the bipolar mitotic-spindle apparatus at the time of cell division, which is essential for the equal separation and distribution of chromosomes to the daughter cells [10]. This process requires a dynamic balance between polymerization and depolymerization processes of microtubules. The treatment of cancer cells with anti-microtubule drugs dis-
turbs this balance inducing interruption of mitosis, thus leading to mitotic arrest and cell death [11].

Most anticancer drugs lack tumor specificity and kill rapidly proliferating tumor cells as well as normal cells of bone marrow, gastrointestinal mucosa and hair leading to severe side effects (myelosuppression, mouth ulcers, diarrhoea, alopecia etc.). Moreover, tumor cells quickly acquire resistance to these agents. Therefore, search for novel and safer anticancer agents is an on-going process. Currently, there is a revival of scientific interest in medicinal plants for lead compounds and/or the generation of their semi-synthetic derivatives. Despite reports on the anticancer potential of *N. oleander* there is no systematic bioassay-guided anticancer activity study on its stem. Keeping this in mind, in the present study *N. oleander* stem methanolic extract, fractions and sub-fractions were evaluated against four human cancer cell lines: HT-144 (melanoma, skin), MCF-7 (adenocarcinoma, breast), NCI-H460 (non-small cell lung cancer) and SF-268 (astrocytoma, CNS). Using immunofluorescence microscopy, the effects of the active compounds derived from *N. oleander* stem were studied on the cytoskeleton and nuclei of NCI-H460 cells.

2. MATERIALS AND METHODS

2.1. Materials and Stock Solutions

Following cell culture grade chemicals were purchased from respective companies given in parentheses: Cy3-labeled β tubulin clone TUB 2.1 monoclonal antibody, 4, 6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), gentamycin sulphate, L-glutamine penicillin-streptomycin solution, poly-L-lysine, RPMI-1640, sulforhamadine B (SRB), trichloroacetic acid, trypsin blue, trypsin-EDTA (Sigma, St. Louis, Mo, USA). Alexa 488 phalloidin, fetal bovine serum (heat inactivated) and zymed clearmount mounting medium (Invitrogen, Grand Island, NY 14072, USA). Amphotericin B, doxorubicin, paclitaxel and vinblastine (MP Biochemicals, Solon, Ohio 44139, USA); tris base (Aldrich, Stenheim, Germany); triton-X-100 (Wako Pure Chemical Industries Ltd., Japan); acetic acid (Riedel-de-Haen, Sigma- Aldrich Gmbh D-30926, Germany); and formaldehyde 4% (Carl Roth, Gmbh Co., Schoemperlenstraße 76185, Karlsruhe, Germany).

The stock solutions of *N. oleander* stem methanolic extract (40 mg /ml), fractions and sub-fractions (20 mg /ml), and pure compounds (20 mM) were prepared in DMSO while doxorubicin stock solution (20 mM) was prepared in DMSO. The stock solutions were stored at -20°C and further dilutions were freshly prepared in RPMI-1640 containing gentamicin (50 µg/ml) to control bacterial contamination.

2.2. Extraction and Isolation

Uncrushed stem of *Nerium oleander* (6 kg) was repeatedly extracted with MeOH (3x) at room temperature. The concentrated syrup residue obtained on removal of the solvent from the combined extract was shaken out with EtOAc and H₂O. The EtOAc layer was extracted with 4% aqueous Na₂CO₃ solution to separate the acidic from the neutral fraction. The EtOAc layer containing the neutral fraction was washed, dried over anhydrous Na₂SO₄, charcoaled and concentrated to a thick syrup. It was divided into petroleum ether soluble and insoluble fractions. The petroleum ether insoluble fraction was subjected to column chromatography (petroleum ether, petroleum ether -EtOAc in order of increasing polarity). The fractions were combined on the basis of their thin layer chromatography (TLC) to yield 15 fractions. Fraction No. 2 (petroleum ether-EtOAc, 8.5:1.5 eluate), 5 (petroleum ether-EtOAc, 8.2 eluate), 6 (petroleum ether-EtOAc, 7.5:2.5 eluate), 8 (petroleum ether-EtOAc, 7.3 eluate) and 10 (petroleum ether-EtOAc, 7.3 eluate) were almost pure and were recrystallized from methanol to yield pure compounds identified as odoroside-A (15 mg) [12], adynerigenin (12 mg) [13], adynerin (30 mg) [13], odoroside-B (10 mg ) [12] and hemidesmin-2 (20 mg) [14], respectively (Fig. 1).

