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- Data Analysis : technical analysis, validation, software use
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RESEARCH ARTICLE

Curcuma xanthorrhiza Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid

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Abstract

BACKGROUND: *Curcuma xanthorrhiza* rhizomes have been demonstrated to have anticancer properties toward various types of cancer cells. The effect of *C. xanthorrhiza* rhizome extract (CXRE) on nasopharyngeal cancer (NPC) cells, including HONE-1 cell line has not been elucidated yet. Therefore, the effect of CXRE on the apoptosis of HONE-1 cells and its possible underlying mechanism are necessary to be explored.

METHODS: *C. xanthorrhiza* rhizomes were minced, dried, extracted with distilled ethanol, filtered, and evaporated to produce CXRE. HONE-1 cells were seeded, starved, and treated with dimethyl sulfoxide (DMSO), Doxorubicin, or various concentrations of CXRE. Treated HONE-1 cells were stained with 4',6'-diamidino-2-phenylindole (DAPI) and the number of viable cells was counted. HONE-1 cells were also collected, lysed, and further processed for immunoblotting analysis to measure Bid activity.

RESULTS: The number of viable HONE-1 cells decreased in concentration- and time-dependent manner. The number of viable cells in 50 and 250 µg/mL CXRE-treated groups were significantly lower compared with that in the DMSO-treated group after 24 h. At 48 h incubation period, the number of viable cells in 10, 50 and 250 µg/mL CXRE-treated groups were significantly lower compared with that in the DMSO-treated group. The number of viable cells in 250 µg/mL CXRE-treatment group was not significantly different compared with that in the Doxorubicin-treated group after 48 h. Bid expression levels in CXRE-treated groups were lower compared with that in the DMSO-treated group.

CONCLUSION: CXRE could induce apoptosis via Bid activation, hence reducing the viability of HONE-1 cells.

KEYWORDS: *Curcuma xanthorrhiza*, nasopharyngeal cancer, HONE-1 cells, apoptosis, Bid

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Introduction

There are 133,354 new nasopharyngeal cancer (NPC) cases and 80,008 deaths worldwide due to this type of cancer in 2020.(1) NPC is considered as the fifth most common cancer in Indonesia after breast, cervix uteri, lung, and liver cancers, with 19,943 new cases and 13,399 deaths.(2) NPC is generally treated with radiotherapy, while

combination of radiotherapy and chemotherapy is used to treat advance-stage NPC.(3-5) Standard treatments have been reported to cause numerous adverse effects, some of which are permanent.(6) Advancement in cancer treatment and the discovery of novel anticancer agents is constantly growing. One of the main focuses of the recent cancer research is the development of anticancer agents from natural substances or their derivatives since they are believed to have a potential to inhibit cancer

development and progression without affecting normal cells.(7,8)

Curcuma xanthorrhiza D.Dietr. is a medicinal plant that belongs to Zingiberaceae family. The rhizome of this plant has been reported to have numerous pharmacological activities, such as antibacterial (9,10), antioxidant (11,12), anti-inflammatory (13,14) and anticancer properties (15). These properties are due to the presence of natural compounds, which are dominated by curcuminoids and terpenoids.(16) Xanthorrhizol, the main compound of *C. xanthorrhiza* that distinguishes this species with other *Curcuma* species, has been demonstrated to show anticancer activities on several types of human cancer cells, including hepatoma (17), oral squamous cell carcinoma (18), promyelocytic leukemia (19), and non-small cell carcinoma.(20)

HONE-1, an NPC cell line, is often used to investigate the cytotoxic effect of compounds obtained from a medicinal plant.(21) This cell line has also been used in research that assesses cytotoxicity of extract obtained from *Curcuma* sp. However, the effect of *C. xanthorrhiza* rhizome extract (CXRE) on NPC cells, including HONE-1 cell line has not been elucidated yet.

The cytotoxicity of compounds found in *C. xanthorrhiza* rhizomes on different types of cancer cells may be related to apoptosis.(17-20,22) One of the signaling pathway that could be activated by these compounds to initiate apoptosis is intrinsic apoptotic pathway, which involves activation of B-cell lymphoma (Bcl)-2 homology 3-interacting domain death agonist (Bid). Apoptotic stimuli induce Bid truncation to form truncated Bid (t-Bid). t-Bid increases mitochondrial membrane permeability, which in turn causes the release of apoptogenic factors from mitochondria to cytoplasm. These apoptogenic factors promote the activation of effector caspases that play a critical role in executing cell death.(19,23) Since *C. xanthorrhiza* has been reported to show anticancer potential in various types of cancer, the effect of CXRE on the apoptosis of HONE-1 NPC cells and its possible underlying mechanism are necessary to be explored.

