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5 α -oleandrin reduce Bcl-2 protein and increase Bax protein expression on Hela cervical cancer cell

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ABSTRACT

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BACKGROUND

The leaves of *Nerium indicum* Mill. have been utilized traditionally to cure cancer. By using *Bioassay guided extraction and isolation* method, three compounds (NiO-1, NiO-2, NiO-3) were isolated from an active fraction of the CHCl₃ extract of *N. indicum* leaves. NiO-2 identified as 5 α -oleandrin is the best cytotoxic compound on HeLa cervical cancer cell *in vitro* among the other two. However mechanism of action of the compound hasn't been evaluated yet. The aims of this study were to determine the mechanism of action at molecular level.

METHODS

The action mechanism of 5 α -oleandrin on HeLa cervical cancer cells was analyzed by staining the cells with Hoechst 33342; the agarose gel electrophoresis was aimed to determine the DNA fragmentation and the western blotting was aimed to determine the Bcl-2 and Bax protein expression.

RESULTS

Incubation of HeLa cervical cancer cell with 5 α -oleandrin at the concentration 3,47x10⁻⁴ mM (24 hours) followed by staining with Hoechst 33342, a broken up light blue color of nucleus was observed (compared with intensive color of untreated control). By gel electrophoresis (at the same concentration of the tested compound), a smear band at about 200 bp was observed. In addition, cells treated with 5 α -oleandrin displayed a decreasing of the Bcl-2 protein expression and increasing of the Bax protein expression

CONCLUSION

5 α -oleandrin induced HeLa cervical cancer cells DNA fragmentation observed by the presence of a smear band at about 200 bp indicative the apoptotic occurrence. 5 α -oleandrin induces apoptosis by reducing the Bcl-2 protein expression but the Bax protein expression increases.

Keywords: *Nerium indicum*, 5 α -oleandrin, cytotoxicity, DNA, anticancer

INTRODUCTION

Cervical cancer is the fourth most common cancer worldwide, among females in 2012. There were estimated about 528 000 new cases of cervical cancer, of which around 85% occurred in less developed regions. Around 266.000 females died of cervical cancer, accounting for 7.5% of all female cancer deaths. About 87% of cervical cancer deaths occurred in the less developed regions.⁽¹⁾ Cervical cancer is still the second most frequent cancer among Indonesian women, thus screening program is still critically important to prevent it. Prevalence of Visual inspection with acetic acid (VIA) test-positive is 4.7% in Jakarta population. The findings of precancerous lesions and cervical cancers are not only between thirty and fifty years old, but also below the thirty years old and after fifty years old.⁽²⁾ Cervical cancer appears as the most frequent cancer among women, and its position was the first rank, followed by breast, ovary, skin, thyroid, rectum, lymph nodes, uterus, colon, nasopharynx.⁽³⁾

Targeted cancer therapies is substance that block the growth and spread of cancer by interfering with molecular targets that are involved in the growth, progression, and spread of cancer. During the past few decades, targeted therapy has emerged as a promising approach for the development of selective anticancer agents.⁽⁴⁾ Several molecular targets for anticancer drug discovery and development have been identified including genes associated with cell cycle control and apoptosis. It is well known that two main apoptosis pathways, the intrinsic and extrinsic pathways, are involved in the regulation of tumorigenesis. Apoptosis is one method the body uses to get rid of unneeded or abnormal cells, but cancer cells have strategies to avoid apoptosis. Apoptosis inducers can get around these strategies to cause the death of cancer cells.⁽⁵⁾ HeLa cervical cancer cell is a cell culture model of cervical cancer, which is generally used for the study because it is quite safe, grows faster and easy to be handled.^(6,7)

