Pharmacogn. Mag.
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ORIGINAL ARTICLE

Inhibition of Phosphorylated c-Jun NH(2)-terminal Kinase by 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone Isolated from *Eugenia aquea* Burm f. Leaves in Jurkat T-cells

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Submitted: 18-01-2017 Revised: 01-03-2017 Published: 11-10-2017

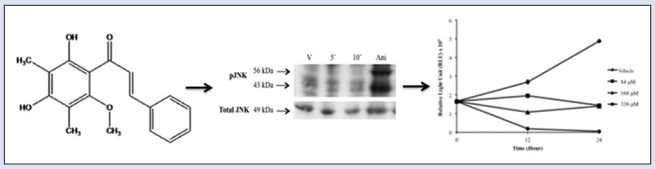
ABSTRACT

Background: Indonesian medicinal plants have been used for their anticancer activity for decades. However, the therapeutic effects of medicinal plants have not been fully examined scientifically. As cancer is a major health problem worldwide, searching for a new anticancer compound has attracted considerable attention. Our previous study found that 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone, an active compound isolated from leaves of Indonesian medicinal plants *Eugenia aquea* Burm f. (Myrtaceae), had anticancer activity in MCF-7 human breast cancer cells through induction of apoptosis. **Objective:** To investigate the molecular mechanism of 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone antiproliferative activity. **Materials and Methods:** Leaves of *E. aquea* were extracted by ethanol, fractionated by ethyl acetate, *n*-hexane, or water, and isolated for its active compound. Jurkat T-cells were treated with 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone for 12 and 24 h, and a cell viability assay and real-time-reverse transcriptase polymerase chain reaction for interleukin-2 (IL-2) mRNA measurement were performed. The effects of active compound to mitogen-activated protein kinases were also examined to investigate the mechanism of its antiproliferative activity. **Results:** 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone inhibited Jurkat T-cell proliferation with a half maximal inhibitory concentration of 59.5 μM. Although IL-2 mRNA expression was slightly increased after treatment, it inhibited c-Jun N-terminal kinase expression but not p38 and extracellular signal-regulated kinase expression. **Conclusions:** Our study indicated that the molecular mechanism mediating the antiproliferative activity of 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone may be attributed to the stimulation of an immunological microenvironment in the cells.

Key words: c-Jun N-terminal kinase, Indonesia, interleukin-2, proliferation

SUMMARY

- 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone was isolated from *Eugenia aquea*. The antiproliferative activity of 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone significantly showed in Jurkat T-cells with a half maximal inhibitory concentration of 59.5 μM through inhibition of c-Jun N-terminal kinase phosphorylation. Interleukin-2 mRNA expression was also slightly increased after treatment with the compound, and this result may be indicated to the stimulation of the immunological microenvironment in T-cells.



Abbreviations used: *E. aquea*: *Eugenia aquea*, IL-2: Interleukin-2, MAPK: Mitogen-activated protein kinase, ERKs: Extracellular signal-regulated kinases, JNKs: c-Jun N-terminal kinases, p38: p38 MAPK, PI3K: Phosphatidylinositol-3 kinase, IC_{50}: Half maximal inhibitory concentration.

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DOI: 10.4103/pm.pm_16_17

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INTRODUCTION

Cancer has become a major health problem worldwide leading to high mortality and morbidity rates in children and adults. Extensive research has been conducted to find novel anticancer agents; natural compounds constitute one potential source of anticancer agents.[1] Cancer progression and suppression can be affected by immune system including both innate and adaptive immune mediators. Understanding the immune system and its microenvironment may lead to the identification of a new biomarker that stimulates the immune system and may also lead to the discovery of novel modulators of immune signaling that can be used in cancer therapy.[2]