Methods

Plant Sample Collection and Extraction

C. xanthorrhiza rhizome samples were collected from Bogor, Indonesia. The rhizomes were identified and extracted in PT. Aretha Medika Utama, Bandung, Indonesia. Briefly, *C. xanthorrhiza* rhizomes were minced, dried, extracted with distilled 70% ethanol for 24 h at room

temperature, and evaporated. The resulting CXRE was then stored at -20°C.

HONE-1 Cell Culture

HONE-1 cells were cultured in RPMI 1640 without L-Gln (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin-Amphotericin B (Gibco). The cells were maintained in an incubator at 37°C with 5% CO₂.

4',6'-diamidino-2-phenylindole (DAPI) Staining

HONE-1 cells were seeded onto coverslips, treated with/without dimethyl sulfoxide (DMSO), 3 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia), or 10, 50, or 250 µg/mL CXRE for 24 or 48 h. CXRE-treated HONE-1 cells were fixed with 70% ethanol for 3 minutes and washed in PBS. HONE-1 cells were then fixed with 0.1% Triton X-100 for 1 minute and stained by applying 1:100 diluted DAPI (Sigma-Aldrich, St. Louis, USA). HONE-1 cells were evaluated and documented under a fluorescence microscope in three replicates. In each slide, viable cell number was counted by using grids by two independent observers.

Immunoblotting

HONE-1 cells were seeded and treated with/without DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h. Treated HONE-1 cells were lysed with a lysis buffer containing 10X radio-immunoprecipitation assay (RIPA) buffer (Abcam, Cambridge, UK) and phenylmethanesulfonyl fluoride (Sigma-Aldrich). Twenty µL lysates were sodium dodecyl sulfate-polyacrylamide gel electrophoresed, followed by the transfer onto the polyvinylidene difluoride membrane. Blocking was performed with 5% skim milk, then the sheets were probed with rabbit polyclonal anti-BID antibody (Cell Signaling Technology) diluted 1:1000 in phosphate-buffered saline (PBS). Then, goat anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) diluted 1:2000 in PBS was added. Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories) was used to visualize the bands while Alliance 4.7 (UVItech, Cambridge, UK) was used to capture and quantify the bands.

Data Analyses

Statistical analysis was performed with IBM SPSS Statistics version 26 (IBM Corporation, Armonk, NY, USA). Shapiro-Wilk test was performed to analyze the normality of the data. To analyze the differences of the number of viable cells between groups at 24 and 48 h, Kruskal-Wallis test followed by post hoc Mann-Whitney U test were used.

Results

CXRE Decreased the Amount of Viable HONE-1 Cells

At 24 and 48 h, the viability of HONE-1 cells in the DMSO-treated group was the highest compared with other groups (Figure 1A, 2A), while the viability of HONE-1 cells in the Doxorubicin-treated group was the lowest (Figure 1B, 2B) as indicated by DAPI staining results. There were 337.83 ± 66.58 and 297.50 ± 81.44 viable HONE-1 cells in the DMSO-treated group at 24 and 48 h, respectively. Meanwhile, there were only 7.00 ± 5.87 and 4.83 ± 2.40 viable cells after Doxorubicin treatment for 24 and 48 h, respectively (Figure 3). Upon CXRE addition, the viability of HONE-1 cells was lower compared with that in the DMSO-treated group, implying that CXRE could reduce the viability of HONE-1 cells (Figure 1C-1E, 2C-2E). The number of viable HONE-1 cells decreased in concentration- and time-dependent manner. The number of viable cells in CXRE-treated groups were significantly lower compared with that in DMSO-treated group ($p < 0.05$), except for 10 $\mu\text{g}/\text{mL}$ CXRE-treated group at 24 h ($p = 0.109$). However, the number of viable cells in CXRE-treated groups were significantly higher compared with that in Doxorubicin-treated group ($p < 0.05$), except for 250 $\mu\text{g}/\text{mL}$ CXRE-treatment group at 48 h ($p = 0.872$). CXRE-treated groups had lower number of viable cells at 48 h than those at 24 h (Figure 3).

CXRE Reduced Bid Expression in HONE-1 Cells

Bid expression levels in 10, 50, and 250 $\mu\text{g}/\text{mL}$ CXRE-treated groups were lower compared with that in the DMSO-treated group. CXRE concentration of 250 $\mu\text{g}/\text{mL}$ showed greater reduction in Bid expression than the other two concentrations. Bid expression level in HONE-1 cells treated with 50 $\mu\text{g}/\text{mL}$ CXRE was slightly higher compared

with those treated with 10 $\mu\text{g}/\text{mL}$ CXRE. Bid expression levels in the CXRE-treated groups were higher compared with that in Doxorubicin-treated group. No band was observed in the Doxorubicin-treated group (Figure 4).

