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EFFECTS OF *Justicia gendarussa* ETHANOLIC EXTRACT ON OSTEOBLASTIC ACTIVITY OF MC3T3-E1 CELL

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Graphical abstract

Abstract

Justicia gendarussa (Acanthaceae) or commonly known as Gendarussa has traditionally been used to treat bone fractures. Bone fracture is a clinical condition that need bone repair and new bone formation. To date, the mechanism of *Justicia gendarussa* acting in enhancing the bone mineralization has not been proven scientifically. The present study aimed to investigate the cytotoxicity and alkaline phosphatase (ALP) activity on osteoblast cells when treated with *Justicia gendarussa* ethanolic leaves extract. MTT and ALP assays were performed on osteoblast cells after being treated with different concentrations of the extract. For cell viability, the result showed that IC_{50} value of the osteoblast cells was 89.1µg/ml. While, ALP assay is used as a biochemical marker for early detection of osteoblast mineralization. The highest amount of ALP activity was at the 37.5 µg/ml when compared to the control. From this study, it shows that *Justicia gendarussa* has potential in enhancing bone mineralization during the bone repair process.

Keywords: Bone mineralization, *Justicia gendarussa*, osteoblast, MTT assay, ALP assay

Abstrak

Justicia gendarussa (Acanthaceae) atau lebih dikenali sebagai Gendarussa telah digunakan secara tradisional untuk merawat tulang patah. Tulang patah adalah keadaan klinikal yang memerlukan penyembuhan dan pembentukan tulang baru. Sehingga kini, mekanisma bagi *Justicia gendarussa* meningkatkan pemineralan tulang masih belum dibuktikan secara saintifik. Kajian ini bertujuan untuk mengkaji kesan sitotoksik (MTT) dan aktiviti 'alkaline phosphatase' (ALP) terhadap sel-sel osteoblast setelah dirawat dengan ekstrak ethanol yang diperolehi dari daun *Justicia gendarussa*. Asai MTT dan ALP telah dijalankan dengan menggunakan sel osteoblast selepas dirawat dengan ekstrak yang berbeza kepekatannya. Asai MTT digunakan untuk kemandirian sel hidup dan hasilnya menunjukkan bahawa nilai IC_{50} untuk sel-sel osteoblast adalah 89.1 µg/ml. Manakala, asai ALP digunakan sebagai penanda biokimia untuk pengesanan awal pemineralan osteoblast. Jumlah tertinggi aktiviti ALP adalah pada 37.5 µg/ml dan berbanding dengan sel osteoblast yang tidak dirawat dengan esktrak, aktiviti ALP tinggi pada kepekatan ini. Kesimpulannya, *Justicia gendarussa* berpotensi dalam meningkatkan pemineralan tulang semasa proses pemulihan.

Kata kunci: Pemineralan tulang, *Justicia gendarussa,* osteoblast, MTT asai, ALP asai

1.0 INTRODUCTION

Justicia gendarussa is belongs to the family of Acanthaceae and commonly known as Gendarussa [1]. It is found in tropical and subtropical throughout Asian countries like India, Malaysia, Indonesia and Sri Lanka [2]. Phytochemical research on the leaves of this plant revealed that alkaloids, amino acids, aromatic amines, flavonoids, triterpinoidal, saponins [1] tannins, justicin and steroids are present in this plant [3]. This plant has been used traditionally to treat chronic rheumatism, inflammations, bronchitis, vaginal discharges, dyspepsia, eye diseases, muscle pain, lumbago, headache, earache, hemiplegia, hair growth promotion, leucoderma, asthma, antiseptic, haemostatic, nasal bleeding, injuries and fever [3-10]. Previous researches have shown that this plant has antimicrobial activity, antihelminth activity, larvacidal and adulticidal activities, in vitro HIV type 1 reverse transcriptase inhibitory activity, antisickling activity on sickle cell, antinociceptive activity, antiangiogenic effect, anti-inflammatory and analgesic activity, antioxidant and hepatoprotective potential and anti-arthritic potential [3,11-21]. However, *J. gendarussa* only has been used traditionally to cure bone fracture [6].

Bone is the body framework and also a structural support for an individual. Bone is able to regenerate and form new osseous tissue whenever the resorption process occur in the bone. Bone fracture is one of the clinical condition that caused either by trauma like accidents and few diseases like osteoporosis, tumor and deficiency of calcium or vitamin D [22]. It can occurs in any type of bones in the body and may cause swelling, protruding bone or blood under skin, numbness, extreme pain, tenderness and paralysis. Basically, there are two types of bone fracture which are hairline or simple fracture and compound fracture. Simple fracture occurs when the skin is still intact in the fractured area while, compound fracture occurs when the skin breaks open due to the broken bones [22]. The treatment for bone fracture can be done by surgical like implant fixation and nonsurgical like usage of drugs [23]. Although there are number of treatment, the healing of bone fracture usually takes a longer period, ranging from six to eight weeks [24].

