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Apoptotic and cytostatic actions of maslinic acid in colorectal cancer cells through possible IKK-β inhibition

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ABSTRACT

Objective: To explore the anti-cancer activity of maslinic acid against colorectal cancer (CRC) cell lines and its possible mechanism.

Methods: The inhibitory effect of maslinic acid was screened against five CRC cell lines (HT-29, HCT 116, SW480, SW48, and LS 174T) *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis and cell cycle analyses were carried out using annexin Ⅴ-FITC/propidium iodide staining and propidium iodide staining, respectively and subjected to fluorescence-activated cell sorting analysis. Protein expression studies of inhibitor of κB kinase-β (IKK-β), checkpoint kinase 1 (Chk1) and cyclin D1 were conducted using the JESS system.

Results: Maslinic acid exhibited growth inhibitory effect in a dose- and time-dependent manner in HT-29 and HCT 116 cell lines. A more prominent apoptosis induced by maslinic acid was observed in HCT 116 cell line. However, in HT-29 cell line, maslinic acid induced cell cycle arrest by inhibiting the G_1-S transition, which was accompanied by the downregulation of cyclin D1. The expression of unphosphorylated IKK-β protein was increased in both (HT-29 and HCT 116) cell lines after maslinic acid treatment.

Conclusions: Maslinic acid inhibits the growth of HT-29 and HCT 116 cells in a different manner, induces cell cycle arrest in HT-29 cells and causes apoptosis in HCT 116 cells partially *via* NF-κB pathway inhibition.

KEYWORDS: Maslinic acid; Colorectal cancer; Apoptosis; Cell cycle arrest; NF-κB pathway; IKK-β

1. Introduction

Cancer, as the emperor of all maladies, is one of the major public health threats, which has taken more than 9.5 million of lives in 2018 globally[1]. Both the incidence and mortality rate of colorectal cancer (CRC) have increased rapidly in the past decades due to population growth and aging, socioeconomic factors and adoption of westernized lifestyle[2]. The current treatments for CRC are resection surgery, cytotoxic chemotherapy and targeted therapies[3]. However, there are many cases where the CRC cancer cells showed tolerance or are resistant to these anti-cancer drugs and subsequently lead to metastasis or recurrence, eventually resulting in mortality. Hence, the development of new approaches for CRC treatment is necessary to provide more treatment options.

The nuclear factor kappa-B (NF-κB) pathway controls diverse cellular activities through a slight variation in the formation of NF-κB dimers that target different gene sequences. In the canonical NF-κB pathway, p50 and RelA (p65) subunits are associated with IκBα, residing in the cytosol under basal condition. The extracellular signals such as tumor necrosis factor-alpha (TNF-α), interleukin

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(IL)-1, and lipopolysaccharides, stimulate the activation of inhibitor of κB kinase-β (IKK-β) complex, relieve the inhibition of IκBα and subsequently free the canonical NF-κB dimer (p50-p65) to translocate to the nucleus and activate the transcription of NF-κB-related genes[4]. The involvement of NF-κB in cancer with elevated activation is supported by substantial evidence. According to Palmer *et al.* in 2017, there is a compromise in the NF-κB regulation that ultimately results in the expression of abnormal cohorts of NF-κB-related genes by cancer cells, instead of the gene mutation in the NF-κB signaling pathway[5,6]. The downstream proteins which are upregulated by NF-κB encompass four of the six core hallmarks of cancer, namely, evading growth suppressor (cyclin D1), resisting cell death (Bcl-2), inducing angiogenesis (VEGF) and activating invasion and metastasis (matrix metalloproteases)[5,6]. In addition, NF-κB is substantially involved in inflammation, one of the two enabling characteristics of cancer, by inducing the expression of cyclooxygenase (COX)-2, inducible nitric oxide synthase, proinflammatory cytokines, and chemokines. Remarkably, these also included TNF-α and IL-1-α, indicating that NF-κB is able to positively regulate its own expression[5]. These proved that the NF-κB cascade contributes to the cellular neoplastic transformation and eventually results in cancer development. Therefore, suppression of NF-κB may be a worthwhile therapeutic strategy approach to inhibit cancer initiation and progression. Many natural products which possessed anti-inflammatory and anti-cancer properties have also been shown to have an inhibitory effect against NF-κB by inhibiting IKK-β, such as wedelolactone, withaferin A, berberine, and nimbolide[7,8]. Hence, this underpins the immediate therapeutic potential of IKKβ inhibitor.

Maslinic acid is also known as crategolic acid with the chemical name of (2α,3β)-2,3-dihydroxyolean-12en-28-oic acid (Supplementary Figure). Maslinic acid is a pentacyclic triterpene acid widely present in numerous dietary plants and fruits including waxy skin of olive[9], spinach[10], basil[11], Chinese hawthorn[12], and glossy privet fruit[13]. Besides, our team successfully isolated maslinic acid from *Coleus tuberosus* Linn (which is locally known as ubi kemili) and determined its anti-tumor-promoting activity in Raji cells[14]. Furthermore, maslinic acid was found to exhibit its anti-cancer effect by targeting the NF-κB pathway in several cancer cell lines such as pancreatic cancer[15], lymphoma[16,17], and gall bladder[18]. These findings suggested the potential of maslinic acid in inhibiting the initiation or progression of diseases that rely on or partially depend on the activation of NF-κB pathway, including CRC since there is evidence that the NF-κB is constitutively activated in CRC[19].

This study aimed to investigate the antitumor effects of maslinic acid against CRC and the possible involvement of the NF-κB pathway. The results of this study will serve as a basis for the *in vivo* analysis of maslinic acid treatment efficacy against the CRC animal models before driving further the development of maslinic acid as a potential therapeutic compound for the treatment of CRC.