Discussion

In the present study, CXRE reduced the viability of HONE-1 cells in concentration- and time-dependent manner, which may be caused by apoptosis induction. A previous study reported that combination of Cisplatin, *C. xanthorrhiza* rhizome ethanolic extract and *Ficus septica* leaves ethanolic extract enhanced apoptosis of human breast cancer cells, as demonstrated by higher cell death percentage when compared with those that were treated with Cisplatin merely.(24) Active compounds found in *C. xanthorrhiza* rhizomes have also been reported to induce apoptosis of several cancer cells. Xanthorrhizol has been demonstrated to promote apoptosis in human hepatoma (17), promyelocytic leukemia (19), and non-small cell carcinoma cells.(20) Curcumin, another important compounds in rhizomes of *C. xanthorrhiza* and other *Curcuma* species (11,25), has been shown to promote apoptosis in NPC cell lines, such as NPC-TW 076 (26), CNE1 and CNE2 (27), as well as other types of cancer, including prostate cancer (22) and acute myeloid leukemia cells.(28) Interestingly, a study reveals that a combination of xanthorrhizol and curcumin synergistically inhibit cell growth by inducing apoptosis in human breast cancer cells.(29)

To confirm whether CXRE promoted apoptosis of HONE-1 cells, the expression levels of Bid were measured. Upon activation of death receptors by apoptotic signals, full length Bid is truncated by cleaved caspase-8 to form truncated Bid (t-Bid), which interconnects intrinsic and extrinsic apoptotic pathways. Hence, upon activation, the amount

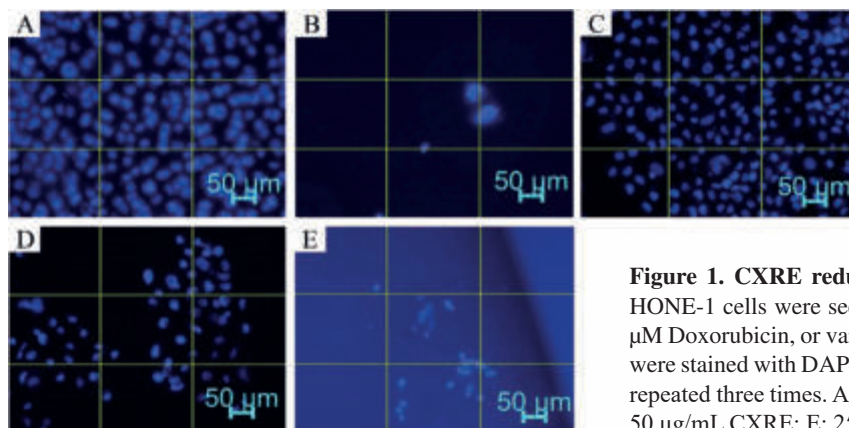


Figure 1. CXRE reduced the viability of HONE-1 cells after 24 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μM Doxorubicin, or various concentrations of CXRE for 24 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: DMSO; B: Doxorubicin; C: 10 $\mu\text{g}/\text{mL}$ CXRE; D: 50 $\mu\text{g}/\text{mL}$ CXRE; E: 250 $\mu\text{g}/\text{mL}$ CXRE.

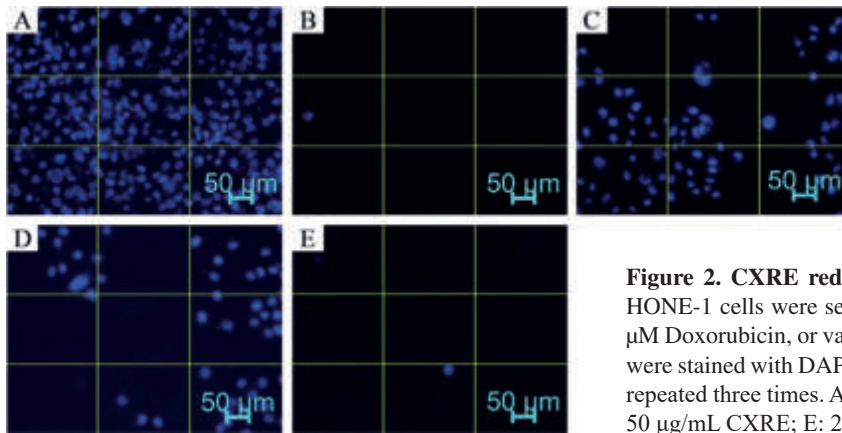


Figure 2. CXRE reduced the viability of HONE-1 cells after 48 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 48 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: DMSO; B: Doxorubicin; C: 10 µg/mL CXRE; D: 50 µg/mL CXRE; E: 250 µg/mL CXRE.