The immune system plays an important role in immune surveillance, recognizing and destroying transformed native cells before they become cancer cells. The immune system could also be affected by immunomodulators that enhance its tumor cells bearing response.[1] Agents or compounds that are able to enhance the immunological microenvironment become potential future therapies for cancer patients. Some mechanisms have been reported for boosting T-cell response such as allocate T-cells stimulation and provide cytokines to stimulate tumor-specific T-cells activation, $CD8^+$ T-cells.[1] One of the cytokines that involve in T-cell proliferation, especially to maintain the balance between the function of T-cell activation and regulation is interleukin-2 (IL-2).[3-6] IL-2 was observed to

Cite this article as: Barliana MI, Diantini A, Subarnas A, Abdulah R, Izumi T. Inhibition of phosphorylated c-Jun NH(2)-terminal kinase by 2′,4′-dihydroxy-6-methoxy-3,5-dimethylchalcone isolated from *Eugenia aquea* Burm f. leaves in jurkat T-cells. Phcog Mag 2017;13:S573-7.

stimulate the activation-induced cell death by promoting the synthesis of interferon gamma (IFN-γ), upregulating the expression of Fas ligand, tumor necrosis factor (TNF) receptor, and downregulating the expression of caspase inhibitor c-FLIP.[7-11] Moreover, IL-2 was shown to increase the activation of dendritic cell-induced $CD8^+$ T-cell through phosphorylation of STAT5 and finally IFN-γ synthesis.[12] Therefore, the use of immunomodulatory agent that regulates IL-2 expression and combats the cancer cells may one of the action mechanisms.[13,14]

Cell proliferation, migration, differentiation, apoptosis, survival, transformation, and inflammation were regulated by mitogen-activated protein kinase (MAPK) family include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38. MAPKs are serine-threonine kinases that activated by several intracellular and extracellular stimuli such as growth factors, cytokines, hormones, and various signaling.[15,16] Therefore, MAPKs have become target for many therapies including cancer.

The phosphatidylinositol-3 kinase (PI3K) is involved in many cell regulatory functions under normal and pathogenic conditions, which is regulating native cells' proliferation and cancer cells' progression. The PI3K isoforms' stimulation also determines the proportion of exterminating cancer cells and immunological system impairment.[17] Therefore, a selective and effective anticancer agent is needed to enhance immune surveillance strategies, instead of influencing immune system impairment. Careful consideration is needed for selecting an anticancer agent with few side effects on the immune defense response against cancer cells.[17]

Many studies have indicated that herbal medicine has promising anticancer activities including the promotion of apoptosis and the enhancement of immunity, with fewer side effects than chemotherapy or hormone therapy.[18] Traditional medicine using medicinal plants, food, or spices has been used by Indonesian people to improve their immune system including *Eugenia aquea* (*E. aquea*).[19,20] *E. aquea* (Myrtaceae), also known as jambu air in Indonesia, contains of Vitamin C and A, calcium, iron, and phosphor and is known to increase immune system or immunomodulatory activity. A previous report showed that *E. aquea* leaves' extract has anticancer property by inhibiting proliferation of MCF-7 human breast cancer cells.[21] The isolated active compound from *E. aquea*, 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone, was reported to inhibit the proliferation of human breast adenocarcinoma, MCF-7 cell line, at a half maximal inhibitory concentration (IC_{50}) of 74.5 μg/mL (250 μM).[22] In the present study, we investigated the molecular mechanism underlying the antiproliferative and immunomodulatory activities of 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcon by determining the antiproliferative property, expression of IL-2 mRNA, and MAPKs involvement in Jurkat T-cells.

MATERIALS AND METHODS

Plant materials

The leaves of *E. aquea* were collected from the Pangandaran Beach Conservation Area (Pangandaran, West Java, Indonesia). The plant species were then determined by the Department of Biology of Universitas Padjadjaran (Bandung, West Java, Indonesia), where specimen is deposited. The leaves were rinsed in tap water and shade-dried.