2. Materials and methods

2.1. Chemical reagents

Fetal bovine serum (FBS) was purchased from Tico Europe (Amstelveen, Netherlands), and Roswell Park Memorial Institute Medium (RPMI 1640) and sodium bicarbonate were from Merck (Darmstadt, Germany). Trypsin-EDTA was obtained from Nacalai Tesque (Kyoto, Japan), penicillin-streptomycin (10 000 U/mL) from Gibco (NY, USA). Phosphate-buffered saline (PBS) from Chemical Solution (Selangor, Malaysia) and MTT reagent from Amresco (OH, USA). The eBioscience™ annexin Ⅴ-FITC apoptosis detection kit [Invitrogen (CA, USA)] and the FxCycle™ PI/RNase staining solution [Life Technologies (CA, USA)] were used. The protein extraction kit, NE-PER Nuclear and Cytoplasmic Extraction Reagents and quantification kit, Pierce® 660nm Protein Assay Kit were purchased from ThermoFisher Scientific (IL, USA). IKK-β antibody (NB100-56509) was purchased from Novus Biologicals (CO, USA). Cyclin D1 (#2978) antibody was obtained from Cell Signalling Technology (Beverly, MA, USA). Chk1 antibody (ab40866) was purchased from Abcam (Cambridgeshire, UK). The 12-230 kDa Jess or Wes separation module 25 capillary cartridge, 12-230 kDa Jess or Wes separation module 13 capillary cartridges and protein normalization module for Jess were obtained from Protein Simple (CA, USA).

2.2. Maslinic acid

Maslinic acid was purchased from TargetMol (Boston, MA, USA) with >99.0% purity determined by HPLC. A stock solution of 30 mM (14.18 mg/mL) dissolved in dimethyl sulphoxide (DMSO) was prepared and stored at –20℃. The stock solution was further diluted with cell culture medium to the desired concentration for the treatment use. Maslinic acid was used as a treatment to the cell cultures dissolved in DMSO to a final concentration of 0.4% (v/v) or below in all the experiments. Vehicle controls were treated with the same amount of DMSO, while negative controls did not receive maslinic acid treatment.

2.3. Cell culture

Human colorectal adenocarcinoma cell lines HT-29 (ATCC no. HTB-38; Duke's class B/grade Ⅱ), SW480 (ATCC no. CCL-228; Duke's class B/stage Ⅱ), SW48 (ATCC no. CCL-231; Duke's class C/stage Ⅲ), LS 174T (ATCC no. CL-188; Duke's class B/stage Ⅱ), colorectal carcinoma cell line HCT 116 (ATCC no. CCL-247; Duke's class D/stage Ⅳ)(CS4, 6, 3 & 5) and normal colon cell, CCD-112CoN (ATCC no. CRL-1541) were used in this study.

The colorectal cells were cultured in cell culture media (HT-29, HCT 116, SW480, LS 174T and CCD-112CoN cells in RPMI 1640 and SW48 cells in DMEM) supplemented with 10% FBS and 1% of penicillin-streptomycin (10 000 U/mL) in a humidified incubator with 5% CO_2 at 37℃.

2.4. Cell proliferation evaluation by MTT assay

All the CRC cells were seeded in 96-well plates at a density of 5×10^3 cells/well in complete RPMI 1640 with 100 μL per well. Maslinic acid solutions were prepared at varying concentrations (0, 20, 40, 60, 80, 100 and 120 μM). The cells were incubated in a humidified environment with 5% CO_2 at 37℃ for 12, 24, 48 and 72 h. Following treatment, 20 μL of MTT solution (5 mg/mL) was added into each well and incubated for another 4 h. The medium and MTT solution were removed from the well and the formazan was resuspended with 100 μL of DMSO. The relative cell viability was assessed by measuring the absorbance at 570 nm (reference wavelength 650 nm) on a microplate reader (The Spark, TECAN, Austria).

2.5. Apoptosis analysis by annexin Ⅴ-FITC/propidium iodide (PI) staining

Two cell lines, HT-29 and HCT 116, were selected for the apoptosis analysis by annexin Ⅴ-FITC/PI staining. A total of 1.5×10^5 cells per well were seeded into 6-well plates with 3 mL of complete RPMI 1640 medium. After 24 h, maslinic acid was added to the cells at their respective IC_{50} concentration and incubated for 24, 48 and 72 h. After treatment, the cells were washed twice with PBS and harvested by trypsinization. The harvested cells were stained with eBioscience™ annexin Ⅴ-FITC apoptosis detection kit by following the recommended protocol before being analyzed with flow cytometry. Briefly, approximately 1×10^5 cells were collected and resuspended in 200 μL of binding buffer (1×). The cells were incubated in 2.5% of annexin Ⅴ-FITC for 10 min at room temperature in the dark. After washing with 200 μL binding buffer (1×), the cells were subsequently added with 5% of PI and prepared for analysis using flow cytometer (FACSCanto™ Ⅱ, BD, Belgium).

2.6. Cell cycle analysis by PI staining assay

The same density of HT-29 and HCT 116 cells were treated with maslinic acid as in the apoptosis analysis. Cells were collected and stained with FxCycle™ PI/RNase staining solution following the suggested protocol. Cells were trypsinized and centrifuged, then 70% ethanol was used to resuspend the cells. The cells were incubated on ice for 30 min for cell fixation. The fixatives were removed by washing the cells with PBS twice and stained with 500 μL of PI/RNase staining solution for each sample. After 30 minutes of incubation in the dark at room temperature, the cells were used for cell cycle analysis using FACSCanto™ Ⅱ.

2.7. Protein expression analysis using capillary electrophoresis immunoassay

The HT-29 and HCT 116 cells were seeded at the same density and treated with maslinic acid in the same way as described above. The intracellular proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents following the recommended protocol. Protein concentration was determined using the Pierce® 660nm Protein Assay Kit. Protein expression was measured by capillary electrophoresis immunoassay (Jess; Protein Simple, CA, USA). Briefly, about 4 ng of cell lysate was mixed with the sample master mix, which was made up of 1× sample buffer, 1× fluorescent molecular weight marker and 40 mmol/L dithiothreitol. The protein samples were vortex-mixed and heated at 95℃ for 5 min. Subsequently, the samples, blocking buffer, primary antibodies, secondary antibodies, horseradish peroxidase-conjugated secondary antibodies, chemiluminescent substrate, and protein normalization solution were added into the plate for automated electrophoresis (375 V for 25 min) and immunodetection by Jess system. Mouse primary antibodies against cyclin D1 (diluted 1:10), IKK-β (diluted 1:10), and Chk1 (diluted 1:50) were used to detect the protein expression. Protein expression was quantified as a peak area.