of Bid is decreased while the amount of t-Bid is increased in the cell. tBid then translocates to mitochondria where it blocks anti-apoptotic activity of Bcl-extra-large (Bcl-XL) and Bcl-2, and activates proapoptotic Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak). This leads to the release of second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein-binding protein with low pI (DIABLO) and cytochrome *c*, which play critical roles in executing cell death.(19,23,30) In the present study, Bid expression levels in CXRE-treated HONE-1 cells were lower compared with those in the DMSO-treated group. Thus, it can be concluded that CXRE stimulated Bid activation.

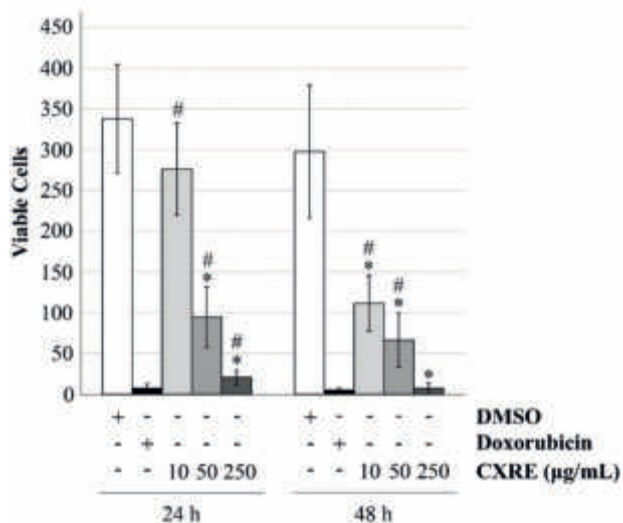


Figure 3. CXRE decreased the number of viable HONE-1 cells in concentration- and time-dependent manner. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 and 48 h. Cell nuclei were stained with DAPI. The number of viable cells in each slide was evaluated by two independent observers as described in Methods. The data were expressed as mean±SD (n=3). * $p < 0.05$ vs. DMSO-treated group; # $p < 0.05$ vs. Doxorubicin-treated group.

The CXRE-induced Bid truncation could be related to its active compounds, xanthorrhizol and curcumin. Xanthorrhizol has been demonstrated to decrease Bid expression in several types of cancer cells, such as hepatoma (17), promyelocytic leukemia cells (19), and colon cancer (31). Curcumin-induced reduction of Bid expression is also reported in several cancer cells, including glioblastoma (32) and B-precursor acute lymphoblastic leukemia cell lines (33).

Xanthorrhizol has been reported to regulate several signaling pathways which modulate apoptosis induction of cancer cells. Xanthorrhizol inhibits proliferation and induces apoptosis of non-small cell carcinoma cells by inhibiting the activation of phosphatidylinositol 3-kinase (PI3K)/Akt/nuclear factor kappa B (NF-κB) pathway, which is involved in controlling cell survival.(20) Interestingly, this compound may also be capable in inducing caspase-independent apoptosis via stimulation of reactive oxygen species (ROS)-

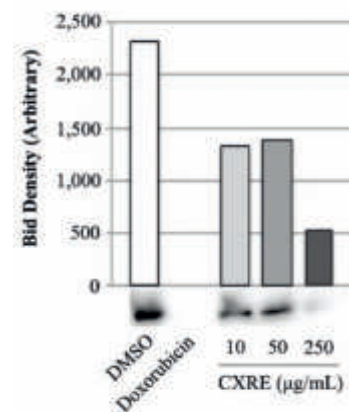


Figure 4. CXRE diminished Bid expression in HONE-1 cells. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h as indicated in the panel. Cells were collected, lysed, and further processed to obtain cell lysate for Western blot analysis as described in Methods.

mediated p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) in human oral squamous cell carcinoma cells.(18)

Since the results of the present study showed that CXRE activated Bid, which in turn leads HONE-1 cells to apoptosis, components and phenomena in apoptosis signaling pathway both upstream and downstream of Bid, such as DNA fragmentation and $\Delta\Psi_m$ attenuation, as well as the expression level of caspases and apoptogenic factors should be examined.

Conclusion

CXRE could induce apoptosis via Bid activation, hence reducing the viability of HONE-1 cells. Taken together, CXRE is suggested to have cytotoxic effect towards NPC cells, and it could be a potential anticancer agent for NPC.