2.3. Cell Lines

HT-144 (melanoma, skin), MCF-7 (adenocarcinoma, breast), NCI-H460 (non-small cell carcinoma, lung) and SF-268 (anaplastic astrocytoma, CNS) human cancer cell lines were maintained in a humid atmosphere with 5% CO₂ at 37°C. The RPMI-1640 medium was supplemented with heat inactivated fetal bovine serum (10%), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin-B (2.5 µg/ml). The cell concentration and viability were determined by using a hemocytometer.

2.4. Growth Inhibitory and Cytotoxicity Assay

In vitro growth inhibition and cytotoxicity were determined by employing sulforhodamine B (SRB) assay [15, 16]. About 1x10⁵/well MCF-7 and NCI-H460 cells were added of 96-well plates while SF-268 and HT-144 cells were added at the density of 1.5x10⁴ and 2x10⁴ cells/well, respectively. After 24 hours’ incubation, different doses of *N. oleander* methanolic extract, fractions, sub-fractions, pure compounds and doxorubicin were added. After 48 hours, cells were fixed with ice-cold trichloroacetic acid (50 µl, 50%) and left for overnight air drying. The cells were stained with SRB dye (100 µl/well). Next day, the bound SRB dye was solubilized in Tris-base solution (10 mM) and optical density measurements were taken at 545 nm in a plate reader. By plotting the percentage of cell growth or cell killing versus the drug concentrations [16], drug concentrations causing growth inhibition and killing of 50% cells i.e. GI₅₀ and LC₅₀ values were calculated graphically.

2.5. Immunofluorescence Microscopy

Alexa Flour 488 phalloidin, Cy3-labeled β tubulin antibody and DAPI were used to visualize F-actin filaments, microtubules and nuclei, respectively [17-19]. Lab-Tek II 8-chambered slides (Nalgene Nunc, USA) were coated with poly-L-lysine to allow firm attachment. About 1x10⁵ NCI-H460 cells/well were added and incubated overnight at 37°C. Next day, the culture medium containing pure compounds (1, 5, 10, 50 and 100 µM), or corresponding vehicle (0.1% DMSO) was added. After incubation for further 24 and/or 48 hours, the cells were fixed with formaldehyde (4%) and the slides washed with freshly prepared phosphate buffered saline (PBS). Permeabilization of the cells was car-
ried out with 1% Triton X-100 in PBS. The cells were incubated initially with Alexa Fluor 488 phalloidin to stain F-actin filaments, then with Cy3-labeled tubulin antibody for microtubules and lastly with DAPI. After extensive washing, the slides were mounted, cover-slipped and viewed under Nikon’s 90i epifluorescence microscope. Nikon DXM1200C cooled CCD camera was used to capture images with DAPI, FITC and TxRed filter cubes. Real-time deconvolution was performed on images by using NIS-elements 3.0 software (AR version) and images were processed with Adobe Photoshop [20].

2.6. Mitotic Index Assay

The cells were identified to be in either interphase or mitotic stage or undergoing cell death. Control interphase nuclei were similar in size, smooth, round or oval shaped. The dead cells showed condensed and/or fragmented nuclei. The mitotic cells exhibited condensed chromosomes without nuclear membrane. Prophase stage was characterized by nuclear envelope breakdown and chromosome condensation. In the metaphase stage of the cell cycle, replicated chromosomes were aligned at the spindle equator. The anaphase...
Table 1. Cytotoxic effects of *N. oleander* stem methanolic extract, fractions and sub-fractions against HT-144, MCF-7, NCI-H460 and SF-268 human cancer cell lines.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>HT-144</th>
<th>MCF-7</th>
<th>NCI-H460</th>
<th>SF-268</th>
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<tr>
<td><strong>(LC&lt;sub&gt;50&lt;/sub&gt;: µg/ml)</strong></td>
<td></td>
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<tr>
<td><strong>A) Extract</strong></td>
<td></td>
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<tr>
<td>Methanolic</td>
<td>1.8 ± 0.14&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>6.0 ± 0.6&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.85 ± 0.03&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>7.5 ± 1.3&lt;sup&gt;a2&lt;/sup&gt;</td>
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<td><strong>B) Fractions</strong></td>
<td></td>
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<tr>
<td>Aqueous</td>
<td>81.3 ± 5.7&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>&gt; 100</td>
<td>17.1 ± 0.9&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>44.3 ± 0.7&lt;sup&gt;b1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl acetate soluble</td>
<td>0.94 ± 0.2&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>6.2 ± 1&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.65 ± 0.1&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>5.1 ± 0.8&lt;sup&gt;c2&lt;/sup&gt;</td>
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<td><strong>C) Sub-fractions</strong></td>
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<tr>
<td>Petroleum ether soluble</td>
<td>94.7 ± 3.6&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>31.5 ± 0.5&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>23.0 ± 0.5&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>61.0 ± 1.4&lt;sup&gt;c2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Petroleum ether insoluble</td>
<td>0.72 ± 0.04&lt;sup&gt;b1,2&lt;/sup&gt;</td>
<td>1.5 ± 0.4&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>0.16 ± 0.006&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>0.37 ± 0.12&lt;sup&gt;c2&lt;/sup&gt;</td>
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</table>