2.8. Statistical analysis

The treatment effect of maslinic acid was analyzed using statistical tests. All quantitative data were summarised as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant. One-way ANOVA test was used for the intergroup comparisons in MTT assay and protein expression studies. Two-way ANOVA test was used for the comparisons between treatment groups in apoptosis and cell cycle analyses which have more than one categorical variable. Tukey's multiple comparison test was used to determine specific differences between results if the result was found to be significant. All the statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, CA, USA).

3. Results

3.1. Growth inhibitory effect of maslinic acid

The effect of maslinic acid was examined upon the proliferation of different colorectal cancer cells (HT-29, HCT 116, SW480, SW48 and LE 174T) and normal colon cells (CCD-112CoN) using MTT assay. The growth of the maslinic acid treated cells was determined by formazan dye uptake by the cells in comparison to vehicle control. As shown in Figure 1A, the decreasing percentage of the proliferative formazan accumulating cell in all the six cell lines indicated maslinic acid inhibited the proliferation of the cells in a dose-dependent manner after 72 hours of incubation. The SW48 obtained the lowest IC_{50} value at (36.05 ± 1.06) μM, followed by LS 174T (45.36 ± 1.80) μM. HT-29 and HCT 116 showed a similar IC_{50} value around 50 μM [HT-29, (49.92 ± 1.33) μM; HCT 116, (49.80 ± 1.62) μM], while SW480 and the normal colon cell, CCD-112CoN obtained relatively higher IC_{50} values of (56.32 ± 1.06) μM and (56.70 ± 0.65) μM, respectively. As indicated in Figure 1B and 1C, maslinic acid resulted in a decrease in the cell proliferative percentage of HT-29 and HCT 116 in a dose- and time-dependent fashion. Both cell lines (HT-29 and HCT 116) showed the lowest IC_{50} values after 72 hours of incubation (Table 1).

Table 1. IC_{50} values of HT-29 and HCT 116 cells after treatment with maslinic acid at 0-120 μM for 12, 24, 48 and 72 h.

Cell lines	IC_{50} (μM)			
	12 h	24 h	48 h	72 h
HT-29	137.83 ± 11.31	110.10 ± 5.03	67.56 ± 0.94	49.92 ± 1.33
HCT 116	81.85 ± 2.09	64.91 ± 2.61	51.62 ± 1.00	49.80 ± 1.62

The results represent mean ± SD of four independent experiments performed in triplicate.

3.2. Apoptotic effect of maslinic acid

The assessment of apoptosis was performed on HT-29 and HCT 116 cells after receiving 50 μM of maslinic acid (with similar IC_{50} obtained after 72 h incubation) for 24, 48 and 72 h. As shown in Figure 2, the cells that stained negative with annexin Ⅴ-FITC and PI (Q3, lower left quadrant) are viable cells. Early apoptotic cells that were stained annexin Ⅴ-$FITC^+$/PI^- can be seen in Q4, the lower right quadrant, so it can be differentiated from late apoptotic cells which stained positive in both dyes that will gather in Q2 (upper right quadrant). The apoptotic cells, including early and late apoptotic cells, accounted for 2.95%, 12.46% and 24.13% of vehicle control cells after 24, 48 and 72 hours of incubation, respectively, in HT-29, which had no significant differences with the proportion of apoptotic cells in the untreated group (3.23%, 12.37% and 21.20%), suggesting normal cell viability without significant apoptosis (P>0.05).

The apoptosis events in HT-29 cells increased in a time-dependent manner with the treatment of maslinic acid. In Figure 2, a decrease of 14.6% of the viable cells following an increase of 14.4% in apoptotic cells (11.9% early apoptosis and 2.5% late apoptosis) can be observed in HT-29 cells after 48 hours of maslinic acid treatment in comparison to vehicle control with no statistically significant difference. Early apoptosis in HT-29 cells induced by maslinic acid can be seen at 72 h after exposure. A significantly higher percentage of (36.53 ± 0.93)% of early apoptotic cells was detected in maslinic acid-treated cells compared with (21.83 ± 9.02)% in vehicle control. An increase in maslinic acid-induced apoptosis was accompanied with a concurrent decrease in viable cells to (59.17 ± 1.88)% in the cells treated with maslinic acid as compared to (71.33 ± 3.50)% in DMSO-treated cells (P<0.05).

The maslinic acid induced a comparatively higher level of apoptosis in HCT 116 than in HT-29 cells. Statistically significant differences in the percentage of early apoptotic and viable cells between DMSO and maslinic acid-treated cells can be observed as early as 24 h, and subsequent 48 and 72 h (Figure 2D-F). After 24 hours of maslinic acid exposure, the percentage of viable cells in Q3 quadrant decreased from (89.70 ± 5.10)% (DMSO treated) to (44.73 ± 3.55)% while apoptotic cells increased from 7.36% (5.83% early apoptosis, 1.53% late apoptosis) to 50.30% (37.33% early apoptosis and 12.97% late apoptosis). The apoptosis event further increased after 72 hours of treatment. The viable cells decreased by 69.14% with a significant concurrent increase with 66.70% and 6.27%, respectively in early and late apoptotic cells, as shown in Figure 2F.

3.3. Cell cycle arrest induced by maslinic acid

In light of the growth inhibition and apoptosis caused by maslinic acid, the cell cycle analysis was performed to further determine the bioactivity of maslinic acid. It is noteworthy that 50 μM of maslinic acid can induce cell cycle arrest in both HT-29 and HCT 116 cell lines as early in 12 hours of treatment. Maslinic acid led to G_0/G_1 cell cycle arrest in HT-29 cells in a time-dependent fashion. As shown in Figure 3, there was a marked increase in the percentage of cells in G_0/G_1 phase (62.24 ± 1.37)%, concomitantly with a decrease in S phase (20.37 ± 0.20)% in the maslinic acid-treated cells as compared to vehicle control. This compound continued to increase the population in G_0/G_1 phases in the following hours (increases of 24.42% for 24 h, 25% for 48 h and 19% for 72 h) with concurrent reduction of cells in S phase (decreases of 20.04% for 24 h, 20.51% for 48 h and 16.27% for 72 h) in HT-29 cells with respect to DMSO-treated cells, suggesting G_0/G_1 arrest.