Authors Contribution

DR and FS prepared study concept and design. DR, FS and JH performed processing and acquisition of data. DR, FS, SJAI and MSD performed analysis and interpretation of results. DR and JH prepared the draft of the manuscript. FS, SJAI and MSD made critical revisions of the manuscript. DR, JH and SJAI assisted in administrative, technical, and material support. FS and MSD performed supervision of the study.

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Curcuma xanthorrhiza Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid

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RESEARCH ARTICLE

***Curcuma xanthorrhiza* Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid**Dewi Ranggani¹, Ferry Sandra^{2*}, Johni Halim¹, Solachuddin Jauhari Arief Ichwan³,
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Abstract

BACKGROUND: *Curcuma xanthorrhiza* rhizomes have been demonstrated to have anticancer properties toward various types of cancer cells. The effect of *C. xanthorrhiza* rhizome extract (CXRE) on nasopharyngeal cancer (NPC) cells, including HONE-1 cell line has not been elucidated yet. Therefore, the effect of CXRE on the apoptosis of HONE-1 cells and its possible underlying mechanism are necessary to be explored.

METHODS: *C. xanthorrhiza* rhizomes were minced, dried, extracted with distilled ethanol, filtered, and evaporated to produce CXRE. HONE-1 cells were seeded, starved, and treated with dimethyl sulfoxide (DMSO), Doxorubicin, or various concentrations of CXRE. Treated HONE-1 cells were stained with 4',6'-diamidino-2-phenylindole (DAPI) and the number of viable cells was counted. HONE-1 cells were also collected, lysed, and further processed for immunoblotting analysis to measure Bid activity.

RESULTS: The number of viable HONE-1 cells decreased in concentration- and time-dependent manner. The number of viable cells in 50 and 250 µg/mL CXRE-treated groups were significantly lower compared with that in the DMSO-treated group after 24 h. At 48 h incubation period, the number of viable cells in 10, 50 and 250 µg/mL CXRE-treated groups were significantly lower compared with that in the DMSO-treated group. The number of viable cells in 250 µg/mL CXRE-treatment group was not significantly different compared with that in the Doxorubicin-treated group after 48 h. Bid expression levels in CXRE-treated groups were lower compared with that in the DMSO-treated group.

CONCLUSION: CXRE could induce apoptosis via Bid activation, hence reducing the viability of HONE-1 cells.

KEYWORDS: *Curcuma xanthorrhiza*, nasopharyngeal cancer, HONE-1 cells, apoptosis, Bid

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Introduction

There are 133,354 new nasopharyngeal cancer (NPC) cases and 80,008 deaths worldwide due to this type of cancer in 2020.(1) NPC is considered as the fifth most common cancer in Indonesia after breast, cervix uteri, lung, and liver cancers, with 19,943 new cases and 13,399 deaths.(2) NPC is generally treated with radiotherapy, while

combination of radiotherapy and chemotherapy is used to treat advance-stage NPC.(3-5) Standard treatments have been reported to cause numerous adverse effects, some of which are permanent.(6) Advancement in cancer treatment and the discovery of novel anticancer agents is constantly growing. One of the main focuses of the recent cancer research is the development of anticancer agents from natural substances or their derivatives since they are believed to have a potential to inhibit cancer

development and progression without affecting normal cells.(7,8)

Curcuma xanthorrhiza D.Dietr. is a medicinal plant that belongs to Zingiberaceae family. The rhizome of this plant has been reported to have numerous pharmacological activities, such as antibacterial (9,10), antioxidant (11,12), anti-inflammatory (13,14) and anticancer properties (15). These properties are due to the presence of natural compounds, which are dominated by curcuminoids and terpenoids.(16) Xanthorrhizol, the main compound of *C. xanthorrhiza* that distinguishes this species with other *Curcuma* species, has been demonstrated to show anticancer activities on several types of human cancer cells, including hepatoma (17), oral squamous cell carcinoma (18), promyelocytic leukemia (19), and non-small cell carcinoma.(20)

HONE-1, an NPC cell line, is often used to investigate the cytotoxic effect of compounds obtained from a medicinal plant.(21) This cell line has also been used in research that assesses cytotoxicity of extract obtained from *Curcuma* sp. However, the effect of *C. xanthorrhiza* rhizome extract (CXRE) on NPC cells, including HONE-1 cell line has not been elucidated yet.

