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Endophytic *Bacillus amyloliquefaciens* surfactin possesses anti-inflammatory potential through acetylcholinesterase and lipoxygenase inhibitory activities

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ABSTRACT

Aims: This study was aimed to investigate the anti-inflammatory and anti-rheumatoid effects of the *Bacillus amyloliquefaciens* derived surfactin.
Methodology and results: Crude and biosurfactant extracts were analyzed using thin-layer chromatography to determine the presence of biosurfactant. Both extracts were evaluated for their inhibitory effects against the acetylcholinesterase and 5-lipoxygenase enzymes. Human synovial cells were induced with TNF-α and IL-1β. The percentages of the cell viability for both normal and induced cells were determined with an MTT assay. Results showed that surfactin was detected in the biosurfactant extract and demonstrated higher inhibitory effects compared to the crude extract against both inhibitory enzymes acetylcholinesterse (IC_{50}=30.60 µg/mL) and lipoxygenase (IC_{50}=110.10 µg/mL). Both crudes showed no cytotoxic effects at the highest concentration used (50 µg/mL) against normal human synovial cells but showed active reactions against the induced cells. The anti-proliferative effects of biosurfactant and crude extracts were in dose-dependent manner.
Conclusion, significance and impact of study: Notably, surfactin obtained from *B. amyloliquefaciens* has shown an inhibitory effect against pro-inflammatory enzymes and cell viability of the induced rheumatoid arthritis cell line. These results highlighted the therapeutic potential of surfactin application as an anti-inflammatory agent for arthritis treatment. Further study is needed to elucidate the mechanisms underlying the anti-inflammatory effect of surfactin.

Keywords: Biosurfactant, endophytes, acetylcholinesterase, lipoxygenase, arthritis

INTRODUCTION

The inflammation of the joints is a symptom of arthritis and rheumatoid arthritis (RA) is the most common. Rheumatoid arthritis affects approximately 1% of the world population and it affects women two or three times more than men (Radhakrishnan *et al.*, 2022). Rheumatoid arthritis is an inflammation of soft tissue when the synovium cell inflamed and thickened, causing destruction to cartilage and joints. Patient's body will develop an immune response by activating T-cells and B-cell, followed by pro-inflammatory cytokine production. These will cause inflammatory changes in many organs such as heart, lungs and blood vessels which lead to premature death (Koper-Lenkiewicz *et al.*, 2022).

A recent study shows uncontrollable production of cytokine related to the cholinergic pathway in arthritis. It is controlled by neural input via an inflammatory reflex which involved in the central and peripheral nervous system. Acetylcholine is a neurotransmitter that carries the electrical signal released by nerve cells to signal and regulate the other muscle cell. However, the accumulation of acetylcholinesterase (AChE) enzyme regulated the level of acetylcholine and rapidly degraded the acetylcholine that, caused interference in electrical signal transmission. Besides that, a patient who suffers from rheumatoid arthritis has a high concentration of lipoxygenase enzyme in the synovium. This enzyme induces TNF-α expression in muscle cells which leads to the inhibition of the proliferation and apoptosis of chondrocytes cell (Bryda and Wątroba, 2018). Although the mechanism of RA pathogenesis remains unclear, cytokines pro-inflammatory especially interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α play an important role in the disease.

Currently approved drugs for RA treatment include NSAISs, DMARDs, corticosteroids, methotrexate, TNF-blocking, interleukin blocking, B-cell depletion and CTLA4-Ig that helps by changing the disease process and giving clinical relief. However, most of them are not effective and has some disadvantage (Zaizar-Fregoso *et al.*, 2022). Recent advances in pathogenesis lead to the

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identification of new potential agents. Biological agents show some evidence for RA treatment and bring hope to rheumatoid arthritis sufferers. The relationship between mangrove and endophytic through mutualism is an interesting field of research.