The leaves of *Nerium indicum* Mill. containing cardiac glycosides possibly having a role in cancer management, cardiac arrhythmias, and anti-viral.⁽⁸⁻¹⁰⁾ Current evidence showed that oleandrin from *N. indicum* inhibited proliferation and suppress the export of fibroblast growth factor-2 in prostate cancer cells, also induce apoptosis in human colorectal cancer cells via the mitochondrial pathway.⁽¹¹⁾ Our previous study showed that the major compound isolated from the leaves of *Nerium indicum* Mill. was identified as 5 β -oleandrin was cytotoxic on several human cancer cells, but this compound was also cytotoxic to normal cells *in vitro*.⁽¹²⁾ Sonawane et al.⁽¹³⁾ stated that safe delivery of effective antitumor agents is the main goal in cancer therapy. This is understandable that only a limited amount of the functional drug reaches tumor cells whereas it also acts on the normal healthy tissues resulting in serious adverse effects. Further isolated compound identified as 5 α -oleandrin (16 β -acetyl-3 β -oleandrosa-14 β -hydroxy-5 α -card-20(22)-enolide), possessed the remarkable cytotoxic effect on HeLa cervical cancer cells (IC₅₀, 8.38 x10⁻⁶ mM), and surprisingly was not cytotoxic to normal cell.⁽¹²⁾ Till today the mechanism of action of 5 α -oleandrin compound hasn't been evaluated yet. Therefore, this study was aimed to determine the mechanism of action at molecular level especially the Bcl-2 and Bax protein expression using western blotting method.

METHODS

Research design

This study was an experimental laboratory study conducted at the Integrated Research and Testing Laboratory (IRTL), Universitas Gadjah Mada, Yogyakarta and also at the Division of Molecular Oncology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Japan. HeLa cervical cancer cell line, treated with 200 ng/mL of 5 α -oleandrin compound was used in this study.

Study subjects

Culture of HeLa cervical cancer cell line, obtained from Prof. Tatsuo Takeya (NAIST, Japan), which were recultured by the Integrated Research and Testing Laboratory (IRTL), Universitas Gadjah Mada Yogyakarta.

Research material

5 α -oleandrin was isolated by Prof. Dr. Mae Sri Hartati W., M.Si., Apt. (Department of Pharmacology and therapy, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia). The compound is presented in Figure 1.

Preparation of stock solution

5 α -oleandrin (1.0 mg) was dissolved in 100,0 μ l of dimethyl sulfoxide (DMSO) (Merck, Germany) and then RPMI-1640 (medium) was added to obtain 10 mg/mL of stock solution.

Preparation of cells culture

HeLa cervical cancer cell line was maintained *in vitro* in RPMI-1640 medium (Sigma Chemical Co., USA) which was supplemented with 10% fetal bovine serum (Gibco Invitrogen, USA), 100 μ g/mL of streptomycin (Gibco Invitrogen, USA), 100 unit/mL of penicillin (Gibco Invitrogen, USA), and 2 mM of glutamin in tissue culture flask. The cells were incubated in 5% of CO₂ incubator set at 37°C.

Preparation of fluorescent staining of apoptotic cells using Hoechst 33342

HeLa cervical cancer cells 1x10⁵ cell/well in RPMI 1640 media were placed on 6 well plate, put on cover slips and incubated overnight at 37°C, 5% CO₂. The medium was removed and added 5 α -oleandrin and 5 β -oleandrin (200 ng/mL) of each well in RPMI 1640 medium 10% FBS plate was incubated for 24 hours at 37°C, 5% CO₂. They were fixed in 4% paraformaldehyde, washed with PBS and stained with 10 mM Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 10 min. The stain was

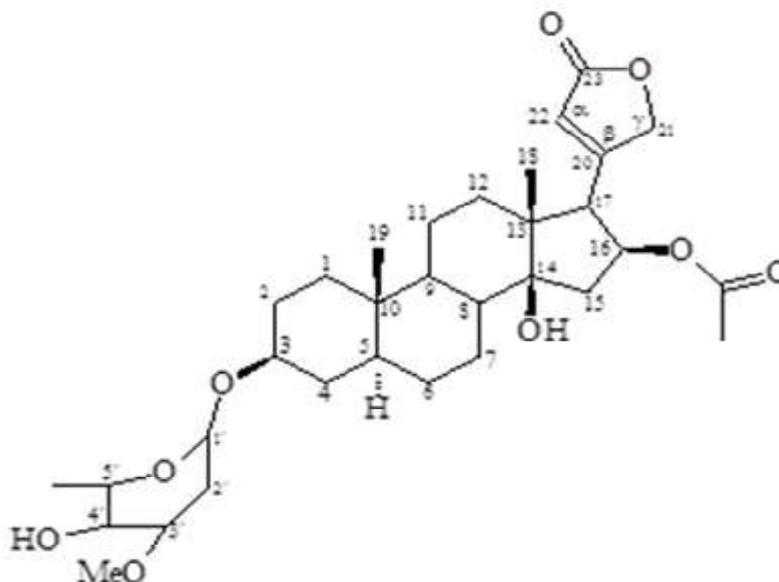
then removed and the cells were covered with mounting media (Immunotech) prior to observation using fluorescence microscopy.