The cytotoxicity of compounds found in *C. xanthorrhiza* rhizomes on different types of cancer cells may be related to apoptosis.(17-20,22) One of the signaling pathway that could be activated by these compounds to initiate apoptosis is intrinsic apoptotic pathway, which involves activation of B-cell lymphoma (Bcl)-2 homology 3-interacting domain death agonist (Bid). Apoptotic stimuli induce Bid truncation to form truncated Bid (t-Bid). t-Bid increases mitochondrial membrane permeability, which in turn causes the release of apoptogenic factors from mitochondria to cytoplasm. These apoptogenic factors promote the activation of effector caspases that play a critical role in executing cell death.(19,23) Since *C. xanthorrhiza* has been reported to show anticancer potential in various types of cancer, the effect of CXRE on the apoptosis of HONE-1 NPC cells and its possible underlying mechanism are necessary to be explored.

Methods

Plant Sample Collection and Extraction

C. xanthorrhiza rhizome samples were collected from Bogor, Indonesia. The rhizomes were identified and extracted in PT. Aretha Medika Utama, Bandung, Indonesia. Briefly, *C. xanthorrhiza* rhizomes were minced, dried, extracted with distilled 70% ethanol for 24 h at room

temperature, and evaporated. The resulting CXRE was then stored at -20°C.

HONE-1 Cell Culture

HONE-1 cells were cultured in RPMI 1640 without L-Gln (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin-Amphotericin B (Gibco). The cells were maintained in an incubator at 37°C with 5% CO₂.

4',6'-diamidino-2-phenylindole (DAPI) Staining

HONE-1 cells were seeded onto coverslips, treated with/without dimethyl sulfoxide (DMSO), 3 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia), or 10, 50, or 250 µg/mL CXRE for 24 or 48 h. CXRE-treated HONE-1 cells were fixed with 70% ethanol for 3 minutes and washed in PBS. HONE-1 cells were then fixed with 0.1% Triton X-100 for 1 minute and stained by applying 1:100 diluted DAPI (Sigma-Aldrich, St. Louis, USA). HONE-1 cells were evaluated and documented under a fluorescence microscope in three replicates. In each slide, viable cell number was counted by using grids by two independent observers.

Immunoblotting

HONE-1 cells were seeded and treated with/without DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h. Treated HONE-1 cells were lysed with a lysis buffer containing 10X radio-immunoprecipitation assay (RIPA) buffer (Abcam, Cambridge, UK) and phenylmethanesulfonyl fluoride (Sigma-Aldrich). Twenty µL lysates were sodium dodecyl sulfate-polyacrylamide gel electrophoresed, followed by the transfer onto the polyvinylidene difluoride membrane. Blocking was performed with 5% skim milk, then the sheets were probed with rabbit polyclonal anti-BID antibody (Cell Signaling Technology) diluted 1:1000 in phosphate-buffered saline (PBS). Then, goat anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) diluted 1:2000 in PBS was added. Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories) was used to visualize the bands while Alliance 4.7 (UVItech, Cambridge, UK) was used to capture and quantify the bands.

Data Analyses

Statistical analysis was performed with IBM SPSS Statistics version 26 (IBM Corporation, Armonk, NY, USA). Shapiro-Wilk test was performed to analyze the normality of the data. To analyze the differences of the number of viable cells between groups at 24 and 48 h, Kruskal-Wallis test followed by post hoc Mann-Whitney U test were used.

Results

CXRE Decreased the Amount of Viable HONE-1 Cells

At 24 and 48 h, the viability of HONE-1 cells in the DMSO-treated group was the highest compared with other groups (Figure 1A, 2A), while the viability of HONE-1 cells in the Doxorubicin-treated group was the lowest (Figure 1B, 2B) as indicated by DAPI staining results. There were 337.83 ± 66.58 and 297.50 ± 81.44 viable HONE-1 cells in the DMSO-treated group at 24 and 48 h, respectively. Meanwhile, there were only 7.00 ± 5.87 and 4.83 ± 2.40 viable cells after Doxorubicin treatment for 24 and 48 h, respectively (Figure 3). Upon CXRE addition, the viability of HONE-1 cells was lower compared with that in the DMSO-treated group, implying that CXRE could reduce the viability of HONE-1 cells (Figure 1C-1E, 2C-2E). The number of viable HONE-1 cells decreased in concentration- and time-dependent manner. The number of viable cells in CXRE-treated groups were significantly lower compared with that in DMSO-treated group ($p < 0.05$), except for 10 $\mu\text{g}/\text{mL}$ CXRE-treated group at 24 h ($p = 0.109$). However, the number of viable cells in CXRE-treated groups were significantly higher compared with that in Doxorubicin-treated group ($p < 0.05$), except for 250 $\mu\text{g}/\text{mL}$ CXRE-treatment group at 48 h ($p = 0.872$). CXRE-treated groups had lower number of viable cells at 48 h than those at 24 h (Figure 3).