Data are the mean ± SEM of three independent experiments analyzed by ANOVA. For comparison of different test agents, least significant difference (LSD) and Duncan’s multiple range tests were used. LC<sub>50</sub> = Lethal concentration of the extract/fraction/sub-fraction that killed 50% cells. The mean absorbance reading (at 545nm) of control cells of the aforementioned cell lines was 2.8 ± 0.12. In columns statistically different values are represented by dissimilar alphabetical superscripts. In rows statistically different values are represented by dissimilar numerical superscripts.

stage began when the chromosomes segregated towards the spindle poles to form two new daughter cells [21]. The telophase stage was evident when the cleavage furrow appeared till the completion of cytokinesis [22]. The mitotic cells in prophase, metaphase, anaphase, or telophase were identified, and their percentages determined for the control cells and after treatment with odoroside B (50 µM) for 24 and 48 hours. The percent mitotic index was calculated by dividing the number of cells in mitosis by the total number of cells and multiplying the result with 100.

2.7. Statistical Analysis

Statistical software SPSS 16 was used to analyze the data which was expressed as mean ± SEM. ANOVA (Analysis of variance) followed by LSD (least significance difference) and Duncan’s multiple comparison tests were used for statistical comparisons between the control and the treated groups and between the various treatments. p values of less than 0.05 were considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001, versus control).

3. RESULTS

3.1. Cytotoxic Effects of Extract, Fractions and Sub-fractions

The methanolic extract, fractions and sub-fractions exhibited significantly maximum cytotoxic effects against NCI-H460 lung cancer cell line as reflected in their lowest LC<sub>50</sub> values (Table 1). The methanolic extract and its ethyl acetate soluble fraction showed similar cytotoxicity against MCF-7 cell line with LC<sub>50</sub> of ~ 6 µg /ml. The petroleum ether insoluble sub-fraction demonstrated maximum cytotoxic activity against all the cell lines. The aqueous fraction exhibited minimum cytotoxicity against MCF-7 cell line (LC<sub>50</sub> > 100 µg/ml).

3.2. Growth Inhibition and Cytotoxicity of Extract, Fraction, Sub-fraction and Pure Compounds

The methanolic extract and the ethyl acetate soluble fraction exhibited similar GI<sub>50</sub> of ~ 0.3 µg/ml to the pure compound adynerin, while the petroleum ether insoluble sub-fraction demonstrated comparable growth inhibition to odoroside A and the reference compound doxorubicin (Table 2). The GI<sub>50</sub> potency order was odoroside A = doxorubicin = PEIS > EA = adynerin = ME > hemidesmin-2 > odoroside B. The petroleum ether insoluble sub-fraction demonstrated ~2-fold greater cytotoxic effects than odoroside A and doxorubicin (LC<sub>50</sub>: 0.16 µg/ml). The potency order for LC<sub>50</sub> was PEIS > odoroside A = doxorubicin > EA = ME > adynerin = hemidesmin-2 > odoroside B.