Traditional herbal medicines which have been used in medical practice might play important role in bone fracture healing [25]. To our knowledge, there is no experimental evidence to support on the mechanism of *J. gendarussa* acting on osteoblast cells for detecting the mineralization process. Thus, the aim of this study is to investigate the cytotoxicity and alkaline phosphatase (ALP) activity of osteoblast cells when treated with ethanolic leaves extract of *J. gendarussa*.

2.0 EXPERIMENTAL

2.1 Plant Materials

J.gendarussa plants were bought from Nursery Pak Ali in Skudai, Johor, Malaysia. These plants were raised in pots containing soil under greenhouse conditions at Faculty of Biosciences and Medical Engineering, UTM.

2.2 Extraction

The young and green leaves of *J.gendarussa* plants were collected and cleaned thoroughly under running tap water. The leaves were cut into small pieces, air dried and powdered. The powdered leaves were macerated with 96% ethanol for three days at room temperature [26]. The liquid extract was filtered using Advantec filter paper (ADVANTEC Toyo Roshi International, Inc., Dublin, CA, USA) and evaporated under reduced pressure using a rotary evaporator (EYELA N-1110, EYELA, Tokyo, Japan) and the procedure above was repeated few times. The sample yields a gummy ethanol extract. The sample was dried in freeze dryer (Beta 2-4 LD plus LT, Martin Christ, Germany) until complete dryness and yield powder form [27].

2.3 Materials

Minimum Essential Media alpha medium (α-MEM), fetal bovine serum (FBS), trypsin and penicillin/streptomycin were purchased from Gibco Company (Bio-Diagnostic Sdn Bhd, Petaling Jaya, Selangor, Malaysia), 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) from Sigma-Aldrich Group (Medigene Sdn Bhd, Puchong, Selangor, Malaysia) and ethanol (96%, Qrec, Malaysia)

2.4 Cell Cultures

The mouse osteoblast cell line (MC3T3-E1) was purchased from American Type Culture Collection (ATCC). MC3T3-E1 cells were cultured in alpha minimal essential medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with mixture of 95% air and 5% CO_2 at 37°C. The medium was changed every 3 to 5 days prior to use [28].

2.5 MTT Assay

Cell optimization was done using different cell densities (2×10^3–1×10^5). Based on the result, 1×10^4 cells/ml seeding density were selected for MC3T3-E1 cell and seeded in 96-well plate [29]. After 24 hours of incubation, the cells were treated with different concentrations of *J. gendarussa* ethanolic leaves extract (7.81–1000 μg/mL) and incubated for 72 hours. MTT (3-(4,5-dimethylthiazol-2,5-diphenyl

tetrazolium bromide) was dissolved in phosphate buffer saline at 5 mg/mL. 20 µL of the solution was added to each well and the plate was incubated at 37°C for 4 hours. Then, medium was discarded and 225 µL of 1N HCl/isopropanol buffer was added to each wells to dissolve the purple crystals [1]. The plate was read at the wavelength of 560 nm using Promega GloMax microplate reader (Promega, Wisconsin, USA) [30]. The results were recorded as percentage of cell viability using the formula given as below [31] .

$$\% \text{ Cell viability} = \frac{(\text{Abs of test} - \text{Abs of blank})}{(\text{Abs of control} - \text{Abs of blank})} \times 100$$

Where, Abs = Absorbance

2.6 Alkaline Phosphatase (ALP) Assay

Cell optimization was done using different cell densities (4×10^4–1×10^5). From the result, 4×10^4 cells/mL seeding density were chosen for MC3T3-E1 cell and seeded in 24-well plate. After 24 hours of incubation, cells were treated with different concentrations of *J. gendarussa* ethanolic extract (9.38–15 µg/mL) for 72 hours. Then, the cells were washed once with phosphate buffer saline and lysed in 0.5 mL of 0.5% Triton X-100 in each well for 30 minutes at room temperature. The lysate were centrifuged at 3000 rpm for 10 minutes and the supernatant were used for the determination of ALP. ALP activity was assayed with a QuantiChrom™ Alkaline Phosphatase Assay Kit (DALP-250; BioAssay System, CA) according to manufacturer's protocol. Optical density was determined at 405 nm by using a BioTek microplate reader (BioTek Instruments, Winooski, VT, USA) [32].

2.7 Statistical Analysis

The results expressed as a mean±standard deviation (SD) from triplicate values. SPSS16.0 software (SPSS 16.0 for Windows Evaluation Version software, SPSS Inc., USA) was used to analyze the data. The normality of the data was determined using the Shapiro-Wilk test. The statistical significance was evaluated using an independent *t*-test for normal data while the Mann - Whitney test was used for non-normal data. A *p*-value less than 0.05 were considered significant [1].