CXRE Reduced Bid Expression in HONE-1 Cells

Bid expression levels in 10, 50, and 250 $\mu\text{g}/\text{mL}$ CXRE-treated groups were lower compared with that in the DMSO-treated group. CXRE concentration of 250 $\mu\text{g}/\text{mL}$ showed greater reduction in Bid expression than the other two concentrations. Bid expression level in HONE-1 cells treated with 50 $\mu\text{g}/\text{mL}$ CXRE was slightly higher compared

with those treated with 10 $\mu\text{g}/\text{mL}$ CXRE. Bid expression levels in the CXRE-treated groups were higher compared with that in Doxorubicin-treated group. No band was observed in the Doxorubicin-treated group (Figure 4).

Discussion

In the present study, CXRE reduced the viability of HONE-1 cells in concentration- and time-dependent manner, which may be caused by apoptosis induction. A previous study reported that combination of Cisplatin, *C. xanthorrhiza* rhizome ethanolic extract and *Ficus septica* leaves ethanolic extract enhanced apoptosis of human breast cancer cells, as demonstrated by higher cell death percentage when compared with those that were treated with Cisplatin merely.(24) Active compounds found in *C. xanthorrhiza* rhizomes have also been reported to induce apoptosis of several cancer cells. Xanthorrhizol has been demonstrated to promote apoptosis in human hepatoma (17), promyelocytic leukemia (19), and non-small cell carcinoma cells.(20) Curcumin, another important compounds in rhizomes of *C. xanthorrhiza* and other *Curcuma* species (11,25), has been shown to promote apoptosis in NPC cell lines, such as NPC-TW 076 (26), CNE1 and CNE2 (27), as well as other types of cancer, including prostate cancer (22) and acute myeloid leukemia cells.(28) Interestingly, a study reveals that a combination of xanthorrhizol and curcumin synergistically inhibit cell growth by inducing apoptosis in human breast cancer cells.(29)

To confirm whether CXRE promoted apoptosis of HONE-1 cells, the expression levels of Bid were measured. Upon activation of death receptors by apoptotic signals, full length Bid is truncated by cleaved caspase-8 to form truncated Bid (t-Bid), which interconnects intrinsic and extrinsic apoptotic pathways. Hence, upon activation, the amount

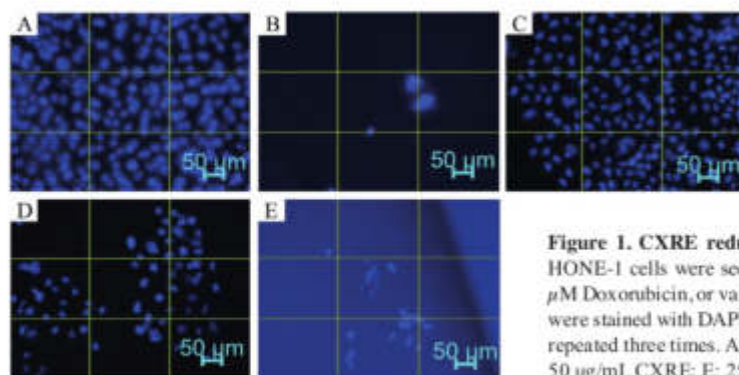


Figure 1. CXRE reduced the viability of HONE-1 cells after 24 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μM Doxorubicin, or various concentrations of CXRE for 24 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: DMSO; B: Doxorubicin; C: 10 $\mu\text{g}/\text{mL}$ CXRE; D: 50 $\mu\text{g}/\text{mL}$ CXRE; E: 250 $\mu\text{g}/\text{mL}$ CXRE.

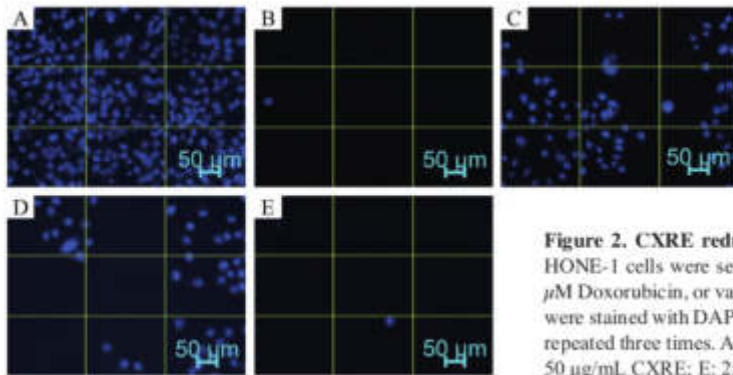


Figure 2. CXRE reduced the viability of HONE-1 cells after 48 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μ M Doxorubicin, or various concentrations of CXRE for 48 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: DMSO; B: Doxorubicin; C: 10 μ g/mL CXRE; D: 50 μ g/mL CXRE; E: 250 μ g/mL CXRE.

