Print ISSN: 2085-3297, Online ISSN: 2355-9179

The Indonesian BIOMEDICAL JOURNAL

Volume 15 Number 1
February 2023

Published by:

PERI
The Prodia Education & Research Institute

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Print ISSN: 2085-3297, Online ISSN: 2355-9179

The Indonesian BIOMEDICAL JOURNAL

Volume 15 Number 1, February 2023

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Print ISSN: 2085-3297, Online ISSN: 2355-9179

Content

The Indonesian Biomedical Journal
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Curcuma xanthorrhiza Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid

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Received date: Jan 6, 2023; Revised date: Jan 19, 2023; Accepted date: Jan 20, 2023

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

Indones Biomed J. 2023; 15(1): 100-5

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% CO_2.

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 µg/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 µg/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 µg/mL CXRE-treated groups were lower compared with that in the DMSO-treated group. CXRE concentration of 250 µg/mL showed greater reduction in Bid expression than the other two concentrations. Bid expression level in HONE-1 cells treated with 50 µg/mL CXRE was slightly higher compared with those treated with 10 µg/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

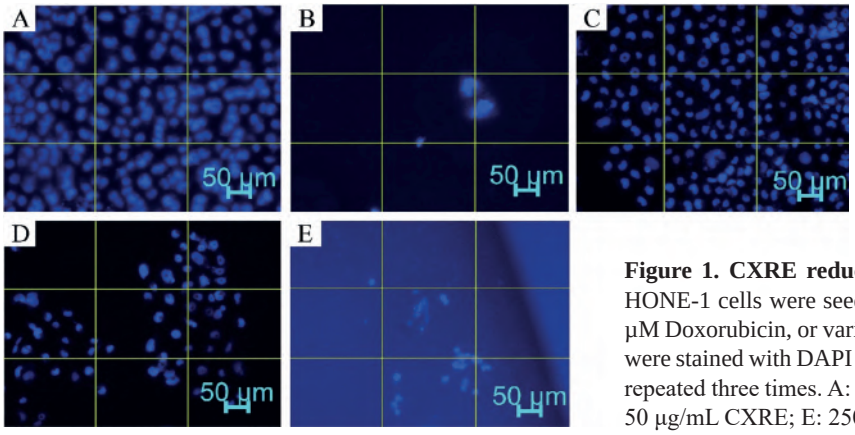

Figure 1. CXRE reduced the viability of HONE-1 cells after 24 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

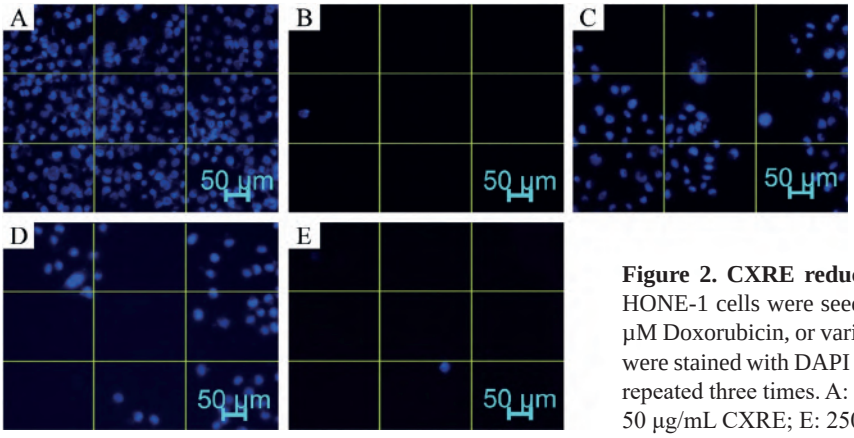

Figure 2. CXRE reduced the viability of HONE-1 cells after 48 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 48 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

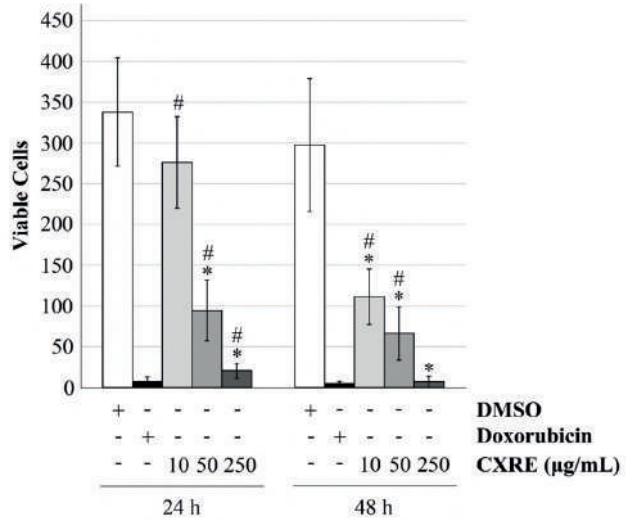

Figure 3. CXRE decreased the number of viable HONE-1 cells in concentration- and time-dependent manner. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 and 48 h. Cell nuclei were stained with DAPI. The number of viable cells 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)-