The DNA distribution analysis showed that, in HCT-116 cells, the

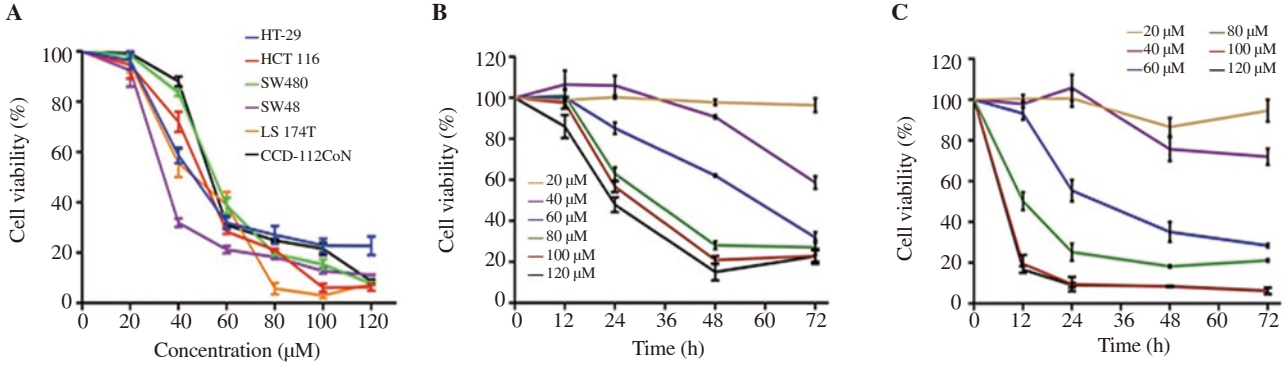

Figure 1. Inhibitory effect of maslinic acid on the proliferation of colorectal cancer cell lines (HT-29, HCT 116, SW480, SW48 and LS 174T) and normal colon cell, CCD-112CoN. (A) Every cell line was treated with maslinic acid at various concentrations for 72 h. (B-C) HT-29 and HCT 116 cells were treated with maslinic acid at 0-120 μM for 12, 24, 48 and 72 h. Each point represents mean ± SD of four independent experiments performed in triplicate.