Extraction, fractionation, and isolation

Dried leaves of *E. aquea* were extracted, fractionated, and isolated according to Subarnas *et al.*[22] Briefly, dried leaves of *E. aquea* were extracted using 95% ethanol at room temperature (3 × 24 h), and then the solvent was evaporated under pressure at 50°C. Concentrated extract was then partitioned into *n*-hexane, ethyl acetate, and water phase. The *n*-hexane fraction was subjected to column chromatography on a silica gel and eluted with *n*-hexane–ethyl acetate mixtures of increasing polarity. The subjected fraction was purified over several silica gel column chromatographs, resulting in a 150.30 mg yellowish crystal of pure active compound. The pure active compound was characterized using ultraviolet, infrared, mass, and nuclear magnetic resonance spectra and identified as 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone with a molecular formula of $C_{18}H_{18}O_4$ as shown in Figure 1.[22]

Cell cultures

Jurkat T-cell leukemia cell lines were cultured in RPMI 1640 (Invitrogen, NY, US) containing 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2. The medium also supplemented with antibiotics, which consisted 1% of penicillin and streptomycin. The active compound was dissolved in dimethyl sulfoxide with a concentration < 0.1% (v/v) in control and treated cells, which did not disrupt the cell conditions.

Cell viability assay

Jurkat T-cells (1.5×10^4/50 μL/well, in a 96-well plate) were treated with 84, 168, and 336 μM of the active compound for 12 or 24 h. After the indicated times, cells were collected, and cell proliferation rate was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI, USA) according to the manufacturer's instructions. Luminescent signals were proportional to the amount of ATP in the cells and detected using a Wallac Arvo HTS Multilabel Counter (Perkin-Elmer, Turku, Finland). The cytotoxicity effect of the active compound was shown as IC_{50}, the concentration of 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone that caused 50% inhibition of cell proliferation.

Real-time reverse transcriptase polymerase chain reaction analysis

Jurkat T-cells were treated with 59.5 μM of the active compound or ionomycin (0.1 μM) for 24 h. Isolation of total RNA was performed using RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK). An analysis of the expression of IL-2 mRNA was performed using a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Qiagen, Mainz, Germany) with the following IL-2 primers: Sense 5′-GAATGGAATTAATAATTACAAGAATCCC-3′ and antisense 5′-TGTTTCAGATCCCTTTAGTTCCAG-3′. Quantitative real-time RT-PCR was performed using a DNA Engine Opticon 2 System (BioRad, Hercules, California, USA), and quantification of the samples was based

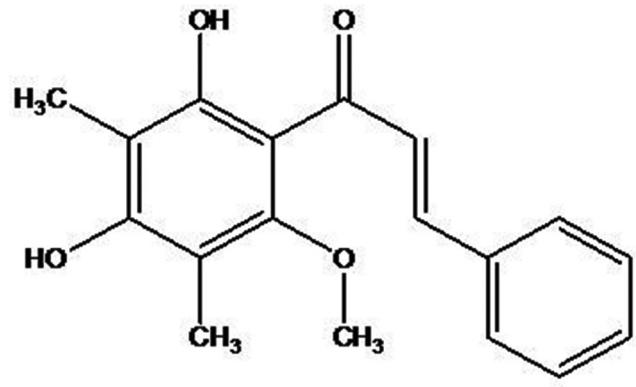


Figure 1: Structure of 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone, the active compound isolated from *Eugenia aquea*

on the threshold cycle or C(t) and normalized to that of an internal control (GAPDH).

Western blot analysis

Jurkat T-cells were treated with various stimulations by 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone and disrupted by sonication in a homogenizing buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4) containing a complete protease inhibitor (Roche, IN, USA). The homogenates were centrifuged at 10,000 g for 10 min. The supernatants were obtained, and protein concentrations were calculated using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., IL, USA). The protein (20 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in Tris-buffered saline (TBS) containing 50% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) and incubated with primary antibodies in TBS containing 10% Block Ace for 1 h at room temperature. MAPK was analyzed. Total or phosphorylated Erk-1/2, p38, and JNK primary antibodies were supplied as a part of a MAP Kinase Activation Sampler Kit (BD Bioscience) and used according to the manufacturer's instructions. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies, and the signals were visualized by ECL Plus Western Blotting Detection System (GE Healthcare).