Instead of harming their host, endophyte provides survival conditions and protection to their host by producing bioactive natural products. This is called mutualistic activity that helps to enhance plant growth and improved their resistance against various biotic and abiotic stresses (Somiari *et al.*, 2022). Endophytes from the marine environment can live in extreme conditions such as high salt salinity and deep seas volcanoes, even the temperature over 100 °C. Hence, the metabolic interaction between them and their hosting plant produce a novel secondary metabolites, enzymes, proteins, antibiotics and salt-tolerant genes that have potential source in medicinally treatment (Khattab and Farag, 2021). These metabolites have attracted a lot of attention and increased interest due to their significant application in therapeutic purposes against numerous diseases, including inflammation, arthritis, diabetes and other diseases. Therefore, this study aims to investigate the potential secondary metabolites surfactin derived from *B. amyloliquefaciens* and their anti-inflammatory and anti-rheumatoid effects against RA.

MATERIALS AND METHODS

Extraction of bioactive compound from *Bacillus amyloliquefaciens*

Liquid-liquid extraction

Bacillus amyloliquefaciens were incubate in 20% of Mueller Hinton agar with 1% of vegetable oil. After seven days of incubation with agitation speed 150 rpm. The fermentation broth sample was collected and centrifuged with 10,000 rpm for 20 min at 4 °C to separate the cell pellets and supernatant. Liquid-liquid extractions were used to further separate the active compound that accumulates in the phases using 2:1 ratio of supernatant to ethyl acetate (Adebajo *et al.*, 2020). Next, separation funnel was shaken up vigorously to allow the compound to separate according to their polarity. The organic solvent was evaporated using a rotary evaporator and residue was obtained that served as the crude extract. The sample was stored in 4 °C.

Acid-acid precipitation

The crude extract of *B. amyloliquefaciens* was treated by acid-acid precipitation to pH 2 using 1 M HCL and the acidified supernatant was left overnight in the cool room for complete precipitation of the biosurfactant formed. Precipitated samples were centrifuged at 10,000 rpm for 20 min at 4 °C and the pellets obtained served as the unpurified biosurfactant extract. Biosurfactant extract pellets were stored in a cool room (4 °C) for biological activities procedure (Adebajo *et al.*, 2020).

Thin layer chromatography

Bacillus amyloliquefaciens crude and biosurfactant extracts obtained were analyzed by thin-layer chromatography to determine the presence of a bioactive compound. The samples were spotted in TLC plate (Silica gel 60, Fluka) and further developed in an appropriate solvent system to give better separation. The mobile phase used was chloroform/methanol 9:1 (v/v). The TLC plate was then sprayed with 10% of sulphuric acid. The sample was compared with standard Surfactin purchased from Sigma Aldrich (S3523) to compare the spot detected (Mainez *et al.*, 2017).

Biological screening

Acetylcholinesterase (AChE) inhibitory assay

The AChE inhibitory effect of potential bacteria was determined (modification of Ellman *et al.*, 1961). Buffer A and buffer B were prepared, and pH was adjusted until it reach pH 8.0. Acetylthiocholine (AChI) was used as substrate, and it was prepared by a mixture of three unit of an enzyme with 14 mL of buffer to make it as 2 units of the enzyme. An experiment began with 50 µL of buffer B were added into each well of 96 well plates. Next, 125 µL of DTMB was added and used for the measurement of AChE activities. AChI was added at 25 µL to initiate the reaction. A sample with different concentrations was added and the plate was measured with wavelength 405 nm. Then, the AChE enzyme was added at 25 µL and measured with a wavelength of 405 nm.

Lipoxygenase

The activity of 15-lipoxygenase (Cayman, USA) assay was examined following as described in the protocol. The experiment began with 100 µL of 1× assay buffer was added into well, which act as blank wells. While for positive control, 90 µL of 15-LO standard and 10 µL of 1× assay buffer was added into the wells. NDGA was used in the inhibitor wells by mixing 90 µL of lipoxygenase enzyme and 10 µL of NDGA. Then, 90 µL of lipoxygenase enzyme was added with 10 µL of solvent into the 100% initial activity wells. Approximately, 90 µL of lipoxygenase enzyme and 10 µL of a sample with different concentration were added into inhibitor wells. After five min of incubation at room temperature, the reaction was initiated by adding 10 µL of Arachidonic acid to each of the wells. The plate was placed on a shaker for 10 min. Next, 100 µL of Chromogen were added to each of the wells to stop enzyme catalysis and develop the reaction. After five min on a shaker, the cover of the 96-well plate was removed and read the absorbance at 490 nm using the multi-plate reader.