3.3. Immunofluorescence Microscopy

Immunocytochemistry was employed to explore the mechanism of antiproliferative effects of the pure compounds isolated from *N. oleander* stem against NCI-H460 cells. F-actin filaments, microtubules and the nuclei were visualized using Alexa 488 phalloidin, Cy3-labeled tubulin antibody and DAPI, respectively. The effects of these compounds were compared with control, and those induced by doxorubicin, paclitaxel and vinblastine. Odoroside A induced cell death at the dose of 1 µM, while odoroside B (50 µM) exhibited remarkable changes in the morphology and percent mitotic stages, hence further studies were conducted on the latter compound.

3.3.1. Cytoskeleton and Nuclear Morphology of Control NCI-H460 Cells

The control NCI-H460 cells were polygonal in shape but rounded at the time of cell division with fine, pointed and thread-like F-actin processes (Fig. 2a, d). The interphase nuclei were smooth, round or oval with 2-3 invaginations
Table 2. Antiproliferative effects of *N. oleander* stem methanolic extract, ethyl acetate soluble fraction, petroleum ether insoluble sub-fraction, pure compounds adynergenin, adynerin, hemidesmin-2, odoroside A, odoroside B and doxorubicin against human lung cancer NCI-H460 cell line

<table>
<thead>
<tr>
<th></th>
<th>GI&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
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<tbody>
<tr>
<td>A) Methanolic extract</td>
<td>0.31 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.85 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>B) Ethyl acetate soluble fraction</td>
<td>0.22 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>C) Petroleum ether insoluble sub-fraction</td>
<td>0.03 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16 ± 0.006&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>D) Compounds</td>
<td></td>
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<tr>
<td>Adynergenin</td>
<td>&gt; 37.3 (&lt;100)</td>
<td>&gt; 37.3 (&lt;100)</td>
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<tr>
<td>Adynerin</td>
<td>0.26 ± 0.02&lt;sup&gt;a&lt;/sup&gt; (0.5 ± 0.03)</td>
<td>3.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt; (5.9 ± 0.2)</td>
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<tr>
<td>Hemidesmin-2</td>
<td>1.12 ± 0.1&lt;sup&gt;a&lt;/sup&gt; (2.9 ± 0.2)</td>
<td>3.5 ± 0.05&lt;sup&gt;a&lt;/sup&gt; (9.0 ± 0.1)</td>
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<tr>
<td>Odoroside A</td>
<td>0.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt; (0.04 ± 0.02)</td>
<td>0.38 ± 0.01&lt;sup&gt;a&lt;/sup&gt; (0.74 ± 0.02)</td>
</tr>
<tr>
<td>Odoroside B</td>
<td>3.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt; (6.7 ± 0.7)</td>
<td>28.0 ± 1.5&lt;sup&gt;c&lt;/sup&gt; (54.0 ± 3.0)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.03 ± 0.003&lt;sup&gt;d&lt;/sup&gt; (0.052 ± 0.005)</td>
<td>0.43 ± 0.07&lt;sup&gt;e&lt;/sup&gt; (0.7 ± 0.12)</td>
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</table>

The values in the parentheses are expressed in µM. GI<sub>50</sub> = Concentration of the compound causing 50% growth inhibition of cells. LC<sub>50</sub> = Lethal concentration of the compound that killed 50% cells. Data are the mean ± SEM of three independent experiments analyzed by ANOVA. Least significant difference (LSD) and Duncan’s multiple range tests were used for comparison. Statistically similar values in a column are represented by same numerical superscripts. The mean absorbance reading (at 545nm) of control NCI-H460 cells was 2.73 ± 0.2.

Fig. (2). Fluorescent staining of F-actin, nuclei and microtubules in control NCI-H460 cells after 48 hours of incubation. NCI-H460 cells showing: (a) F-actin, (b) nuclei and (c) microtubules at 20x magnification. At 60x magnification: (d) F-actin, Arrows showing (e) the nucleus with DNA in the telophase stage of mitosis, and (f) microtubules of the interphase network and the separating microtubule bundles after formation of two daughter cells. Scale bar: 20 µm.
and 2–4 nucleoli. The condensed chromatin visible in anaphase mitotic stage is marked by an arrow (Fig. 2e). Thread-like, fine microtubules were arranged in a circular ring around the nucleus forming a regular network in the periphery (Fig. 2f). Highly fluorescent bundles of microtubules forming the mitotic spindles and connecting the two daughter cells at the end of cell division were clearly visible (Fig. 2f).