Statistical analysis

The result was statistically determined by Student's *t*-test for its significant differences. $P < 0.05$ was considered statistically significant.

RESULTS

2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone inhibited Jurkat T-cell proliferation

2',4'-Dihydroxy-6-methoxy-3,5-dimethylchalcone was shown to inhibit Jurkat T-cell proliferation after treatment for 12 and 24 h in various concentrations [Figure 2]. Treatment with 84 μM of the active compound began to show significant inhibition of Jurkat T-cell proliferation after 12 h of incubation, and this effect was shown to be greater in 168 and 336 μM of treatment. Following analysis showed that active compound of *E. aquea* has IC_{50} of 59.5 μM (17.73 μg/mL) after 24 h treatment.

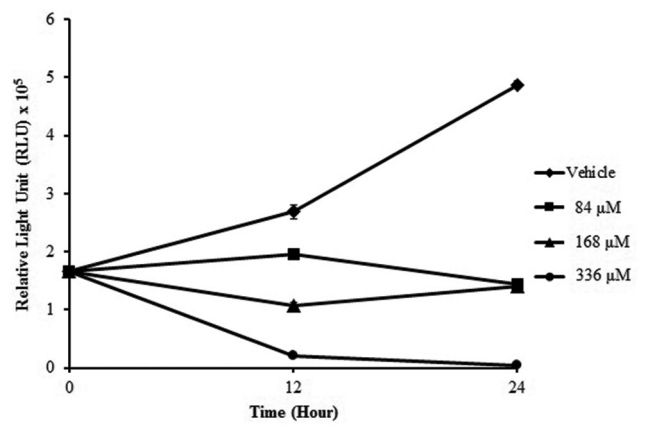


Figure 2: Inhibition of Jurkat cells proliferation after treatment with 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone in various concentrations for 12 or 24 h

Interleukin-2 mRNA expression was slightly increased by 2',4'-dihydroxy-6-methoxy-3, 5-dimethylchalcone

IL-2 mRNA expression was measured after Jurkat T-cells were treated with 59.5 μM of active compound for 24 h [Figure 3]. IL-2 is a T-cell growth factor that induces T-cell proliferation. The expression of IL-2 mRNA was slightly stimulated by the active compound of *E. aquea* compared with vehicle-treated Jurkat T-cells. Ionomycin was used as a positive control which enhanced IL-2 intracellular production. In this experiment, ionomycin did not have a significant effect on Jurkat T-cell proliferation.

The c-Jun NH(2)-terminal kinase pathway affected in 2',4'- dihydroxy-6- methoxy-3,5-dimethylchalcone- treated Jurkat T-cells

Jurkat T-cells were treated with 59.5 μM of 2', 4'- dihydroxy-6-methoxy -3,5-dimethylchalcone in 0, 5, and 10 min [Figure 4]. The compound inhibited JNK expression after 5 min and diminished its expression after 10 min of incubation, but this inhibitory effect was not observed for p38 and ERK expression. Anisomycin was used as a positive control which induced JNK signaling upon activation. Total JNK was also observed as an internal control.

DISCUSSION

Isolated active compound from herbal medicines, 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone, showed an antiproliferative activity such as in *Cleistocalyx operculatus* (Myrtaceae).[23,24] The present study showed that the active compound of *E. aquea*, 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone, has an antiproliferative effect on Jurkat cell lines at 84 μM, which has an IC_{50} of 59.5 μM after 24 h treatment. After 12 h of treatment with 84 μM of the active compound, Jurkat cell proliferation was decreased significantly compared to vehicle. Nevertheless, the IC_{50} level of the active compound in Jurkat cells was lower than that in MCF-7 human breast cancer cells. This data showed that the active compound was more selective and sensitive as an antiproliferative agent in Jurkat cells. 2',4'-dihydroxy-6-methoxy-3, 5-dimethylchalcone has a potential activity as an antiproliferative agent; nevertheless, confirmation using primary T-cells should be done in the future.