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Cell viability screening assay using MTT cell proliferation assay

The effects of extracts on cell viability were examined to confirm their best concentration to be further used in cell proliferation. Briefly, 100 µL of human synovial cells were seeded into each well of 96-well plates with a density 7.0 × 10^4 and grown at 37 °C in a humidified incubator with 5% CO_2. After 3 days, different concentrations of potential crude and biosurfactant bacteria were from 12.5 µg/mL to 100 µg/mL and added to the cell. Treated cells were then incubated for 24 h at 37 °C in 5% incubator.

The plate was then assayed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) source Sigma Aldrich, USA. Briefly, 20 µL of MTT solution was added into each well and incubated at 37 °C. After 4 h, 150 µL of DMSO was added into each well. The absorbance was determined at 570 nm. Wells consisted of an untreated cell and MTT solution was used as a positive control.

Percentage of cell viability = (Absorbance of treated cell/ Absorbance of untreated cell) × 100%

Then, 100 µL of human synovial cells were seeded into each well as before. Both crude and biosurfactant extracts were grown in a synoviocyte medium. Next day, the media were discarded and filled with a medium consist of TNF-α (10 ng/mL)/IL-1β (5 ng/mL). The concentration of cytokine pro-inflammatory represents the maximum activities in induced cell culture. Both induced cells were grown for two days. After 4 days, cells were treated either with the absence (untreated control) or with the presence of different concentrations of crude and biosurfactant extract from 6.25 µg/mL to 50 µg/mL concentrations. Methotrexate acts as (positive control). Treated cells were then incubated for 72 h at 37 °C in 5% CO_2 incubator before were analyzed using MTT assay.

RESULTS AND DISCUSSION

Lipopeptides surfactin were detectable in biosurfactant extract samples. It was produced by the incubation of bacteria *B. amyloliquefaciens* with minimal salt concentration and the presence of vegetable oil as a carbon source. The growth medium starts changing colour as media turn to milky white and oil starts to mix and form a foam. This is due to the presence of lipopeptides as secondary metabolite products of bacteria. Lipopeptides production was induced when cells were critically exhausted with essential nutrients; as in this study, we minimize the salt concentration; hence the bacteria started to consume the carbon source available. These will indirectly secrete the production of lipopeptides surfactin during the transition from exponential to stationary (Pueyo *et al.*, 2014).

TLC is a method to analyze the presence of a chemical compound by analyzing the mixture of crude extract through separation using the proper solvent system. It is the initial method to determine the number of compounds in the mixture and identify the potential compound before proceeding to the purification method. Silica gel acts stationary phase and solvent system with a calculated ratio as a mobile phase that flows through the stationary phase while carrying the compound in the sample extract. Standard of surfactin from *Bacillus subtilis* (Sigma-Aldrich) was purchased to confirm the presence of spot surfactin. Hence, the biosurfactant extract of *B. amyloliquefaciens* was used as a sample to compare with the standard of surfactin. $CHCl_3$:MeOH; 9:1 were used as a solvent system and 10% of H_2SO_4 was chosen as spraying reagent for biosurfactant precipitate (Kanta *et al.*, 2022). Both standard surfactin (control) and biosurfactant extract of *B. amyloliquefaciens* were appeared at R_f:0.86 (Figure 1).

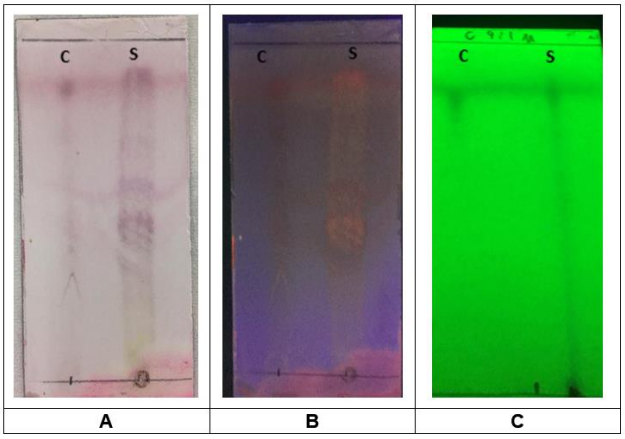

Figure 1: Thin layer chromatography (TLC) analysis of the biosurfactant extract of *B. amyloliquefaciens* with R_f value: 0.86. A: Under visible light, B: Under UV long wavelength and C: Under UV short wavelength. C: Control (Standard surfactin) and S: Sample (Biosurfactant extract).