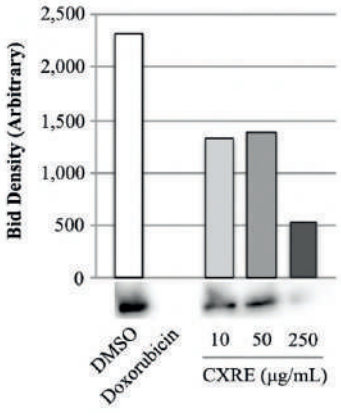

Figure 4. CXRE diminished Bid expression in HONE-1 cells. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h as indicated in the panel. Cells were collected, lysed, and further processed to obtained cell lysate for Western blot analysis 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 ΔΨm 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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The Indonesian Biomedical Journal, Vol.15, No.1, February 2023, p.1-105 Print ISSN: 2085-3297, Online ISSN: 2355-9179

RESEARCH ARTICLE

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

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Received date: Jan 6, 2023; Revised date: Jan 19, 2023; Accepted date: Jan 20, 2023

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

Indones Biomed J. 2023; 15(1): 100-5

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

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by Ferry Sandra

Submission date: 17-Jan-2024 08:39AM (UTC+0700)
Submission ID: 2272236039
File name: V15N1A10.pdf (1.58M)
Word count: 3793
Character count: 20892

The Indonesian Biomedical Journal, Vol.15, No.1, February 2023, p.1-105 Print ISSN: 2085-3297, Online ISSN: 2355-9179

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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.

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Introduction

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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% CO_2.

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 µg/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 µg/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 µg/mL CXRE-treated groups were lower compared with that in the DMSO-treated group. CXRE concentration of 250 µg/mL showed greater reduction in Bid expression than the other two concentrations. Bid expression level in HONE-1 cells treated with 50 µg/mL CXRE was slightly higher compared with those treated with 10 µg/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

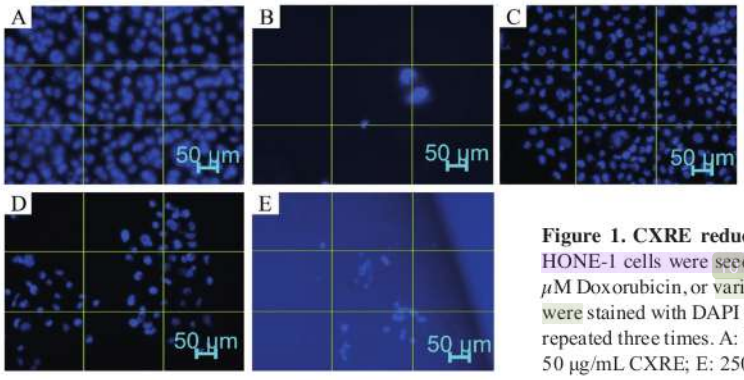

Figure 1. CXRE reduced the viability of HONE-1 cells after 24 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

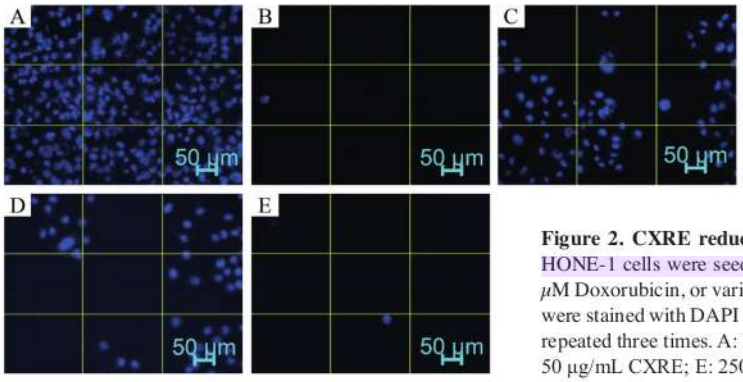

Figure 2. CXRE reduced the viability of HONE-1 cells after 48 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μM Doxorubicin, or various concentrations of CXRE for 48 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