Thus, *E. aquea* was shown to inhibit Jurkat cell proliferation. In the present study, the active compound of *E. aquea* stimulated IL-2 mRNA expression after treatment with 59.5 μM 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone for 24 h. This finding

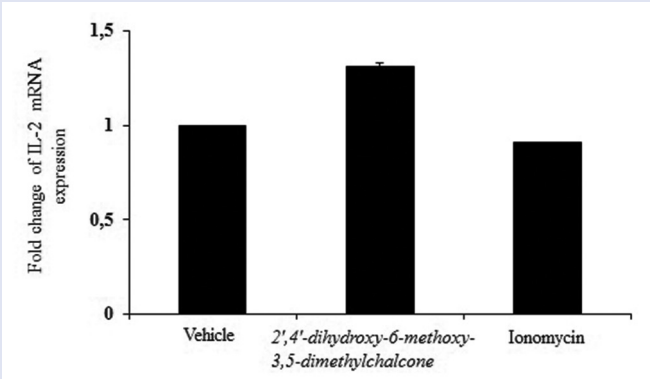
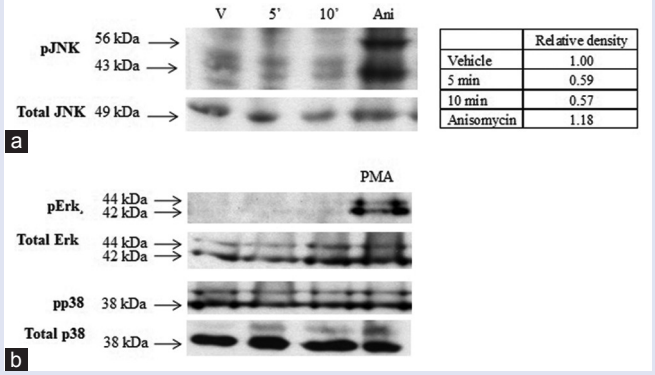


Figure 3: The active compound of *Eugenia aquea* stimulated interleukin-2 mRNA expression in Jurkat cells after a 24 h treatment of vehicle, 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone, or ionomycin



	Relative density
Vehicle	1.00
5 min	0.59
10 min	0.57
Anisomycin	1.18

Figure 4: The active compound of *Eugenia aquea* inhibited phosphorylation of c-Jun N-terminal kinase at indicated times by Western blot analysis and densitometer (a) but not extracellular signal-regulated kinase or p38 (b)

indicated that 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone also modulates the immune system by increasing IL-2 mRNA expression. In this study, we used an IC_{50} concentration (59.5 μM) of the active compound, which caused 50% cell death, indicating robust antiproliferative activity of the compound. A lower concentration of the active compound might have shown different effects such as a possible increase in Jurkat cell proliferation and greater stimulation of IL-2 mRNA expression. Therefore, 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone might promote two paradoxical processes; one involving antiproliferative activity and the other leading to improvement of the cell's microenvironment and immunosurveillance. In this study, we investigated IL-2 expression in mRNA level as preliminary research for 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone activity in modulating T-cell proliferation. Expression of IL-2 should be performed in protein level by ELISA for further determination.