Determination of biological activities

Acetylcholinesterase (AChE) inhibitory assay

Acetylcholinesterase is an enzyme responsible in the termination of impulse transmission by rapidly catalyzing the hydrolysis of neurotransmitter ACh into choline and acetic acid. Hydrolysis occurs in the central and peripheral nervous system in the cholinergic pathway. These will lead to the accumulation of acetylcholine, hyperstimulation of nicotinic and muscarinic receptors, and start to trigger the secretion of other pro-inflammatory mediators. Study about the acetylcholine in the cholinergic pathway was very limited understanding; however, the inactivation of acetylcholinesterase inhibitors were proven to suppress the inflammatory activities (Su *et al.*, 2022). Hence, this present study was a target to inactivate acetylcholinesterase enzyme activities. Acetylcholinesterase inhibitor assay is a colourimetric assay determined by hydrolysis of ACh as a

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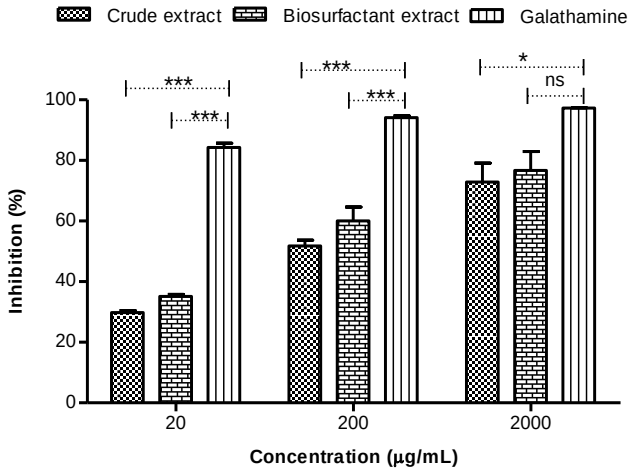

Figure 2: Inhibition (%) of acetylcholinesterase enzyme by crude and biosurfactant extract from *Bacillus amyloliquefaciens* and Galathamine as a positive control (2000, 200 and 20 µg/mL). The percentage inhibition of the positive control, Galathamine (1000 µg/mL) was 97.33 ± 0.09. Value are means ± SEM (n=3); ns=p>0.05, **p<0.05 and ***p<0.001; One-way ANOVA was performed, followed by Tukey's multiple tests for analysis.

substrate by acetylcholinesterase (AChE) enzymes in the reaction. The colour of the product will be colourless if there is no hydrolysis of ACh; however, hydrolysis of ACh by AChE will react with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to produce thiocholine as a by-product of reaction and forming a yellow colour product which indicates the unsuccessful result (Sucianti *et al.*, 2022).

Statistical analysis showed that the mean percentage of inhibition value for both crude and biosurfactant extracts of *B. amyloliquefaciens* is significantly different with the Galanthamine (Figure 2). Different concentrations of extracts starting with 2000, 200 and 20 µg/mL were used as a preliminary test to determine their inhibition activity. The different percentage of inhibition for both crude and biosurfactant extracts are not much different, where the crude extract able to inhibit up to 72% (IC_{50}:50.71 µg/mL) and biosurfactant extract inhibits 76% (IC_{50}:30.60 µg/mL) at concentration 2000 µg/mL. Both of extracts able to inhibit the AChE enzyme up to 50%, which can be concluded that surfactin from *B. amyloliquefaciens* is the responsible compound in suppressing inflammation. This is similar to the study by Jamshidi-Aidji *et al*. (2019), where surfactin compound extracted through acid precipitation from ethyl acetate crude extract of *Bacillus* sp. shows strong AChE inhibition. The IC_{50} result obtained for the AChE inhibitory activity of the potential bacteria in the present study is 50.71 µg/mL, which is similar to that obtained by Elgorashi *et al.* (2004) where all the IC_{50} of the sample value were ≤490 ± 7 µM (140 µg/mL). The concentration of AChE inhibitory activity of ≤500 µM is considered active (Adewusi *et al.*, 2012).