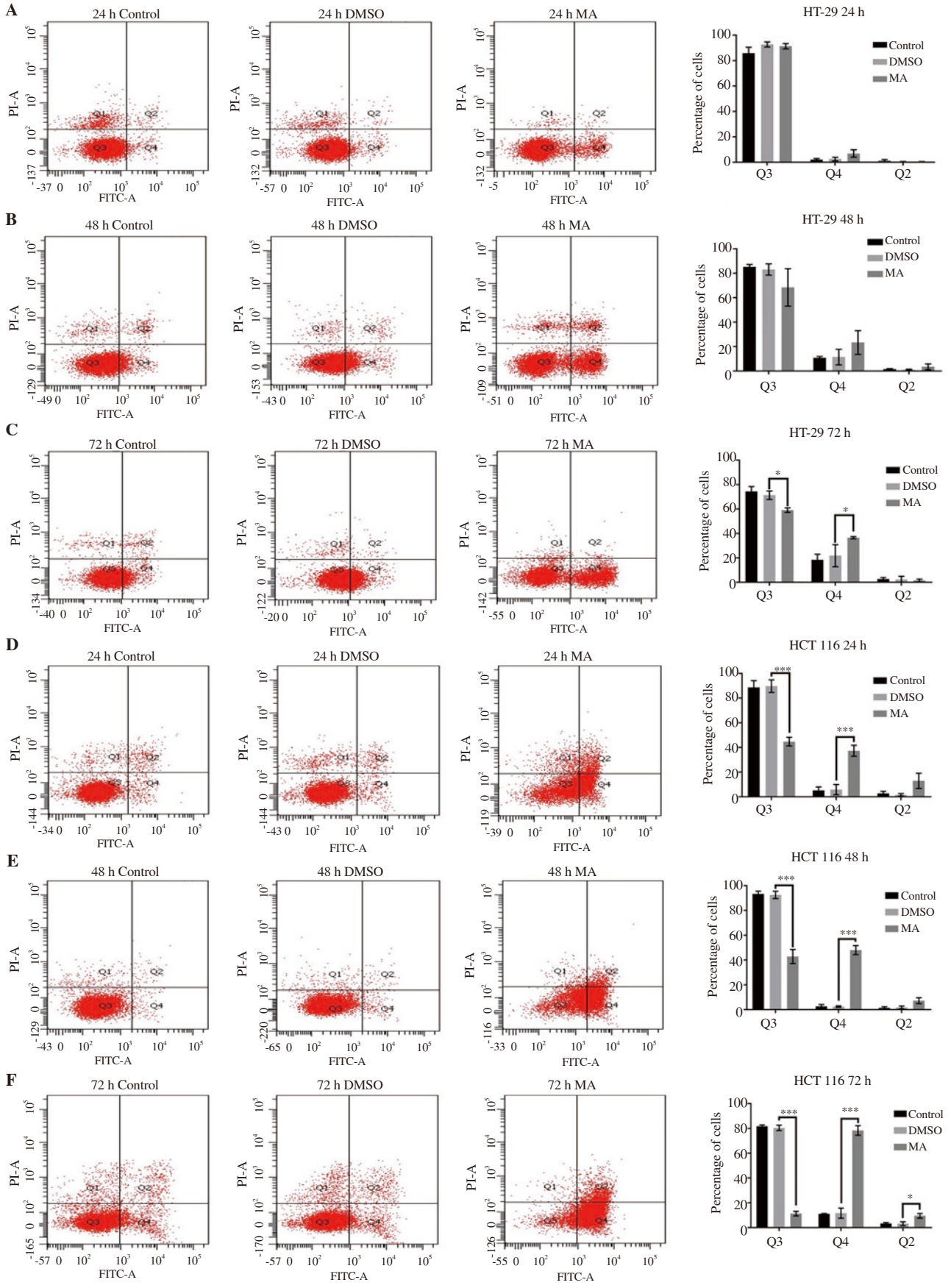

Figure 2. Time-course flow cytometry analysis of HT-29 (A-C) and HCT 116 (D-F) cells stained with annexin Ⅴ-FITC/PI after maslinic acid treatment at 50 μM. The bar graphs are the percentage of cells in different treatment groups in the quadrant 3 (Q3), quadrant 4 (Q4) and quadrant 2 (Q2) which represent viable, early and late apoptotic cells, respectively. Values represent mean ± SD of three independent experiments $^{*}P < 0.05$, $^{***}P < 0.001$ with respect to the vehicle control group. MA: maslinic acid.

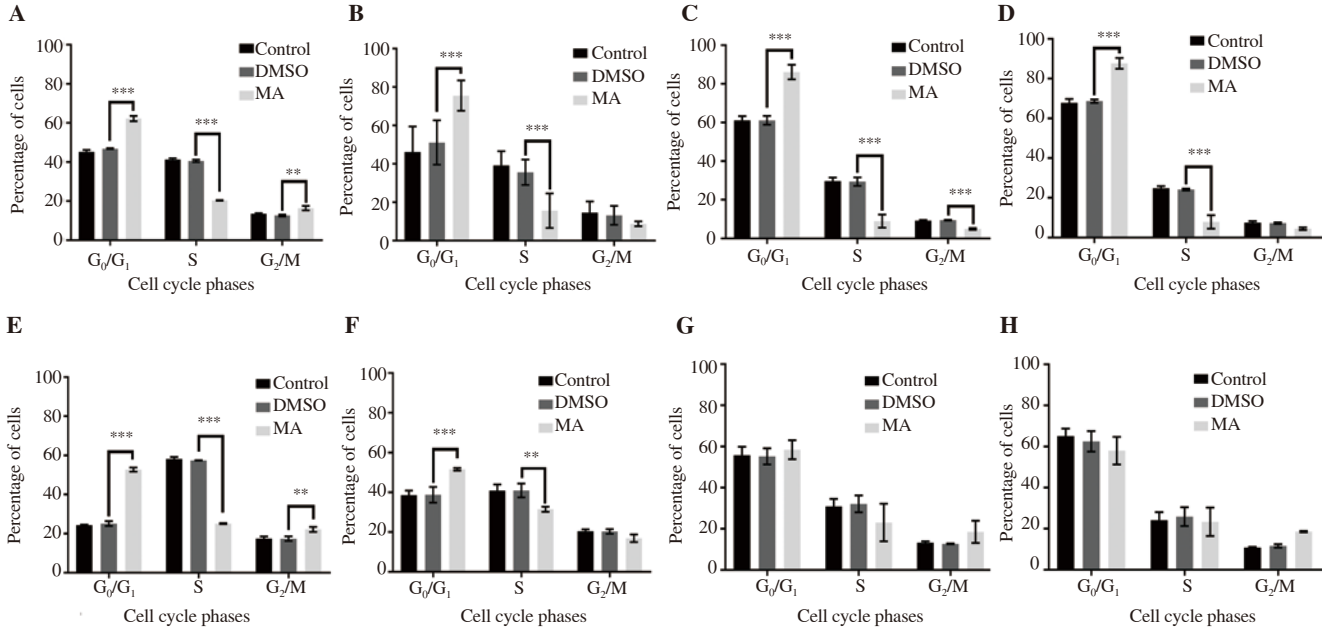

Figure 3. Time-course flow cytometry analysis of PI staining after exposure of HT-29 (A-D) and HCT 116 (E-H) cells to maslinic acid at 50 μM concentration for 12, 24, 48, and 72 h. The bar graphs show the percentage of cells in different treatment groups arrested in the G_0/G_1 phase, S phase and G_2/M phase. Values represent mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to the vehicle control group.