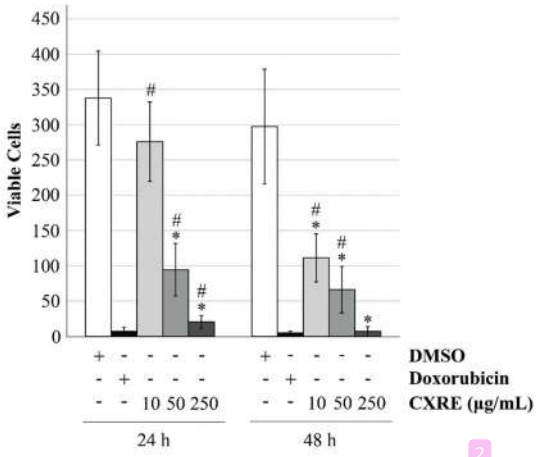

Figure 3. CXRE decreased the number of viable HONE-1 cells in concentration- and time-dependent manner. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μM Doxorubicin, or various concentrations of CXRE for 24 and 48 h. Cell nuclei were stained with DAPI. The number of viable cells 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)-

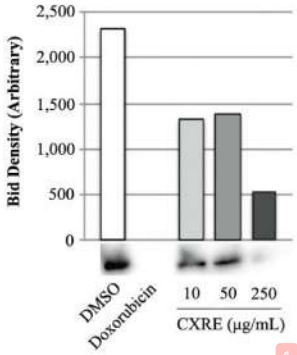

Figure 4. CXRE diminished Bid expression in HONE-1 cells. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h as indicated in the panel. Cells were collected, lysed, and further processed to obtained cell lysate for Western blot analysis 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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1	K.M. Li, X. Sun, H.K. Koon, W.N. Leung, M.C. Fung, R.N.S. Wong, Maria L. Lung, C.K. Chang, N.K. Mak. "Apoptosis and expression of cytokines triggered by pyropheophorbide-a methyl ester-mediated photodynamic therapy in nasopharyngeal carcinoma cells", Photodiagnosis and Photodynamic Therapy, 2006 Publication	1%
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InaBJ V15N1A10 - Curcuma xanthorrhiza Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid

GRADEMARK REPORT

FINAL GRADE

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GENERAL COMMENTS

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UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2023009 Editor Decision Round 1 - Revisions Required

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, 17 Jan, 2023 at 11:46 AM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "***Curcuma xanthorrhiza* Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid Activation**".

Our decision is: **Revisions Required.**

Find the file attached to see detailed comments from reviewers. Please make sure you read all the comments and revise the manuscript based on the suggestions given.

Revise this manuscript thoroughly before **January 31, 2023**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes.

When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/2217, or simply send us an email of your revised manuscript and response letter.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

--
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Manuscript Review Form

Reviewer	:	Reviewer 1
Manuscript #	:	M2023009
Manuscript Title	:	*Curcuma xanthorrhiza* Rhizome Extract Induces Apoptosis inHONE-1 Nasopharyngeal Cancer Cells Through Bid Activation

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	V	
	Notes:		
2.	Are the title and abstract of the manuscript appropriate?	V	
	Notes: The abstract is clear, written in a good English structure but can be made more interesting		
3	Do the title and abstract reflect the study result/content?	V	
	Notes:		
4.	Is the significance of the study well explained at the Background?	V	
	Notes:		
5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists?	V	
	Notes: The number of viable cells in each slide was counted by two independent observers. However, the cell count has not been clearly described		

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6.	Are the results, ideas, and data presented in this manuscript important enough for publication?	V	
	Notes:		
7.	Are all figures and tables necessarily presented?	V	
	Notes:		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?	V	
	Notes: 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). Please recheck the references, In order to explain further about its apoptotic pathway.		
9.	Are the conclusions and interpretations valid and supported by the data?	V	
	Notes:		
10.	Is the manuscript clear, comprehensible, and written in a good English structure?	V	
	Notes: The manuscript is clear, but can it be made to be more interesting, and written in a good English structure?		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

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Reviewer's Recommendation (Please tick only one option)	√
Accept Submission (No significant alterations suggested)	
Revisions Required (Suggest changes to the manuscript as specified in this review)	V
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	

Further Reviewer's Comments Regarding Disposition of the Manuscript:

Date and Sign:
January 10, 2023

Reviewer 1

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Manuscript Review Form

Reviewer	:	Reviewer 2
Manuscript #	:	M2023009
Manuscript Title	:	*Curcuma xanthorrhiza* Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid Activation

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	✔	
	Notes: This study explored effect of CXRE on the apoptosis of HONE-1 cells via Bid expression		
2.	Are the title and abstract of the manuscript appropriate?	✔	
	Notes:		
3	Do the title and abstract reflect the study result/content?	✔	
	Notes:		
4.	Is the significance of the study well explained at the Background?	✔	
	Notes:		
5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists?		✔
	Notes: 1) Why are HONE-1 cells starved for 12 hours prior to treatment? 2) Why use DMSO as a HONE-1 cells therapy agent? What is the concentration of DMSO used in this study? 3) Why use DAPI staining to identify viable cells? DAPI staining (nuclei stained) is applied to observe changes in nuclear morphology (condensed and fragmented nuclei). Cell numbers were reduced due to nuclear shrinkage, chromosome condensation and apoptotic bodies. 4) Why is the HONE-1 cell treatment for the Bid expression carried out only for 6 hours?		
6.	Are the results, ideas, and data presented in this manuscript important enough for publication?		✔
	Notes: 1) The data had large standard deviation, what were this caused by? 2) Lines 125-126: "the number of viable cells in 250 µg/mL CXRE-treatment group		