Anti-leukemia from natural compound using Jurkat T-cell was also reported from leaves extract of *Lantana camara* (Verbenaceae) through antioxidant properties such as radical scavenging, xanthine oxidase inhibition, and nitrites scavenging activities.[25,26] Our findings are similar to the reported activity of *Lycium barbarum* polysaccharides (LBP) isolated from *Lycium barbarum* (Solanaceae) fruit, which also show antiproliferative activities. In addition, high doses (≥5 g/L) of crude *L. barbarum* extract stimulate p53-induced apoptosis in hepatocellular carcinoma.[27] LBP has also been observed to increase both IL-2 and TNF-γ mRNA and protein expression in human peripheral blood mononuclear cells.[28] The dual activity of LBP as both an antiproliferative agent and immunomodulator suggests great potential for LBP as an anticancer agent and adjuvant to cancer therapy.[29]

IL-2 is a pleiotropic cytokine that has been used as an antitumor target since the 1980s, and in 1998, IL-2 was approved by the US Food and Drug Administration for metastatic melanoma and renal cell carcinoma therapy.[30,31] IL-2 can activate $CD8^+$ T-cell, natural killer (NK) cells, naïve $CD4^+$ T-cells into T helper-1 (Th1) and T helper-2 (Th2), regulatory T-cells (Tregs) and cell death, regulate T-cell differentiation, and inhibit T helper-17 (Th17).[32-35] From these important roles of IL-2, an agent that could stimulate mRNA expression and secretion of IL-2 have a potency as a target for cancer immune therapy.

In general, IL-2 was induced in T-cell proliferation. This phenomenon was not observed in present data since Jurkat T–cells' proliferation was inhibited by 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone followed by slightly increasing of IL-2 mRNA expression. These data were in accordance with the effect of OK-432. OK-432 (Picibanil) is a streptococcal preparation which has an antitumor activity by inhibiting proliferation of cancer cells through direct inhibition of RNA synthesis and also stimulation of immune cells. In immune cells' stimulation, OK-432-induced IL-2 expression responsible for stimulating dendritic cells maturation, activity of NK, response of Th1, and cytotoxic T lymphocyte (CTL).[36] The involvement of 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone in stimulating the immunological microenvironment might be started from this IL-2-induced activity, which has potential to stimulate other immune cells' activation such as NK and CTL.

Traditional herbal medicine has been used to cure many diseases by modulating the immune system. The underlying mechanisms for the pharmacological and immunomodulatory activities of herbal medicine have not been fully elucidated. Recently, researchers have started to investigate and confirm the medicinal benefit of traditional herbal medicine. An *in vivo* study examining the effect of *Tinospora crispa* (Menispermaceae) stem bark ethanol extract in Wistar Kyoto rats indicated dose-dependent increases in chemotactic activity of neutrophils, T- and B-cell proliferation, and several Th1 (TNF-α, IL-2, and IFN-γ) and Th2 cytokines (IL-4).[37] A traditional Chinese medicine containing the decoction of Radix astragali and Radix angelicae sinensis, called Danggui Buxue Tang, was shown to increase cell proliferation by stimulating the secretion of IL-2, IL-6, and IL-10, as well as phosphorylation of ERK after 10 min of incubation.[38]

Our present study showed that the active compound of *E. aquea* inhibited the phosphorylation of MAPK and JNK but not p38 and Erk. These data were in line with the results from the proliferation assay result, in which 59.5 μM of 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone treatment caused 50% death of Jurkat T-cell population [Figure 2]. These results are also consistent with a previous study, suggesting a potential underlying mechanism for *E. aquea*'s antiproliferative activity[22] although the concentration of the active compound was different due to a different type of cell types being used. Nevertheless, further examination should be performed to confirm the involvement of JNK in antiproliferative effect of 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone using JNK inhibitors.

MAPK/receptor tyrosine kinase/Ras GTPase signaling pathways are important in controlling many biological processes, especially for metazoan development. Most of the essential cellular processes were controlled by MAPKs including cell cycle, cell migration, cell metabolism, survival, cell proliferation, and differentiation.[39]

A previous report by Zhu *et al.* observed that 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone isolated from *C. operculatus*, a Chinese herbal medicine, inhibited phosphorylation of Akt and MAPK in human vascular endothelial HDMEC cells in the presence of vascular endothelial growth factor (VEGF). The 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone was able to inhibit tumor growth by inhibiting the angiogenesis but only after induction of VEGF.[40] Our results also showed that 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone inhibited the proliferation of Jurkat cells by inhibiting phosphorylation of JNK.