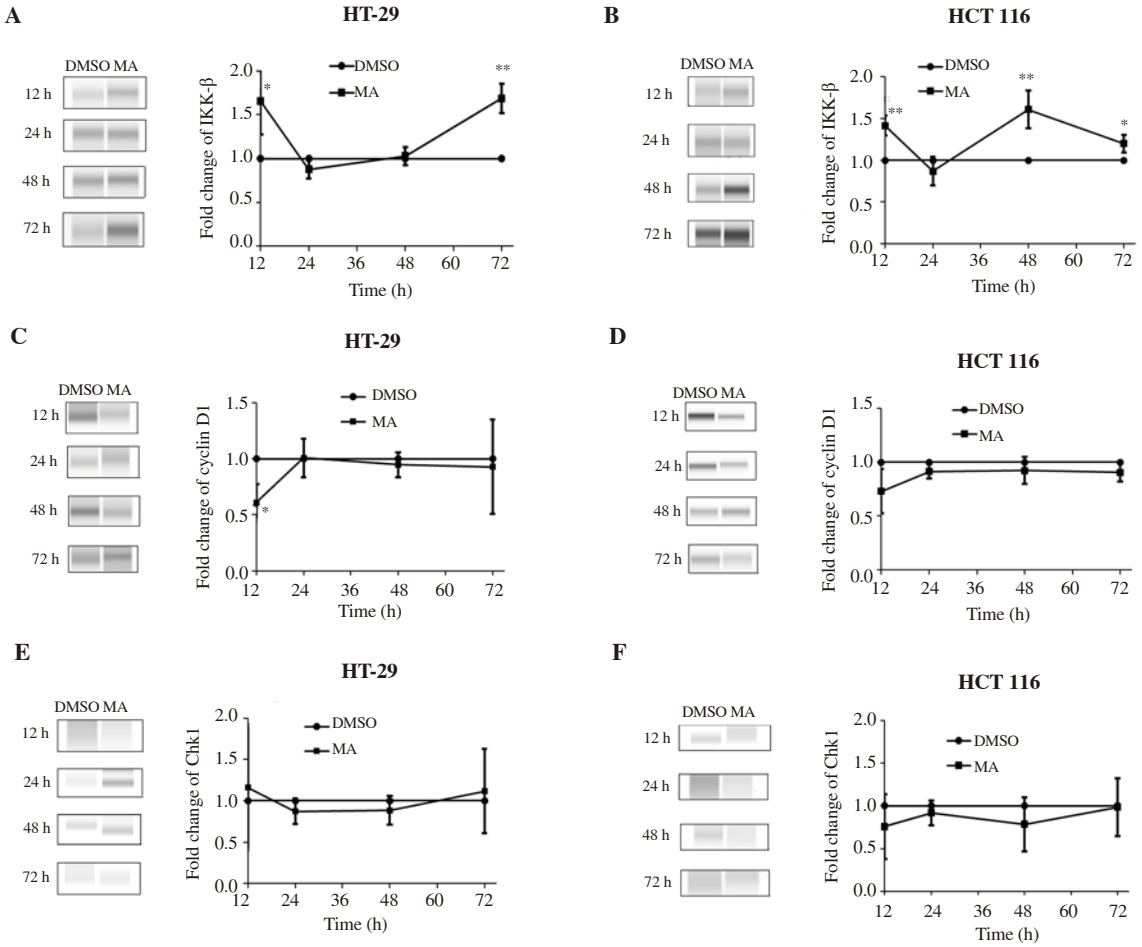

Figure 4. Protein expression of IKK-β (A-B), cyclin D1 (C-D) and Chk1 (E-F) in HT-29 and HCT 116 cells in different time points. Both cell lines were treated with maslinic acid at their IC_{50} for 12, 24, 48 and 72 h. The fold change of the protein expression levels in treated cells (maslinic acid) with respect to the vehicle control (DMSO) is shown in the line graphs against 4 different time points. Values represent mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to the vehicle control group. IKK-β: inhibitor of κB kinase-β; Chk1: checkpoint kinase 1.

effect of cell cycle arrest induced by maslinic acid could only be seen in 12 and 24 h (Figure 3). More than 50% of maslinic acid-treated cells were distributed within G_0/G_1 phase, and only around 30% or below of proliferating cells were detected in S phase (Figure 3), whereas in 48 and 72 h, there were no significant differences in terms of the cell distribution in different cell cycle phases.

3.4. NF-κB pathway inhibition of maslinic acid

Since the NF-κB pathway is constitutively activated in CRC and several NF-κB inhibitors have been proven to be useful for CRC therapeutics, the expression of IKK-β was studied. The IKK protein complex functions, at its phosphorylated state, to inhibit the IκB molecules that sequesters the transcription factor, NF-κB, in order to inhibit the nuclear localization. After 12 hours of maslinic acid treatment, the expression of IKK-β was significantly increased in both HT-29 (1.65-fold) and HCT 116 cells (1.41-fold) with respect to the vehicle control (Figure 4A and B). No changes were found in the subsequent 12 h for both cell lines. However, the increase was detected in HCT 116 after 48 hours of exposure to maslinic acid (1.6-fold) and reduced to 1.2-fold in 72 h with respect to the vehicle control cells. A similar trend can be observed in HT-29, whereby the IKK-β protein regained its expression (1.68-fold at 72 h) after 60 h, with 24 h delay as compared to HCT 116 cells.

As maslinic acid has been proven to induce G_0/G_1 arrest in both cell lines, the expression of cyclin D1 was assessed at varying time points (12, 24, 48 and 72 h). As shown in Figure 4C-D, cyclin D1 showed lower expression in HT-29 (0.61-fold, $P<0.05$) and HCT 116 cells (0.73-fold, $P>0.05$) after 12 hours treatment in comparison to DMSO-treated samples. The expression of this protein in treated cells remained similar to vehicle control until 72 h in both cell lines. In addition, maslinic acid did not cause any significant changes to the expression level of Chk1 protein in both HT-29 and HCT 116 cell lines (Figure 4E and F).

4. Discussion

Maslinic acid exhibited growth inhibitory effects against the 5 CRC cell lines in the dose- and time-dependent fashion. The IC_{50} concentration of maslinic acid against HT-29 cells decreased along the treatment hours tested and reached the lowest concentration of approximately 50 μM at 72 h. This observation is, however, opposed to the findings reported by Rufino *et al.*[20], which claimed that maslinic acid has more potent effect with a shorter exposure period [24 h, IC_{50}: (30±0.5) μM]. The differences in the finding might be due to the behavior of cells cultured in different media and the source of the compound extracted. In addition to the inhibitory property of maslinic acid against the 5 CRC cell lines (HT-29, HCT 116, SW480, SW48 and LS 174T), the triterpene compound also exerts growth inhibitory effect on the normal colon cells, CCD-112CoN. The results are in contrast to the previous studies that reported "normal" cell lines, including non-tumoral intestine cell lines IEC-6 & IEC-18[21], human bronchial epithelial cells HBE[11], human oesophageal squamous epithelial cells Het-1A, normal human lung fibroblast cells MRC-5, normal human liver cells L-02[22,23], human umbilical vein endothelial cell line and primary cultures of kidney proximal tubular epithelial cells[24] were insensitive to maslinic acid.

The outcome of MTT assay can result from the inhibitory or cytotoxic effect of the compound against the cells. In the case that the compound exerts the cytotoxic effects, the treated cells will have lesser viable cells; thus lower absorbance reading compared to the control group. On the other hand, if the compound exhibits growth inhibitory effect against the cells, the treated cells which are non-proliferating will produce lesser amount of formazan crystal in comparison to the control group which was actively proliferating, thus giving a lower absorbance value. Hence, the two CRC cell lines, HT-29 and HCT 116 were selected for the subsequent apoptosis and cell cycle analyses. Since the activation of transcription factor NF-κB is claimed to be directly involved in the development of colitis-associated CRC[25], the NF-κB status in the CRC cell lines for the following analyses was considered. According to Sakamoto and colleagues, the SW48 cell line which showed the most significant sensitivity to maslinic acid in this study, however, did not have the constitutive activation of NF-κB. Instead, the constitutive activation of this transcription factor was confirmed in the HT-29 and HCT 116 cell lines, which had similar IC_{50} values, *via* electrophoretic mobility shift assay[19]. There are no studies indicating the activation of NF-κB in the LS 174T cell line. Thus, this cell line was not selected for further analyses, although it had a lower IC_{50} value as compared to HT-19 and HCT 116 cell lines.