of Bid is decreased while the amount of t-Bid is increased in the cell. tBid then translocates to mitochondria where it blocks anti-apoptotic activity of Bcl-extra-large (Bcl-XL) and Bcl-2, and activates proapoptotic Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak). This leads to the release of second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein-binding protein with low pI (DIABLO) and cytochrome c, which play critical roles in executing cell death.(19,23,30) In the present study, Bid expression levels in CXRE-treated HONE-1 cells were lower compared with those in the DMSO-treated group. Thus, it can be concluded that CXRE stimulated Bid activation.

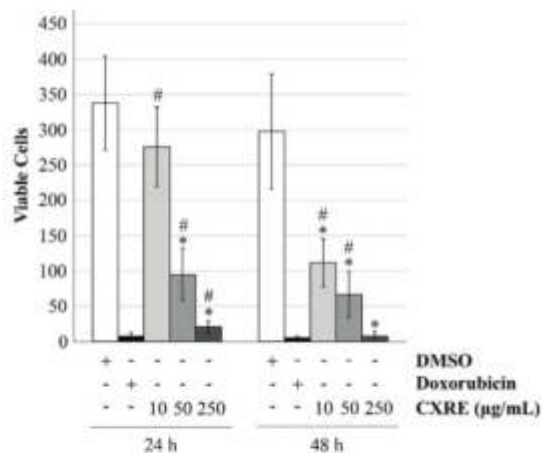


Figure 3. CXRE decreased the number of viable HONE-1 cells in concentration- and time-dependent manner. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μ M Doxorubicin, or various concentrations of CXRE for 24 and 48 h. Cell nuclei were stained with DAPI. The number of viable cells in each slide was evaluated by two independent observers as described in Methods. The data were expressed as mean \pm SD (n=3). * p <0.05 vs. DMSO-treated group; # p <0.05 vs. Doxorubicin-treated group.

The CXRE-induced Bid truncation could be related to its active compounds, xanthorrhizol and curcumin. Xanthorrhizol has been demonstrated to decrease Bid expression in several types of cancer cells, such as hepatoma (17), promyelocytic leukemia cells (19), and colon cancer (31). Curcumin-induced reduction of Bid expression is also reported in several cancer cells, including glioblastoma (32) and B-precursor acute lymphoblastic leukemia cell lines (33).

Xanthorrhizol has been reported to regulate several signaling pathways which modulate apoptosis induction of cancer cells. Xanthorrhizol inhibits proliferation and induces apoptosis of non-small cell carcinoma cells by inhibiting the activation of phosphatidylinositol 3-kinase (PI3K)/Akt/nuclear factor kappa B (NF- κ B) pathway, which is involved in controlling cell survival.(20) Interestingly, this compound may also be capable in inducing caspase-independent apoptosis via stimulation of reactive oxygen species (ROS)-

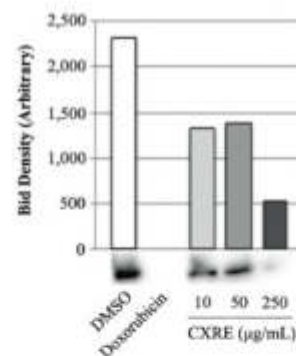


Figure 4. CXRE diminished Bid expression in HONE-1 cells. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μ M Doxorubicin, or 10, 50, or 250 μ g/mL CXRE for 6 h as indicated in the panel. Cells were collected, lysed, and further processed to obtain cell lysate for Western blot analysis as described in Methods.

mediated p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) in human oral squamous cell carcinoma cells.(18)

Since the results of the present study showed that CXRE activated Bid, which in turn leads HONE-1 cells to apoptosis, components and phenomena in apoptosis signaling pathway both upstream and downstream of Bid, such as DNA fragmentation and $\Delta\Psi_m$ attenuation, as well as the expression level of caspases and apoptogenic factors should be examined.

Conclusion

CXRE could induce apoptosis via Bid activation, hence reducing the viability of HONE-1 cells. Taken together, CXRE is suggested to have cytotoxic effect towards NPC cells, and it could be a potential anticancer agent for NPC.

Authors Contribution

DR and FS prepared study concept and design. DR, FS and JH performed processing and acquisition of data. DR, FS, SJAI and MSD performed analysis and interpretation of results. DR and JH prepared the draft of the manuscript. FS, SJAI and MSD made critical revisions of the manuscript. DR, JH and SJAI assisted in administrative, technical, and material support. FS and MSD performed supervision of the study.

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