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	was not significantly different compared to that in the Doxorubicin-treated group". ➢ Why are no bands observed on Doxorubicin-treated group?		
7.	Are all figures and tables necessarily presented?	✔	
	Notes:		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?	✔	
	Notes: Lines 154-155: "Bid activation reduces the amount of Bid" ➢ Caspases-8/-10 cleaves Bid to produce tBid, which activates a crosstalk pathway between death receptors and mitochondria. ➢ tBid translocates to mitochondria where it blocks anti-apoptotic activity of Bcl-2 and Bcl-XL, and activates Bax and Bak. ➢ This leads to release of cytochrome c and Smac/Diablo and activation of the mitochondrial pathway of apoptosis.		
9.	Are the conclusions and interpretations valid and supported by the data?	✔	
	Notes:		
10.	Is the manuscript clear, comprehensible, and written in a good English structure?	✔	
	Notes:		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

➢ It is necessary to analyze the effect of CXRE on growth, proliferation, or apoptosis of normal cell

Reviewer's Recommendation (Please tick only one option)	√
Accept Submission (No significant alterations suggested)	
Revisions Required (Suggest changes to the manuscript as specified in this review)	✔
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	

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Further Reviewer's Comments Regarding Disposition of the Manuscript:

- This is a good paper with the potential to achieve a substantial contribution

Date and Sign:
January 14th, 2023

Reviewer 2

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

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 expression.

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 to 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

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) The 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 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 Biomolecular Biomedical Research Center, PT. Aretha Medika Utama, Bandung, Indonesia. Briefly, *C. xanthorrhiza* rhizomes were minced and dried. The dried material was extracted with distilled 70% ethanol at room temperature for 24 h, filtered, and evaporated using a rotatory evaporator. 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% CO_2.

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. DAPI staining was performed in three replicates and the number of viable cells in each slide was counted by two independent observers.

Comment [BK1]: 1) Why use DMSO as a HONE-1 cells therapy agent?
2) What is the concentration of DMSO used in this study?

Comment [BK2]: 1) DAPI staining (nuclei stained) is applied to observe changes in nuclear morphology (condensed and fragmented nuclei).
2) Cell numbers were reduced due to nuclear shrinkage, chromosome condensation and apoptotic bodies.

Immunoblotting

HONE-1 cells were seeded to 96-well plate 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 separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk in phosphate-buffered saline (PBS), the sheets were probed with rabbit polyclonal anti-BID antibody (Cell Signaling Technology) diluted 1:1000 in PBS. Then, goat anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) diluted 1:2000 in PBS was added. The bound antibodies were visualized using Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories). All visualized bands were documented using Alliance 4.7 (UVItech, Cambridge, UK) and quantified using UVIband software (UVItech).

Comment [BK3]: 1) Why use DMSO as a HONE-1 cells therapy agent?
2) What is the concentration of DMSO used in this study?

Comment [BK4]: 1) Why is the HONE-1 cell treatment for the Bid expression carried out only for 6 hours?
2) Why was the treatment duration not carried out for 24 and 48 hours, as in the DAPI staining?

Data Analyses

Statistical analysis was performed with IBM SPSS Statistics version 26 (IBM, Armonk, NY, USA). Shapiro-Wilk test was performed to analyze the normality of the data. Kruskal-Wallis

test followed by *post hoc* Mann-Whitney U test was used to analyze the differences of the number of viable cells between groups at 24 and 48 h. Data was expressed as mean ± standard deviation (SD) and $p<0.05$ were considered as statistically significant.

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-E, 2C-E). The number of viable HONE-1 cells decreased in concentration- and time-dependent manner. At 24 h incubation period, the number of viable cells in 10 µg/mL CXRE-treated group was not significantly different compared with that in the DMSO-treated group ($p=0.109$). 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 ($p<0.05$). Furthermore, the number of viable cells in all CXRE-treated groups were significantly higher compared to that in the Doxorubicin-treated group ($p<0.05$). Meanwhile, at 48 h incubation period, the number of viable cells in all CXRE-treated groups were significantly lower compared with that in the DMSO-treated group ($p<0.05$). The number of viable cells in 10 and 50 µg/mL CXRE-treated groups were significantly higher when compared to the Doxorubicin-treated group ($p<0.05$), while the number of viable cells in 250 µg/mL CXRE-treatment group was

Comment [BK5]: The data had large standard deviation, what were this caused by?