CONCLUSIONS

In the present study, we observed that 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone has antiproliferative activities in Jurkat cells through the inhibition of JNK phosphorylation using a low dose of IC_{50}. We also found that the compound induced IL-2 mRNA. Although IL-2 expression is involved in T-cell proliferation, this effect was not observed in our study. Further investigations are needed since similar activity of this compound might observe in other cells of different types of cancer and to evaluate the detailed molecular mechanism through which 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone

increases the immunological microenvironment of T-cells. This information may lead to the discovery of novel immune signaling modulators to be employed in cancer therapy.

Financial support and sponsorship

This research was funded by Grant-in-aid from Universitas Padjadjaran Fundamental Research Grant for MIB.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Abbas AK, Lichtman AH, Pillai S. Cellular and Molecular Immunology. 7th ed. Philadelphia, USA: Elsevier Saunders; 2012.
2. Markman JL, Shiao SL. Impact of the immune system and immunotherapy in colorectal cancer. J Gastrointest Oncol 2015;6:208-23.
3. Smith KA. Interleukin-2: Inception, impact, and implications. Science 1988;240:1169-76.
4. Dutcher J. Current status of interleukin-2 therapy for metastatic renal cell carcinoma and metastatic melanoma. Oncology (Williston Park) 2002;16 11 Suppl 13:4-10.
5. Pahwa S, Morales M. Interleukin-2 therapy in HIV infection. AIDS Patient Care STDS 1998;12:187-97.
6. Nelson BH. IL-2, regulatory T cells, and tolerance. J Immunol 2004;172:3983-8.
7. Cousens LP, Orange JS, Biron CA. Endogenous IL-2 contributes to T cell expansion and IFN-gamma production during lymphocytic choriomeningitis virus infection. J Immunol 1995;155:5690-9.
8. Refaeli Y, Van Parijs L, Alexander SI, Abbas AK. Interferon gamma is required for activation-induced death of T lymphocytes. J Exp Med 2002;196:999-1005.
9. Lenardo MJ. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. Nature 1991;353:858-61.
10. Refaeli Y, Van Parijs L, London CA, Tschopp J, Abbas AK. Biochemical mechanisms of IL-2-regulated fas-mediated T cell apoptosis. Immunity 1998;8:615-23.
11. Zheng L, Trageser CL, Willerford DM, Lenardo MJ. T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death. J Immunol 1998;160:763-9.
12. Herr F, Lemoine R, Gouilleux F, Meley D, Kazma I, Heraud A, *et al.* IL-2 phosphorylates STAT5 to drive IFN-gamma production and activation of human dendritic cells. J Immunol 2014;192:5660-70.
13. Hoyer KK, Dooms H, Barron L, Abbas AK. Interleukin-2 in the development and control of inflammatory disease. Immunol Rev 2008;226:19-28.
14. Liu CP, Kuo YC, Shen CC, Wu MH, Liao JF, Lin YL, *et al.* (S)-armepavine inhibits human peripheral blood mononuclear cell activation by regulating Itk and PLCgamma activation in a PI-3K-dependent manner. J Leukoc Biol 2007;81:1276-86.
15. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. Oncogene 2007;26:3279-90.
16. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 2010;1802:396-405.
17. Dituri F, Mazzocca A, Giannelli G, Antonaci S. PI3K functions in cancer progression, anticancer immunity and immune evasion by tumors. Clin Dev Immunol 2011;2011:947858.
18. Lee JH, Koung FP, Cho CK, Lee YW, Yoo HS. Review of tumor dormancy therapy using traditional oriental herbal medicine. J Pharmacopuncture 2013;16:12-20.