Lipoxygenase assay

Leukotrienes (LT) is an inflammatory mediator produced by the instability of Arachidonic acid (AA). This lead to the production of excess cytokine pro-inflammatory mediators that lead to an autoimmune disorder in RA. The conversion of LT from AA was stimulated by the enzyme 5-lipoxygenase (5-LO). Thus, inhibition of 5-LO enzyme activities was the focus target to suppress the LT production and autoimmune disorder. As shown in Figure 3, the percentage inhibition of biosurfactant extract showed maximum inhibition up to 58% (IC_{50}: 110.1 µg/mL) at a concentration 1 mg while compared to crude extract, able to inhibit up to 53% (IC_{50}: 194.7 µg/mL). The reaction of both crude extract and biosurfactant extract considers in moderately similar to the with the purified compound of biosurfactant extract from *Acinetobacter* M6 strain which has higher inhibition up until 49% activity (Karlapudi, 2016).

Cell viability assay

Biosurfactant extract and their crude extract were tested to confirm their percentage of cell life at the best concentration to further used in cell proliferation assay against TNF-α and Interleukin-1β (IL-1β). As shown in Figure 4, both biosurfactant and crude extracts show highly significant differences with hydrogen peroxide at four different concentrations 100, 50, 25 and 12.5 µg/mL in the synovial cell. The percentage of cell viability in the synovial cell line at high concentration (100 µg/mL) shows the crude extract was not able to reach 50% of cell viability as compared to biosurfactant extract. However, both extracts start to have less toxicity and able to reduce cell mortality at a concentration of 50 µg/mL. Hence, it is indicate that the cell viability was greater than 60% in the absence or presence of extracts, suggesting that extracts with a concentration of up to 50 µg/mL had no toxicity on synovial cells. Therefore, both extracts with a concentration of 50, 25 and 12.5 µg/mL was used in the subsequent experiments. The non-cytotoxic effect of

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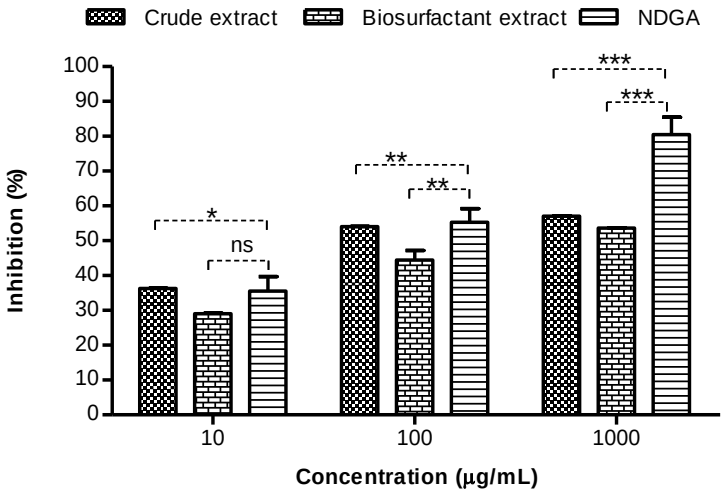

Figure 3: Inhibition (%) of lipoxygenase enzyme by crude and biosurfactant extract from *Bacillus amyloliquefaciens* and Nordihydroguaiaretic acid (NDGA) as a positive control (1000, 100 and 10 µg/mL). The percentage inhibition of the positive control, NDGA (1000 µg/mL) was 80.43 ± 2.91. Value are means ± SEM (n=3); ns=p>0.05, **p<0.05 and ***p<0.001; One-way ANOVA was performed, followed by Tukey's multiple tests for analysis.