Preparation of gel electrophoresis analysis

DNA fragmentation was assessed by gel electrophoresis of extracted genomic DNA from HeLa cervical cancer cells as described by Mishra et al.⁽¹⁴⁾ with slight modifications. Experimental treatment using 5 β -oleandrin (no. 3), 5 α -oleandrin (no.4), and NiO₃ (no.5) 200 ng/mL of each in RPMI 1640 medium 10% FBS, was incubated for 24 hours at 37°C, 5% CO₂; DNA as a marker (no.1) and negative control (no.2). After treatment using those samples, cells were lysed in a buffer, then isopropanol (0,5 mL) was added. The precipitate (DNA) was transferred to a new eppendorf containing 500 μ L 10 mM Tris HCl, 0.1 mM EDTA, pH 7.5, and then incubated overnight at 37 °C stirred slowly. A total of 13 ml DNA was added into 2 μ L of loading buffer mixed until homogeneous mixture was obtained. The mixture was inserted into the wells and run for 75 minutes at 50 volts. After electrophoresis was completed, the gel was transferred into a clean plastic, ethidium bromide was added and observed under UV light.

Preparation of western blot analysis

The Bcl-2 and Bax Protein expression was assessed by western blot analysis as described by Hidayat et al.⁽¹⁵⁾ and Flanagan et al.⁽¹⁶⁾ with slight modifications. After treatment with 5 α -oleandrin (3,47x10⁻⁴ mM) and followed by incubation for period of time, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8), 450 mM NaCl, 1% triton X-100, 5 mM EDTA, 1% (v/v) of protease inhibitor cocktail, and 1% (v/v) phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO). Protein (30–40 mg per lane) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked by 5% non-fat dry milk in Tris-buffered saline and

Figure 1. 5 α -oleandrin

0.1% Tween-20 (TBS-T) before incubation with a designated primary antibody (Pharmingen, San Diego, CA). After washing the membrane with TBS-T, the residue was incubated in secondary antibody and the immunoreaction was visualized using an ECL western blotting kit (Amersham Corp., Arlington Heights, IL).

RESULTS

5 α -oleandrin induces chromatin condensation in HeLa cervical cancer cells

To observe whether 5 α -oleandrin could induce the chromatin condensation in HeLa cervical cancer cells, we assessed by Hoechst 33342 staining.

The result indicated that the nuclei of HeLa cervical cancer cells presented the changing morphology of the cells like chromatin condensation in response to an treatment with 5 β -oleandrin and 5 α -oleandrin (200 ng/mL) for 24 hours incubation. These results is typical of cell apoptosis (Figure 2)

5 α -oleandrin induces DNA fragmentation response in HeLa cervical cancer cells

To investigate whether 5 α -oleandrin could induce DNA fragmentation in HeLa cervical cancer cells, we assessed by gel electrophoresis of extracted

genomic DNA from HeLa cervical cancer cells. Experimental treatment using 200 ng/mL of 5 β -oleandrin (no.3); 5 α -oleandrin (no.4); and NiO₃ (No.5) of each was incubated for 24 hours is presented in Figure 3.

The 5 α -oleandrin showed a clear observed by the presence of a smear band at about 200 bp on agarose gel, however this band was not observed when 5 β -oleandrin at equal concentration was applied. NiO₃ compound is one of other two compound isolated from the leaves of *N. indicum* Mill at the equal concentration with 5 α -oleandrin (200 ng/mL) showed a smear band similar to 5 α -oleandrin, but this band was not as clear as showed by 5 α -oleandrin.

5 α -oleandrin decreased Bcl-2 protein and increased Bax protein activities in HeLa cervical cancer cells.

To examine the Bcl-2 and Bax protein expression in 5 α -oleandrin-induced apoptosis, we used by western blot analysis. The data presented in Figure. 4 demonstrate that treatment of HeLa cervical cancer cell with 5 α -oleandrin ($3,47 \times 10^{-4}$ mM) compared with β -actin, incubation for period of time, it can be seen that Bcl-2 expression decreases compared to untreated control. Protein band of treated cells was similar to that of Bcl-2 protein at 26 kDa.