19. Widyowati R, Rahman A. Chemistry compound and antimicrobe activity of Garcinia celebica extract against *Staphylococcus aureus*, *Shigella dysenteriae* and *Candida albicans*. Majalah Farmasi Airlangga 2010;8:23-7.
20. Kumar PS, Febriyanti RM, Sofyan FF, Luftimas DE, Abdulah R. Anticancer potential of *Syzygium aromaticum* L. in MCF-7 human breast cancer cell lines. Pharmacognosy Res 2014;6:350-4.
21. Subarnas A, Diantini A, Abdulah R, Zuhrotun A, Yamazaki C, Nakazawa M, *et al.* Antiproliferative activity of primates-consumed plants against MCF-7 human breast cancer cell lines. E3 J Med Res 2012;1:38-43.
22. Subarnas A, Diantini A, Abdulah R, Zuhrotun A, Hadisaputri YE, Puspitasari IM, *et al.* Apoptosis induced in MCF-7 human breast cancer cells by 2',4'-dihydroxy-6-methoxy-3,5-dimethylchalcone isolated from *Eugenia aquea* Burm f. leaves. Oncol Lett 2015;9:2303-6.
23. Yu WG, Qian J, Lu YH. Hepatoprotective effects of 2',4'-dihydroxy-6'-methoxy-3', 5'-dimethylchalcone on CCl4-induced acute liver injury in mice. J Agric Food Chem 2011;59:12821-9.
24. Ye CL, Qian F, Wei DZ, Lu YH, Liu JW. Induction of apoptosis in K562 human leukemia cells by 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone. Leuk Res 2005;29:887-92.
25. Mahdi-Pour B, Jothy SL, Latha LY, Chen Y, Sasidharan S. Antioxidant activity of methanol extracts of different parts of *Lantana camara*. Asian Pac J Trop Biomed 2012;2:960-5.
26. Badakhshan MP, Sreenivasan S, Jegathambigai RN, Surash R. Anti-leukemia activity of methanolic extracts of *Lantana camara*. Pharmacognosy Res 2009;1:274-9.
27. Chao JC, Chiang SW, Wang CC, Tsai YH, Wu MS. Hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells. World J Gastroenterol 2006;12:4478-84.
28. Gan L, Zhang SH, Liu Q, Xu HB. A polysaccharide-protein complex from *Lycium barbarum* upregulates cytokine expression in human peripheral blood mononuclear cells. Eur J Pharmacol 2003;471:217-22.
29. Tang WM, Chan E, Kwok CY, Lee YK, Wu JH, Wan CW, *et al.* A review of the anticancer and immunomodulatory effects of *Lycium barbarum* fruit. Inflammopharmacology 2012;20:307-14.
30. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 1982;155:1823-41.
31. Jiang T, Zhou C. The past, present and future of immunotherapy against tumor. Transl Lung Cancer Res 2015;4:253-64.
32. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? Nat Rev Immunol 2010;10:225-35.
33. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. Cell 2010;140:845-58.
34. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol 2003;21:713-58.
35. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775-87.
36. Ryoma Y, Moriya Y, Okamoto M, Kanaya I, Saito M, Sato M. Biological effect of OK-432 (Picibanil) and possible application to dendritic cell therapy. Anticancer Res 2004;24:3295-301.
37. Ahmad W, Jantan I, Kumolosasi E, Bukhari SN. Immunostimulatory effects of the standardized extract of *Tinospora crispa* on innate immune responses in Wistar Kyoto rats. Drug Des Devel Ther 2015;9:2961-73.
38. Gao QT, Cheung JK, Li J, Jiang ZY, Chu GK, Duan R, *et al.* A Chinese herbal decoction, Danggui Buxue Tang, activates extracellular signal-regulated kinase in cultured T-lymphocytes. FEBS Lett 2007;581:5087-93.
39. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000;103:211-25.
40. Zhu XF, Xie BF, Zhou JM, Feng GK, Liu ZC, Wei XY, *et al.* Blockade of vascular endothelial growth factor receptor signal pathway and antitumor activity of ON-III (2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone), a component from Chinese herbal medicine. Mol Pharmacol 2005;67:1444-50.