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not significantly different compared to that in the Doxorubicin-treated group (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 µg/mL CXRE-treated groups were lower compared with that in the DMSO-treated group. CXRE concentration of 250 µg/mL showed greater reduction in Bid expression than the other two concentrations. Bid expression level in HONE-1 cells treated with 50 µg/mL CXRE was slightly higher compared with those treated with 10 µg/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).

Comment [BK6]: "the number of viable cells in 250 µg/mL CXRE-treatment group was not significantly different compared to that in the Doxorubicin-treated group"
Why are no bands observed on Doxorubicin-treated group?

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 to 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). Therefore, Bid activation reduces the amount of Bid and increases the amount of t-Bid in the cell.(23,30) In the present study, Bid expression levels in CXRE-treated HONE-1 cells were lower compared to those in the untreated and sham groups. Thus, it can be concluded that CXRE stimulated Bid activation.

Comment [BK7]:
"Bid activation reduces the amount of Bid"

- Caspases-8/-10 cleaves Bid to produce tBid, which activates a crosstalk pathway between death receptors and mitochondria.
- tBid translocates to mitochondria where it blocks anti-apoptotic activity of Bcl-2 and Bcl-XL, and activates Bax and Bak.
- This leads to release of cytochrome c and Smac/Diablo and activation of the mitochondrial pathway of apoptosis.

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)-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 ΔΨm 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.

Comment [BK8]: It is necessary to analyze the effect of CXRE on growth, proliferation, or apoptosis of normal cell

Author 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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Figures

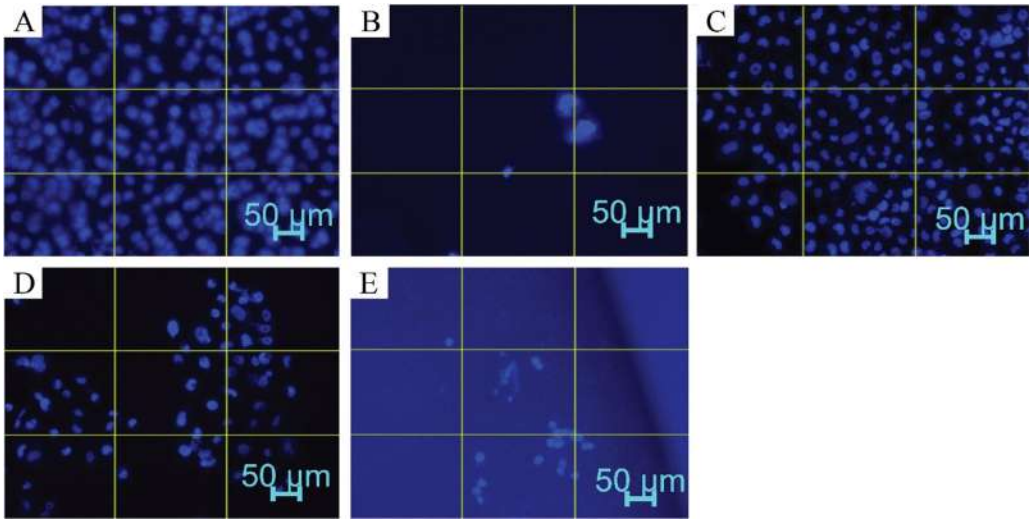

Figure 1. CXRE reduced the viability of HONE-1 cells after 24 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

Comment [BK9]: Why are HONE-1 cells starved for 12 hours prior to treatment?