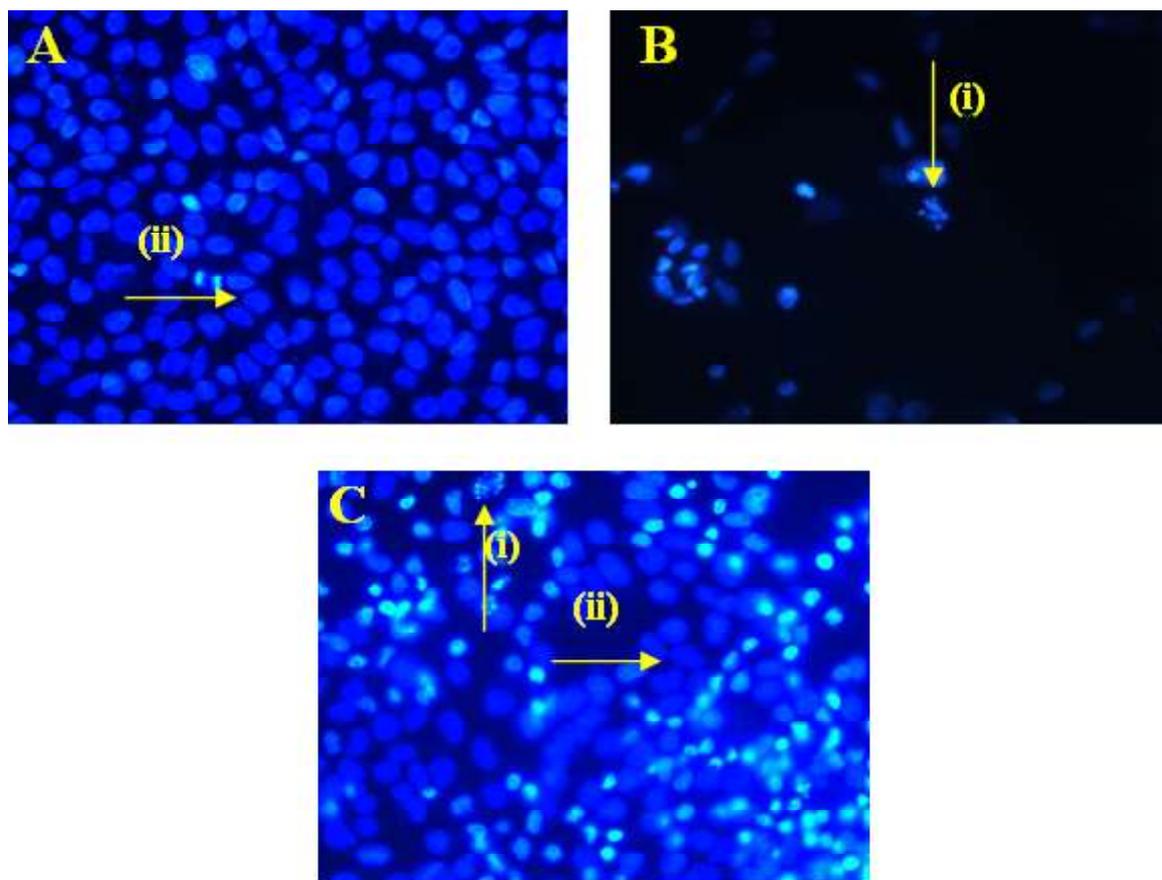


Figure 2. HeLa cervical cancer cell treated with 200 ng/mL, control (A), 5b-oleandrin (B), 5a-oleandrin (C) incubation for 24 hours, staining with Hoechst 33342, fluorescens microscop (100x). Note: (i) Chromatin condensation; (ii) Normal cells

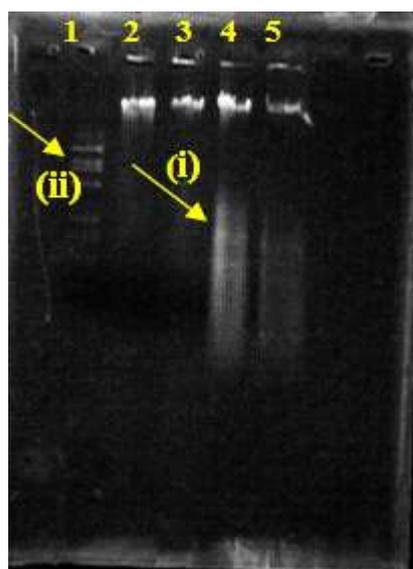


Figure 3. Gel electrophoresis of HeLa cervical cancer cells treated with 200 ng/mL incubation for 24 hours (1) DNA Marker 180 bp; (2) Control; (3) 5b-oleandrin; (4) 5a-oleandrin; (5) NiO3; Note: (i) DNA Fragmentation (ii). DNA leader

Control

The 5a-oleandrin at the same concentration ($3,47 \times 10^{-4}$ mM) treated HeLa cervical cancer cells increases Bax protein expression presented in Figure 4. Protein band of treated cells was similar to that of Bax protein at 23 kDa.