3.3.2. Changes Induced by Doxorubicin, Paclitaxel and Vinblastine

Fig. (3) shows the effects of doxorubicin, paclitaxel and vinblastine at 1 µM on F-actin, nuclei and microtubules of NCI-H460 cells after 24 hours of incubation. There was no appreciable change in the morphology of F-actin in the pres-
ence of doxorubicin (Fig. 3a). However, since doxorubicin is a fluorescent drug and binds to DNA, hence nuclei were visible in the FITC channel also (Fig. 3a). Few nuclei appeared irregular, condensed and fragmented (Fig. 3b). Moreover, the fine network of microtubules, as seen in control cells, was not visible (Fig. 3c). Tubulin stabilizing agent paclitaxel induced long, thick microtubule bundle formation (Fig. 3f), whereas vinblastine caused tubulin paracrystals formation (Fig. 3i). After treatment with both aforementioned drugs, no detectable change in F-actin was observed (Fig. 3d, g). Most of the nuclei treated by either paclitaxel or vinblastine appeared fragmented and apoptotic bodies were clearly visible as marked by arrows (Fig. 3e, h).

Fig. (4). Fluorescent staining of F-actin, nuclei and microtubules in NCI-H460 cells after 24 hours of incubation with odoroside B. NCI-H460 cells (a-c) at 20x, (d-i) at 60x magnification in the presence of odoroside B: At 50 µM: (a) F-actin, Arrows showing (b) many nuclei in prophase stage of mitosis, and (c) microtubules (d) F-actin with rounding of cell at the time of cell division, Arrows showing (e) two nuclei in the prophase stage of mitosis, and (f) mitotic spindle. At 100 µM: (g) F-actin, (h) nuclei, and (i) Arrow showing irregular microtubule bundles. Scale bar: 20 µm.
3.3.2. Changes in Cytoskeletal and Nuclear Morphology Induced by Odoroside B

The fluorescence microscopy showed no appreciable change in the morphology of F-actin, interphase nuclei and microtubules after 24 and 48 hours’ treatment with odoroside B (50 µM) as compared to the aforementioned control (Fig. 4d, e, f; 5a, b, c). After 24 hours’ incubation with odoroside B (50 µM), an increase in the mitotic cells in the prophase stage could be clearly seen as marked by an arrow at 20x magnification (Fig. 4b). When the cells were incubated with higher concentration of odoroside B (100 µM) for 24 hours, the microtubules appeared as thickened, irregular, highly disorganized and entangled fibers (Fig. 4i). After 48 hours, these aforementioned changes were more pronounced (Fig. 5f). Additionally, the treated cells lost their processes and became rounded (Fig. 5d). Nuclear fragmentation was visible and apoptotic bodies were seen as marked by an arrow (Fig. 5e).

3.3.3. Effects of Odoroside B on Cell Number and Percent Mitotic Index

The average number of control cells counted in single field at 20x magnification after 24 hours (240 ± 15) was significantly reduced (67%) after treatment with odoroside B (50 µM) (Fig. 6a). A significant increase in the mean percent mitotic index of control (5.4) vs. treated cells (9.7) was observed (Fig. 6b). The corresponding mean percentages of control mitotic cells after 24 hours’ incubation were about 29, 18 and 53 in prophase, metaphase and anaphase/ telophase stages. However, 24 hours of odoroside B (50 µM) treatment induced a significant increase in prophase stage (96.8%) accompanied by a significant decline in metaphase (1.6%) and anaphase/telophase stages (1.6%) (Fig. 6c). A significant decline was observed in the total number of the cells after incubation for 48 hours (p < 0.001) (Fig. 6a), however, the percent mitotic index was slightly reduced from 4.83 in the control to 3.1 in the treated cells (Fig. 6b).
4. DISCUSSION AND CONCLUSIONS

The search for substances of plant origin with anticancer properties have prompted studies on all parts of *N. oleander* including roots [20]. To our knowledge, the present study provides the first evidence of the antiproliferative activity of *N. oleander* stem methanolic extract, fractions and sub-fractions against the aforementioned cell lines. Moreover, this is the first report of the anticancer activity of the pure compounds: adynerin, hemidesmin-2, odoroside A and odoroside B derived from *N. oleander* stem against NCI-H460 cell line. The current investigation reports that *N. oleander* stem methanolic extract, fractions and sub-fractions exhibited potent cytotoxic effects against all the four human cancer cell lines tested, however, they were more cytotoxic against NCI-H460 cells. In the present study, odoroside A (5β) (GL_{50}: 0.04; LC_{50}: 0.74 μM) was the most potent compound exhibiting comparable activity to the reference compound doxorubicin against NCI-H460 cell line, while its stereoisomer odoroside B (5α) was ~70-fold less cytotoxic (LC_{50}: 54 μM) emphasizing the importance of stereochemistry for biological activities.