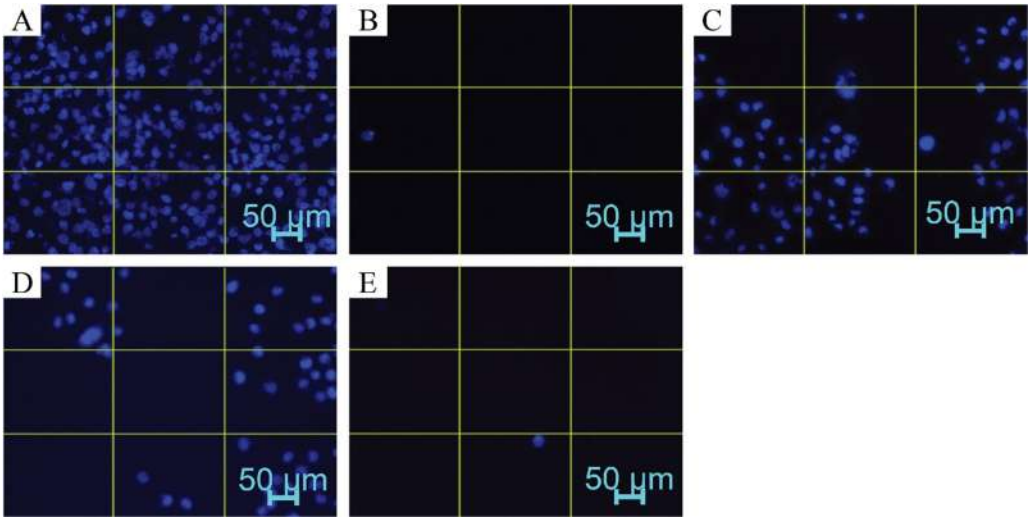

Figure 2. CXRE reduced the viability of HONE-1 cells after 48 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 48 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

Comment [BK10]: Why are HONE-1 cells starved for 12 hours prior to treatment?

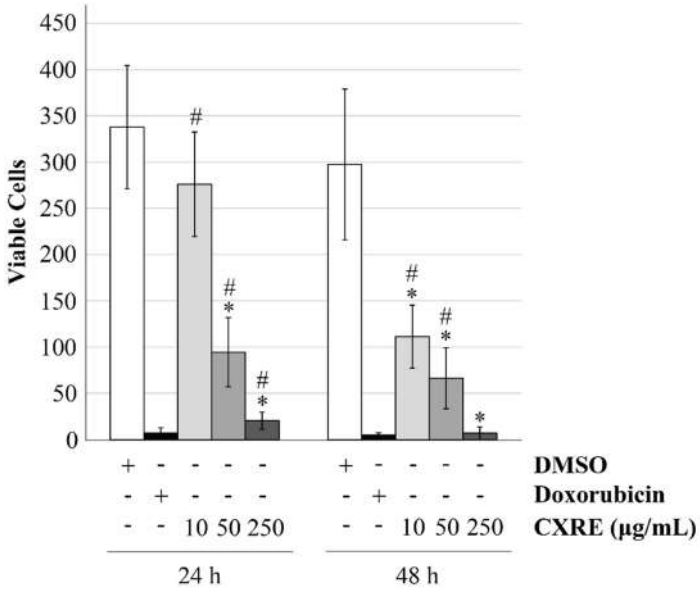

Figure 3. CXRE decreased the number of viable HONE-1 cells in concentration- and time-dependent manner. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 and 48 h. Cell nuclei were stained with DAPI. The number of viable cells 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.

Comment [BK11]: Why are HONE-1 cells starved for 12 hours prior to treatment?

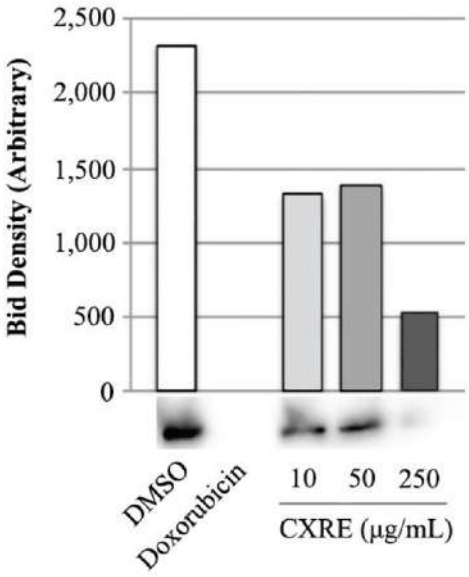

Figure 4. CXRE diminished Bid expression in HONE-1 cells. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h as indicated in the panel. Cells were collected, lysed, and further processed to obtained cell lysate for Western blot analysis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

Comment [BK12]: Why are HONE-1 cells starved for 12 hours prior to treatment?

UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2023009 Editor Decision Round 1 - Revisions Required

Ferry Sandra <ferry@trisakti.ac.id> Thu, Jan 19, 2023 at 9:59 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of The Indonesian Biomedical Journal,

Thank you for sharing the review outcomes. Attached is the revised manuscript M2023009 titled "*Curcuma xanthorrhiza* Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid". I have made the necessary revisions based on the feedback received.

Thank you.

Regards,
Ferry Sandra
[Quoted text hidden]
--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

Round 1 Revision from Author.docx
611K

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

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

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) The 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% CO_2.

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, 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-E, 2C-E). 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 μg/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 µg/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 µg/mL CXRE-treated groups were lower compared with that in the DMSO-treated group. CXRE concentration of 250 µg/mL showed greater reduction in Bid expression than the other two concentrations. Bid expression level in HONE-1 cells treated with 50 µg/mL CXRE was slightly higher compared with those treated with 10 µg/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 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-X_L) 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.

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)-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.