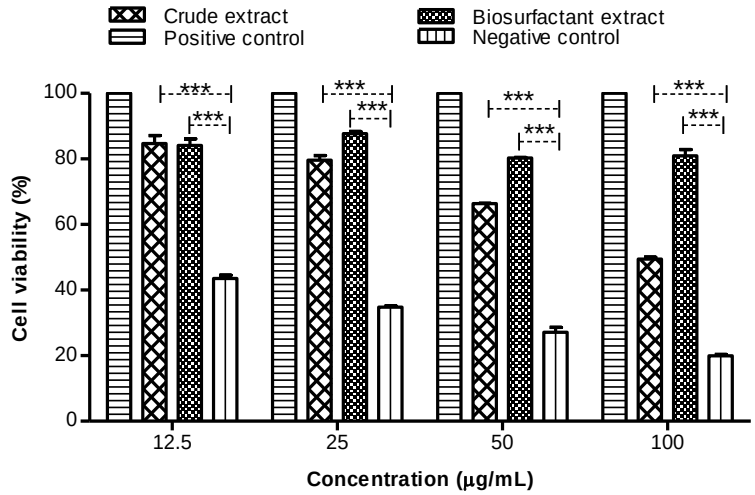

Figure 4: Percentage of cell viability (%) of the human synovial cell by crude and biosurfactant extract from *Bacillus amyloliquefaciens*, blank media as positive control and hydrogen peroxide as a negative control (100, 50, 25 and 12.5 µg/mL). The percentage inhibition of the positive control was 100 ± 0. Value are means ± SEM (n=3); ns=p>0.05, **p<0.05 and ***p<0.001; One-way ANOVA was performed followed by Tukey's multiple test for analysis.

surfactin from *Bacillus* sp. is in line with studies of Duarte *et al.* (2014) who reported that surfactin was toxic to cancer cell without affecting the normal cell to remain healthy.

Cell proliferation assay against induced human synovial cell

Tumour necrosis factor-α (TNF-α)

TNF-α causes synovial inflammation. It was found as the necrotic cytokine triggers cell death by apoptosis (Pratiwi and Sahid, 2018). IL-1β is well known as potent growth-promoting factor that promote cell tumour-like cell proliferation in RA. Hence, these present studies were targeted to suppress the TNF-α and IL-1β by observing the percentage of cell viability. The treatment activities of both extract when comparing with the positive control, Methotrexate (MTX) which is the current drug used to treat RA. MTX inhibits the proliferation of cells and suppresses TNF-α and IL-1β activities by reducing the number of activated T lymphocytes (Witte, 2015).

According to Figure 5, the percentage of cell viability of induced synovial cells is 26% cell viability. The cell

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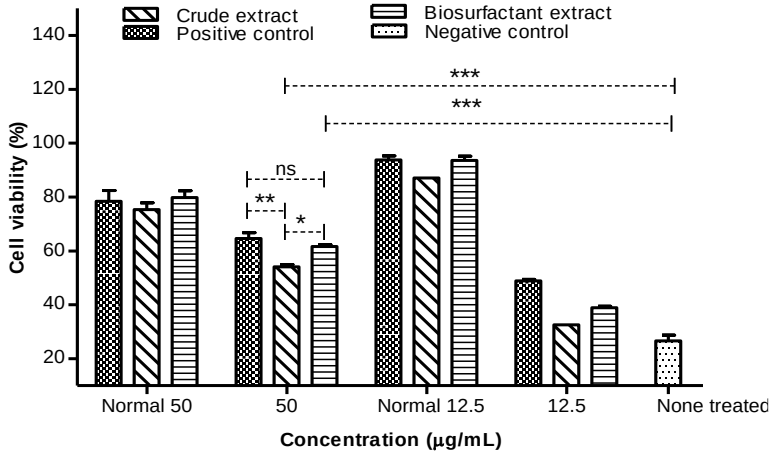

Figure 5: The percentage of cell viability against tumor necrosis factor-alpha (TNF-α) using HS cell by crude extract and biosurfactant extract from *Bacillus amyloliquefaciens*, methotrexate as a positive control with concentration normal 50 (normal cell without TNF-α), 50, normal 12.5 (normal cell without TNF-α) and 12.5 µg/mL. Value are means ± SEM (n=3); ns=p>0.05, **p<0.05 and ***p<0.001; One-way ANOVA was performed, followed by Tukey's multiple tests for analysis.