As expected, the results of the western blot assays demonstrated that 5a-oleandrin-induced apoptosis was associated with the upregulation of cyt C and Bax, the down regulation of Bcl-2 proteins in HeLa cervical cancer cells. Overall, these results indicate that 5 \pm -oleandrin-induced apoptosis is associated with the mitochondrial apoptosis-related signaling pathway.

DISCUSSION

Incubation of HeLa cervical cancer cells with 5 α -oleandrin (200 ng/mL) for 24 hours

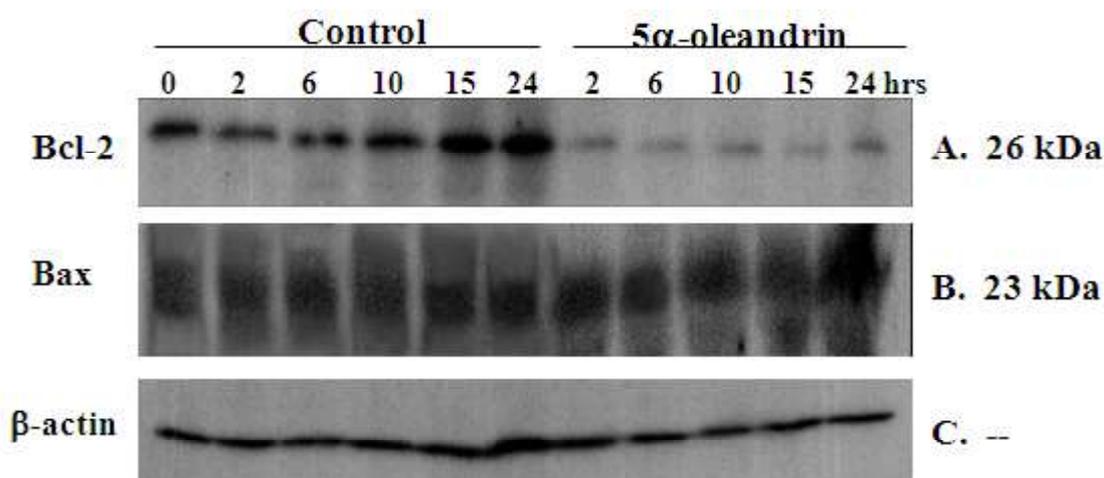


Figure 4. Bcl-2 protein expression (A); Bax (B); and β -actin (C) HeLa cervical cancer cells treated with 5 α -oleandrin (3.47×10^{-4} mM), incubation for period of time

followed by staining with Hoechst 33342 (Figure 2), showed a clear broken up light blue color of nucleus occurred. The color produced was different to the color intensity of the untreated control. The staining Hoechst 33342 is able to enter the living cells without causing toxicity to the cells, and that fluorescence occurs due to complex formation with native DNA. The fluorescence occurs due to interaction between DNA chromatin of the cells and Hoechst 33342. This method is characterized as the non-toxic specific vital stain for DNA. Chromatin condensation can therefore be used to distinguish apoptotic cells from healthy cells or necrotic cells.⁽¹⁷⁾ Apoptosis has a key role in the mechanism by which chemotherapy drugs cause cancer cell death. Apoptotic can be recognized from the changing morphology of the cells, plasma membrane destruction, and chromatin condensation.⁽¹¹⁾