Author 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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Figures

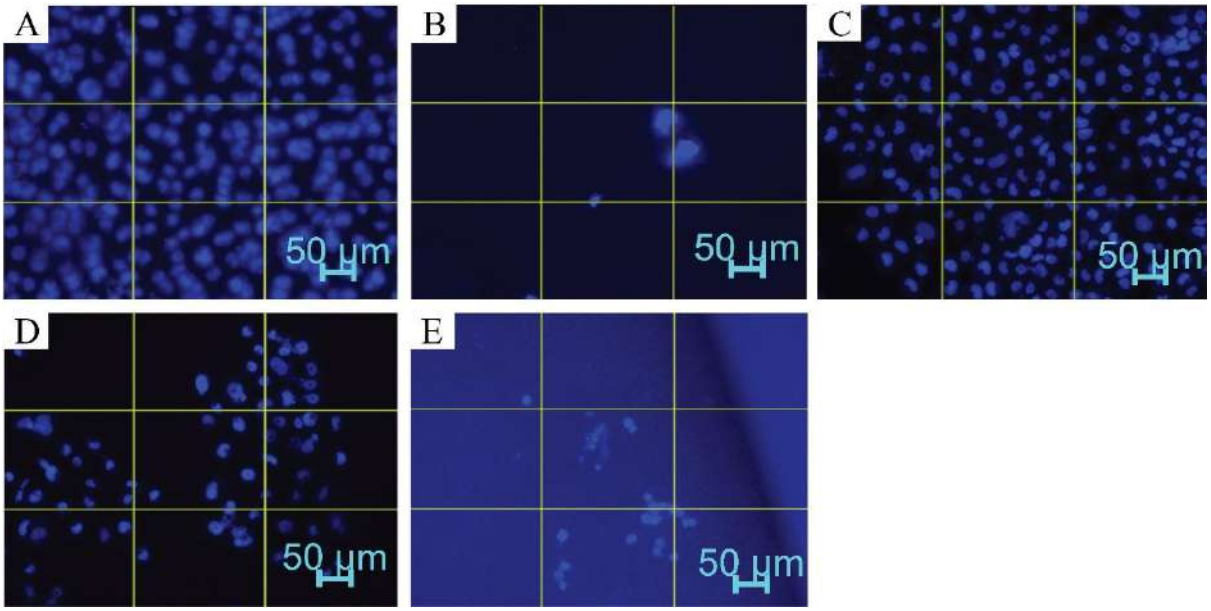

Figure 1. CXRE reduced the viability of HONE-1 cells after 24 h. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μM Doxorubicin, or various concentrations of CXRE for 24 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times. A: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

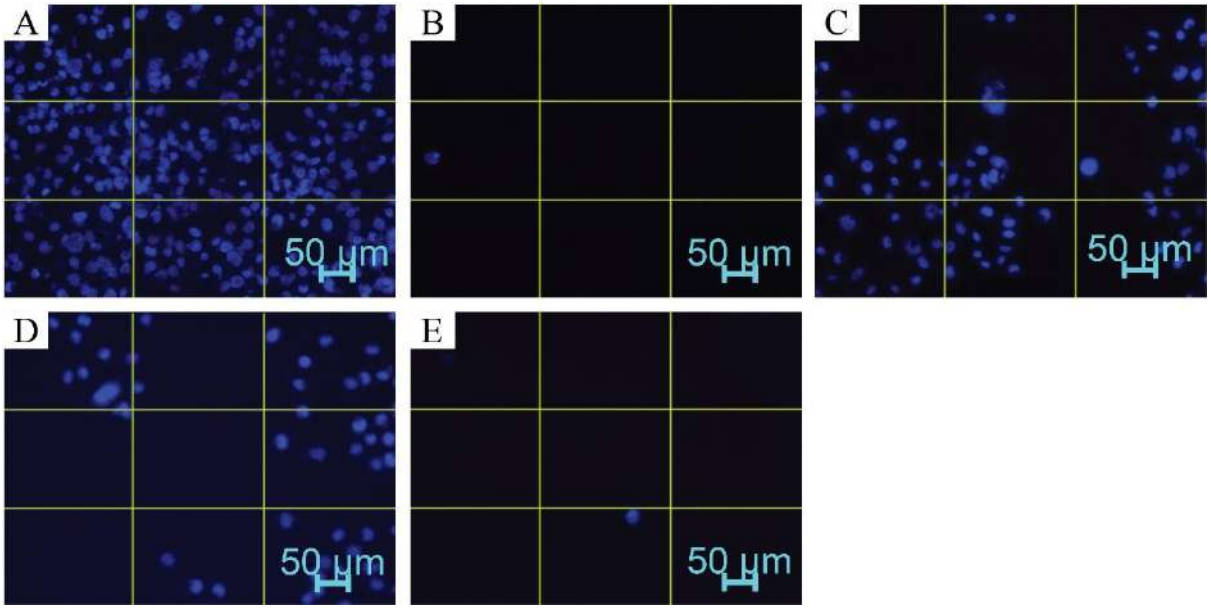

Figure 2. **CXRE reduced the viability of HONE-1 cells after 48 h.** HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 μM Doxorubicin, or various concentrations of CXRE for 48 h. Cell nuclei were stained with DAPI as described in Methods. These experiments were repeated three times.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