As reported in the review[26], the author categorized the natural compounds with the anti-CRC effect based on their molecular mechanism and regulatory network involved, namely, proliferation-, migration and invasion-, apoptosis-, autophagy- and angiogenesis-based regulation. Among these, the induction of apoptosis or cell cycle arrest is the most common mechanism. The inhibition of proliferation caused by maslinic acid in HCT 116 cells might be mediated by apoptosis. In contrast, for HT-29 cells, maslinic acid suppressed the proliferation largely by interfering the cell cycle process. The microscopic observation supports these hypotheses on the cells after maslinic acid treatment (not shown in this study). On one hand, the number of floating cells substantially increased in the treated HCT 116 cells as compared to the vehicle control. On the other hand, the number of viable (adherent) HT-29 cells in vehicle control group outnumbered the treated group.

Noticeably from the results obtained from FACS analysis, maslinic acid induced early apoptosis in HCT 116 cells in a time-dependent manner. However, there was no significant increment in the late apoptosis (secondary necrosis) as well as primary necrosis, which was presented in quadrant 2 and 1, respectively. An increase in the early apoptotic cells instead of late apoptotic allows the efferocytosis to take place in a multicellular organism, whereby the apoptosing cells were phagocytosed by the scavengers and cleared *via* protected

heterolysis. This avoids the deleterious effect caused by the autonomous proteolysis in the secondary necrosis, which results in the leakage of cellular component that can induce inflammation[27]. In fact, the event of apoptosis can also be observed in the maslinic acid-treated HT-29 cells; however, with a more extended treatment period of 72 h in contrast to HCT 116 cells where apoptosis took place in early stages. This is in accordance with the results obtained by Reyes and colleagues who claimed maslinic acid induces the late activation of apoptosis in HT-29 cells *via* JNK-p53 dependent mechanism[21,28]. The delay in activation might be due to the events of enterocyte differentiation and cell cycle arrest, the latter of which has been proven in this study and will be discussed later, prior to apoptosis activation. Maslinic acid is proven to increase the activity of differentiation marker, alkaline phosphatase in HT-29 cells as reported before[21]. The differentiation, cell cycle arrest and late induction of apoptosis could be well explained by the overexpression of the functional, though mutated, p53 protein, whereby p53 regulates these cellular activities by controlling the expression of p21, Bcl-2 and Bax proteins as claimed by Reyes-Zurita and colleagues[21,28,29]. However, in HCT 116 cells which have wild-type p53, an earlier response was induced by maslinic acid, whereby apoptosis took place at early stages at 24 h or maybe earlier. Maslinic acid has been reported to induce apoptosis in HCT 116 cells mediated by caspase 3 *via* the AMPK-mTOR pathway[30]. Hence, maslinic acid seems to be a more potent inducer of apoptosis in HCT 116 cells in comparison to HT-29 cells.

The cell cycle arrest effect of maslinic acid in HCT 116 cells can be observed as early as 12 h but is quickly replaced by apoptosis, whereas the cytostatic effect induced by maslinic acid is more prominent in HT-29 cells. The cell population in G_0/G_1 phase in the treated HT-29 cells increased concomitantly with the decrease in S phase, indicating maslinic acid might interfere with the cell cycle regulation by inhibiting G_1-S transition. This hypothesis is supported by the result obtained in the protein expression study, where the expression of cyclin D1 is significantly decreased by 0.61-fold at 12 h. The result is in agreement with previous studies which also reported downregulation of cyclin D1 after maslinic acid treatment in other cancer cell lines, such as pancreatic cancer and gall bladder cancer lines[15,18]. Maslinic acid has also been proven to suppress the cyclin D1 overexpression and induce p21 levels to prevent the cell cycle progression in Raji cells[17]. Cyclin D1 regulates the G_1-S phase transition by controlling the pRb phosphorylation, which is the rate-limiting factor of cell cycle progression[31]. In addition, maslinic acid is reported to induce cell cycle arrest by targeting different proteins, for instance, lamin B1 and PNCA[20,24]. Cyclin D1 is one of the transcriptional targets of NF-κB[17]. The increased expression of unphosphorylated IKK-β can be observed in both HT-29 and HCT 116 cells after 12 hours of maslinic acid treatment in comparison to the vehicle control. This indicated the possible inhibition of NF-κB activation, whereby the increase of inactive form of IKK-β is likely to be accompanied with the reduction of the phosphorylated IKK-β. Thus, it showed that maslinic acid treatment is able to cause the shifting of IKK-β proteins to the unphosphorylated state. The expressions were increased again at 72 h and 48 h in HT-29 cells and HCT 116 cells, respectively. The fluctuation in the expression of IKK-β can be explained by the dynamic expression patterns of the protein upon receiving the extracellular signal, which is partly induced by maslinic acid in this study. Together with the reduced cyclin D1 expression, these suggest that maslinic acid could induce cell cycle arrest at least in part *via* NF-κB pathway inhibition. It has been reported that maslinic acid is able to enhance the antitumor property of TNF-α by inhibiting the NF-κB-mediated survival signaling pathway, including downregulating the NF-κB activity and its downstream gene that facilitate the tumor cell proliferation, anti-apoptosis, invasion and angiogenesis process in pancreatic cancer[15]. In addition, maslinic acid can potentiate the anti-cancer activity of gemcitabine against gall bladder cancer by suppressing the NF-κB activation and its downstream gene products in the *in vitro* and *in vivo* model. Furthermore, maslinic acid has also been reported to possess chemopreventive activity against lymphoma by reducing the expression of COX-2 *via* NF-κB and AP-1 pathways in Raji cells[16].