Similar result was also obtained from the DNA fragmentation, treated HeLa cervical cancer cells with 5 α -oleandrin (200 ng/mL) for 24 hours (Figure 3), showed a clear observed by the presence of a smear band at about 200 bp on agarose gel. This band is as a signal or indicative of the apoptotic occurrence; however, this band was not observed when 5 β -oleandrin at equal concentration was applied. This indicated that 5 β -oleandrin did not induce HeLa cervical cancer cells DNA fragmentation, it was suggested that

the cells was lysis due to the cytotoxicity of 5 β -oleandrin. NiO3 is one of other two compound isolated from the leaves of *N. indicum* Mill at the equal concentration with 5 α -oleandrin (200 ng/mL) showed a smear band similar to 5 α -oleandrin, but this band was not as clear as showed by 5 α -oleandrin. Biochemically apoptotic can be recognized by DNA fragmentation, internucleosomal of the DNA fragments which are variable in between 180-200 base pair (bp) to form DNA ladder on the electrophoresis agarose gel. DNA fragmentation is one reason of tumorigenesis, and most of tumors have genetic DNA defects, while normal cells usually does not have these problems.⁽¹⁸⁾ The DNA ladder formation on the electrophoresis agarose gel indicates that apoptotic occurs *in vitro*.

The mechanism of action study was conducted at molecular level by determining the Bcl-2 and Bax protein expression. Bcl-2 protein is a trans membrane protein having molecular weight around 26 kDa produced by gen bcl-2 (6kb). Bcl-2 antagonists are novel compounds that sensitise cancer cells to apoptosis by mimicking BH3-only proteins. In the intrinsic apoptotic pathway, Bcl-2 family proteins, which include the anti-apoptotic proteins bcl-2 and bcl-xl, as well as the pro-apoptotic proteins bax, bak, bad and bim, are primary up-stream molecules that respond to apoptosis signals.^(16,19) When cells

are stimulated by apoptosis signals, the aforementioned apoptosis-associated proteins rapidly react to the stress and dramatically induce ROS generation. Meanwhile, a high concentration of ROS in mitochondria may reduce the MMP that enhances the mitochondrial permeability, and induce cytochrome c released from the mitochondria to the cytoplasm. In the cytoplasm, cytochrome c is involved in the formation of cytochrome c/Apaf-1/caspase-9-containing apoptosome, which subsequently results in the activation of caspase-9 and caspases-3. Activation of caspase-3, the last step of the cascade, leads to the degradation of specific cellular substrates and induces cell apoptosis.⁽¹⁹⁾

The protein bands yield was visualized by Bcl-2 antibody following transfer to polyvinylidene difluoride (PVDF) membrane (figure 4), it can be seen that Bcl-2 expression decreases due to addition of 5 α -oleandrin ($3,47 \times 10^{-4}$ mM) for 2 - 24 hours compared to untreated control. Protein band of treated cells was similar to that of Bcl-2 protein at 26 kDa. The decreasing of the Bcl-2 expression level was suggested to be indicative to the death of cancer cells.

Similar procedure for Bcl-2 protein was applied in the Bax protein expression of the cells. The 5 α -oleandrin ($3,47 \times 10^{-4}$ mM) treated HeLa cervical cancer cells increases Bax protein expression (Figure 4). Protein band of treated cells was similar to that of Bax protein at 23 kDa. It was reported that the presence of a molecular antiapoptotic (Bcl-2) can inhibit the activation of Bax following a death signal. Our results study showed that 5 α -oleandrin significantly up-regulated the expression of bax but down-regulated the level of bcl-2 with the increase of treating time, which implied an increase of bax/bcl-2 ratio in 5 α -oleandrin-treated HeLa cervical cancer cell line.

Other study on cardiac glycosides inhibited cell proliferation renal cell carcinoma by increasing the number of cells in the G2/M cell cycle phase. This results may provide a basis to

study combined treatment consisting of cardiac glycosides and radio-/chemotherapy for RCC in the future.⁽²⁰⁾ 5 α -oleandrin is a polyphenolic component of cardiac glycosides.

CONCLUSION

The 5 α -oleandrin induced HeLa cervical cancer cells DNA fragmentation observed by the presence of a smear band at about 200 bp indicative the apoptotic occurrence. 5 α -oleandrin induced apoptosis by reducing Bcl-2 protein expression and increasing Bax protein expression.

CONFLICTS OF INTERESTS

Authors declare that there are no conflicts of interest.

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CONTRIBUTORS

MSH contributed to the preparation of manuscript and research design, data collection, analysis and interpretation. SM and TT contributed to revision of the manuscript especially on the mechanism of molecular action. IGG and SW contributed to revision of the manuscript especially on the isolated compounds from *N. indicum*. MSH wrote the manuscript and all the authors read and approved the final manuscript.



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