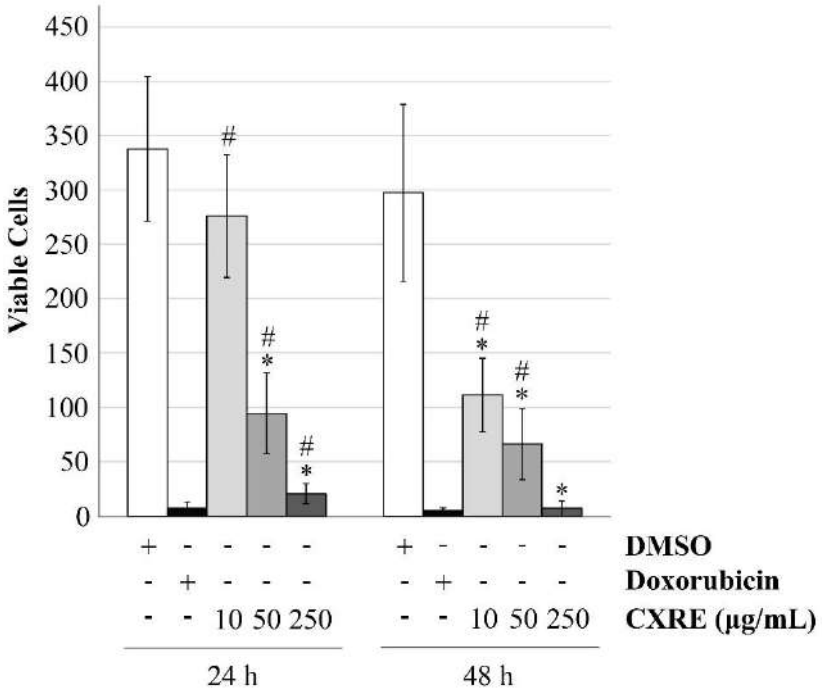

Figure 3. CXRE decreased the number of viable HONE-1 cells in concentration- and time-dependent manner. HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or various concentrations of CXRE for 24 and 48 h. Cell nuclei were stained with DAPI. The number of viable cells 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.

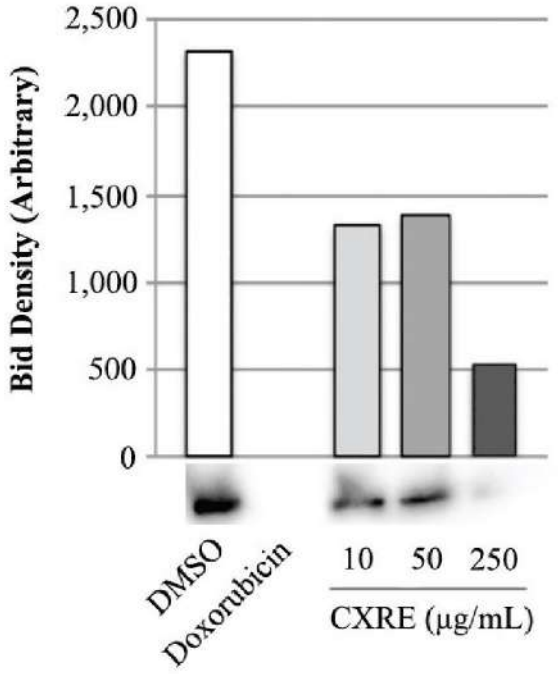

Figure 4. **CXRE diminished Bid expression in HONE-1 cells.** HONE-1 cells were seeded, starved for 12 h, and treated with DMSO, 3 µM Doxorubicin, or 10, 50, or 250 µg/mL CXRE for 6 h as indicated in the panel. Cells were collected, lysed, and further processed to obtained cell lysate for Western blot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

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[InaBJ] M2023009 Editor Decision - Manuscript Accepted

Secretariat of InaBJ <secretariatinabj@gmail.com> Fri, Jan 20, 2023 at 7:56 AM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "***Curcuma xanthorrhiza* Rhizome Extract Induces Apoptosis in HONE-1 Nasopharyngeal Cancer Cells Through Bid**."

Our decision is to: **Accept Manuscript.**

Your manuscript will be sent to our publisher for typesetting and you should receive the proofreading in due course.

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

--

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