There is a study done by Peragón and colleagues in 2015 on the incorporation of maslinic acid in HT29 and HepG2 cells[32]. The study found that maslinic acid exhibited a sigmoid saturation curve when the results were expressed as the amount of maslinic acid uptake by cells with respect to maslinic acid concentration. The response was slow in the initial stage, followed by a higher response observed in the subsequent stage when the concentration of maslinic acid was increased and eventually achieved a plateau where the response has saturated regardless of the increasing maslinic acid concentration. The authors claimed that the uptake of maslinic acid by HT-29 cells depends on a specific receptor-carrier present in the cell membrane. The HT-29 cells were less responsive at the initial state when the binding affinity of maslinic acid to the receptor was low at a low concentration. The cooperative behaviour augmented when the concentration increased, allowing the entry of maslinic acid into the cells. The degree of response would reach a maximum depending on the availability of active receptor carrier existing at the plasma membrane and give a saturation response when all the receptors were occupied[32]. The finding is in agreement with the result obtained from the current study, where the percentage of viability of HT-29 cells maintained at the level above 20% started from 60 μM. In contrast, maslinic acid has shown to have a linear relationship with HepG2 cells that did not show a saturation sign, suggesting the direct diffusion of maslinic acid through the lipid bilayer of HepG2 cells, since maslinic acid is highly lipophilic[32]. HepG2 cells are liver cancer cells that exhibit epithelial morphology, similar to the colonic epithelial cells HCT 116. This suggests that there is a possibility where HCT 116 cells utilized a similar transport route as HepG2 cells to uptake the maslinic acid present in the culture medium. If that is the case, the cell viability curve of HT-29 and HCT 116 cells obtained from MTT assay could be explained by the different modes of maslinic acid uptake by these two types of CRC cell lines. The percentage of viability of HCT 116 cells continued to decrease to below 5% with the increasing maslinic acid concentration present in the medium. Hence, this suggests that there

is a higher amount of maslinic acid absorbed by HCT 116 cells as compared to HT-29 cells where the viability remained above 20%. Moreover, maslinic acid is said to be a non-toxic compound *in vitro* that is supported by the high IC_{50} values against HT-29 and HCT 116 cell lines and the apoptosis activation instead of necrosis. In addition to apoptosis, maslinic acid can trigger cell cycle arrest, as shown in this study, suggesting that the triterpene compound can induce a cytostatic effect against CRC cell lines. On the other hand, the safety assessment of maslinic acid has also been tested *in vivo* by Sánchez-González and colleagues in 2013. The study suggested that maslinic acid provided a sufficient safety margin with a single high dose (1 000 mg/kg) or repeated daily oral administration (50 mg/kg) for 28 d in Swiss CD-1 mice. The maslinic acid treatment did not induce any signs of morbidity or mortality as well as toxicity in hematological, biochemical and histopathological evaluation[33]. Therefore, for safety concern, it can be concluded that maslinic acid is not a toxic compound in both *in vitro* and *in vivo* and has the potential to become a promising agent for CRC treatment that warrants further in-depth study.

All things considered, this study suggests the potential of maslinic acid in restraining the proliferative capacity of CRC cell lines, HT-29 and HCT 116 cells by inducing the apoptosis and cell cycle arrest *via* the increased expression of unphosphorylated IKK-β. This, in turn, minimized the transcriptional activation of NF-κB and resulted in the downregulation of the downstream genes that promote anti-apoptosis and cell cycle progression. Future studies are warranted to confirm the involvement of NF-κB in the maslinic acid-induced anti-cancer effect in the CRC cell lines and the possible signaling pathways. This includes studying the translocation of NF-κB transcription factor (p65) between cytoplasm and nucleus as well as other proteins which regulate its transcriptional activation such as TNF-α and IκB.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

YML, LYF, HHG and ASBK contributed to the development of the conception and design of the work. KXO and MS performed the experimental studies, data collection and data analysis. Both YML and KXO contributed to the manuscript preparation. YML, MS, LYF, HHG and ASBK performed the manuscript review. YML, ASBK and KXO contributed to the final version of the manuscript. YML as the main PI supervised the project.

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Apoptotic and cytostatic actions of maslinic acid in colorectal cancer cell lines through possible IKK-β inhibition

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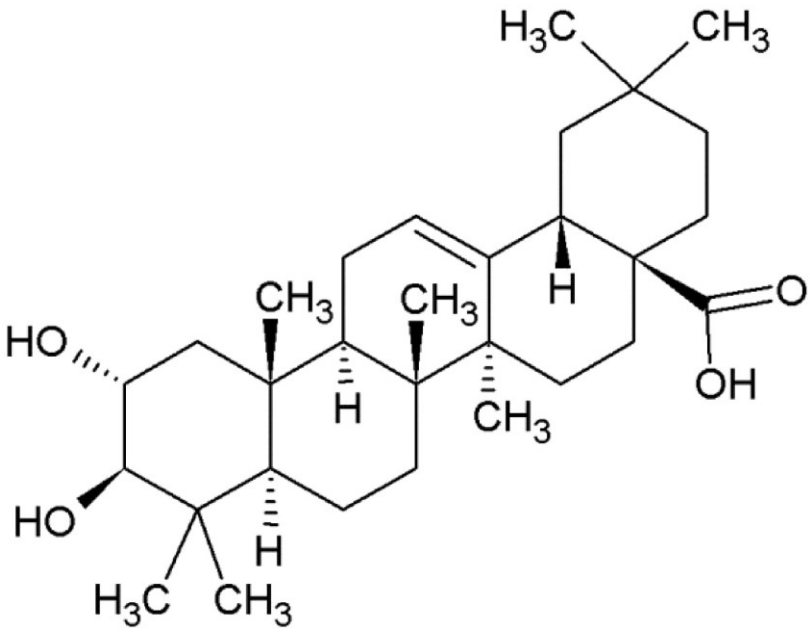

Supplementary Figure 1 Chemical structure of maslinic acid [(2α,3β)-2,3-dihydroxyolean-12en-28-oic acid].