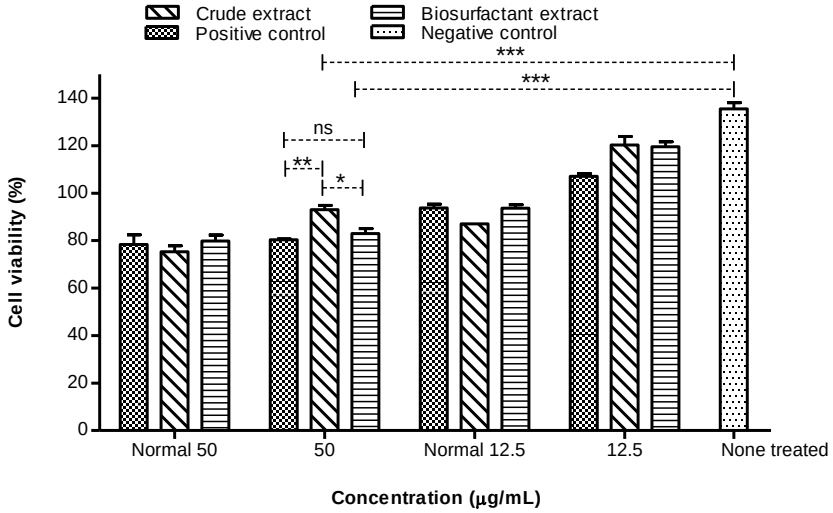

Figure 6: The percentage of cell viability against interleukin-1 beta (IL-1β) using human synovial cell by crude extract and biosurfactant extract from *Bacillus amyloliquefaciens*, methotrexate as a positive control with concentration normal 50 (normal cell without IL-1β), 50, normal 12.5 (normal cell without IL-1β) and 12.5 µg/mL. Value are means ± SEM (n=3); ns=p>0.05, **p<0.05 and ***p<0.001; One-way ANOVA was performed, followed by Tukey's multiple tests for analysis.

death experienced was caused by TNF-α activity. Hence, suppressing the activity of TNF-α in this study were chosen to reduce cell death. Figure 5 indicated that the treatment with both exracts could reduce cell apoptosis. Crude and biosurfactant extracts at concentration 50 and 12.5 µg/mL shows a positive reaction against TNF-α thattheir percentage of cell viability was up between 75% to 93%. Lipopolysaccharides (LPS), also known as endotoxin responsible for the production of TNF-α in the inflammatory study. Studies conducted by Hwang *et al.* (2007) show that surfactin treatment significantly suppressed the production of TNF-α by neutralizing the activities of LPS in the molecular study. The ability of surfactin to reduce TNF-α activities in line with the present study that shows there is no significant difference at 50 µg/mL treatment of biosurfactant extract with the positive control, MTX. At this concentration, biosurfactant extract that consists of surfactin is able to neutralize cell apoptosis with a percentage cell viability of up to 60%. However, the crude extract only has moderate activity against TNF-α with a percentage cell viability of 54%.

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Interleukin-1β (IL-1β)

Surfactin has cytotoxicity effects against tumor cell lines, including human T cell leukemia Jurkat cells with IC_{50} value 1.45 µg/mL and have non-toxicity effects against normal cells with concentration up to 30 µg/mL after 24 h (Takahashi *et al.*, 2006). These, similar with the present study, shows that there is no significance difference between MTX and biosurfactant activities at concentration 50 µg/mL. Biosurfactant extracts able to inhibit the cell proliferation in the induced IL-1β synovial cell to 84% percentage of cell viability compared to none treated cells were proliferated up to 135% percentage of cell viability (Figure 6).

CONCLUSION

Bacillus sp. is a new potential bacteria in the anti-inflammatory study. *B. amyloliquefaciens* were extracted from their biosurfactant. TLC profiling reveals there is the presence of lipopeptides surfactin in the biosurfactant extract. Biological assay screening against AChE and LO enzymatic inhibitory assay shows that biosurfactant extract suppressed both inhibitory enzymes better than the crude extract. The activities of both extracts at the cell proliferation were dose-dependent manner against induced synovial induced with TNF-α and interleukin-1β. Biosurfactant extract shows more potent activity compare to the crude extract in cell proliferation assay at a concentration 50 µg/mL. The activity of TNF-α was neutralized and cell apoptosis has been reduced as the percentage of cell viability was increased. Interestingly, the biological activity of biosurfactant extract also actively suppresses the abnormal of cell growth in IL-1β cell-induced. The percentage of cell viability was reduced to normal. Hence, the active inflammatory activities in this study are believed to be due to the presence of surfactin in both biosurfactant extract and crude extract. Further study is needed to elucidate the mechanism underlying the anti-inflammatory effects of surfactin.

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