The petroleum ether insoluble sub-fraction was most cytotoxic (LC_{50}: 0.16 μg/ml) demonstrating ~2-fold better cell killing activity than the standard anticancer drug doxorubicin against NCI-H460 cell line. The additive or synergistic effects of compounds residing in the sub-fraction could have been responsible for its greater cytotoxicity as seen in traditional Chinese medicine (TCM), where composite and complex remedies are used which possibly act in a synergistic fashion to increase therapeutic effects and reduce adverse side effects on healthy tissues [24].

It is well known that certain drugs like paclitaxel and vinblastine exert their anti-tumor actions by disrupting the dynamics of microtubule assembly, thereby disturbing the formation and function of the mitotic spindle apparatus in dividing cells, arresting cells in mitosis and induction of cell death [25]. It is essential that all the chromosomes are attached to the mitotic spindle before the onset of anaphase; this process is strictly controlled by the mitotic or the spindle assembly checkpoint [26]. The microtubule-disrupting agents possibly arrest cells in mitosis by triggering the mitotic checkpoint that ensures proper attachment of chromo-
somos to the mitotic spindle before the onset of anaphase [27]. Paclitaxel induces mitotic arrest by promoting stabilization of microtubules [28], whereas vinblastine acts by depolymerizing the microtubules [29]. In the present investigation, odoroside B induced mitotic arrest with ~90% of NCI-H460 mitotic cells in the prophase stage along with a substantial decline (90%) in the metaphase and anaphase/telophase stages indicating that it probably interferes with either the function or the structure of the microtubules or both. Dose and time dependent changes were seen at higher concentration (100 μM); the microtubules appeared as thick, irregular and disorganized fibers. Moreover, after 48 hours of treatment, cell rounding and nuclear fragmentation indicated cell death.

It is established that the addition of paclitaxel stabilizes the microtubules and increases the polymerization of tubulin, while the reverse is seen after the addition of vinblastine [30]. Western blotting can be employed to determine whether odoroside B induces polymerization or depolymerization of tubulin in NCI-H460 cells, as demonstrated for H1-1, 2, 4 oxadiazolo[4,3-a]quinoxalin-1-one that prevents microtubule polymerization in HeLa cells [31].

It is being suggested that the aforementioned effects of odoroside B on cell cycle progression be substantiated by DNA flow cytometry which can also evaluate the distribution of actively dividing cells in the cell cycle and the percentage of apoptotic and non-apoptotic cells in each phase of the cell cycle [32]. Moreover, to confirm the mode of cell death induced by odoroside B, measurement of mitochondrial membrane potential and release of cytochrome c providing an early indication of the initiation of cellular apoptosis should be carried out [33]. When it is confirmed that the cell death induced by odoroside B is due to the apoptotic process, then Western blot analysis could also be done to examine the involvement of specific caspasases and/or the cleavage of poly (ADP-ribose) polymerase [34].

These results indicate that N. oleander stem methanolic extract, fractions and sub-fractions possess significant antiproliferative effects against HT-144, MCF-7, NCI-H460 and SF-268 human cancer cell lines justifying the traditional use of this plant. The pure compounds (adynerin, hemidesmin-2, odoroside A and odoroside B) were cytotoxic against NCI-H460 cell line. The importance of stereochemistry for biological activities was evident by 70-fold greater cytotoxicity exhibited by odoroside A (5β) against NCI-H460 cell line than its isomer odoroside B (5α). It is also being suggested that the antiproliferative effects of odoroside B were mainly due to its interaction with microtubules and induction of cell death. Therefore, it is concluded that N. oleander is a potentially valuable candidate for cancer chemotherapy.

CONSENT FOR PUBLICATION
Not applicable.

CONFLICT OF INTEREST
There is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could have inappropriately influenced, or be perceived to influence our work.

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REFERENCES
Antiproliferative Effects of Nerium oleander Stem and Mitotic Arrest