3.0 RESULTS AND DISCUSSION

In this study, cytotoxicity and ALP activity of *J.gendarussa* ethanolic leaves extract were evaluated using MTT and ALP assay. MTT assay is the colorimetric method in which yellow MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to form purple formazan crystal [33].

Based on Table 1, the percentage of viability significantly decreases with the increasing of *J. gendarussa* ethanolic leaves extract concentration. The result indicates that the cytotoxic effect increases with the *J. gendarussa* ethanolic extract concentration. IC50 value is a 50 % reduction in viability of cells [1] and it is determined by plotting a line graph of percentage of viability against Log (concentration of extract) [31]. Using the graph, the Log value of the 50 % of cell viability is determined and antilog of that value gives the exact IC_{50} value [31]. The IC_{50} value of *J. gendarussa* towards MC3T3-E1 cells is 89.1 µg/ml. Previous study stated that, IC_{50} value below than 20 µg/ml is toxic to the cell, ranging between 21- 40 µg/ml is less toxic and not toxic if the IC_{50} value is above than 41 µg/ml [1]. The ethanolic extract of *J.gendarussa* is considered not toxic to the osteoblast cell since the IC_{50} value is above 41 µg/ml.

Table 1 Percentage of cell viability of MC3T3-E1 cells treated with different concentrations of *J gendarussa* ethanolic leaves extract

Concentration (µg/ml)	Control	7.81	15.63	31.25	62.5	125	250	500	1000
		***	**	***	***	*	***	***	*
% of cell viability	100 ±0.008	72.71 ±0.001	70.94 ±0.009	64.99 ±0.009	61.29 ±0.001	40.96 ±0.005	26.34 ±0.009	16.02 ±0.318	8.68 ±0.012
IC_{50} (µg/ml)					89.1				

Values are mean ± STDEV for three replicates; *p<0.05, **p<0.01 and ***p<0.001 compared with control

Figure 1 shows the morphology and confluency of the MC 3T3-E1 cells in different concentration of *J. gendarussa* ethanolic extract. Based on the figure, the confluency of the cells decreases when the concentration of the extract increases. This indicates a concentration dependent manner of the *J.gendarussa* ethanolic extract towards the osteoblasts. Thus, the cells are able to grow and maintain their morphology at lower concentration (500 µg/mL – 7.81 µg/mL). At the highest concentration (1000 µg/mL), morphology of the MC3T3-E1 cells changed into a rounded shape when compared to the control.

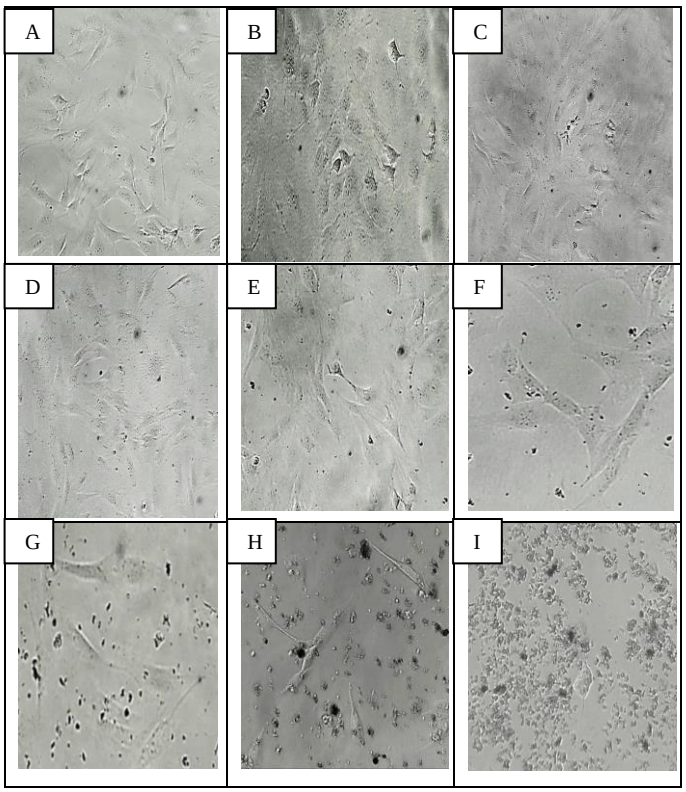

Figure 1 Morphology of MC 3T3-E1 cells after 72 hours treated with different concentration *J. gendarussa* ethanolic extract (200× magnification). (A) control, without any treatment; (B) *J. gendarussa* at 7.81 µg/mL; (C) *J. gendarussa* at 15.63 µg/mL; (D) *J. gendarussa* at 31.25 µg/mL; (E) *J. gendarussa* at 62.5 µg/mL; (F) *J. gendarussa* at 125 µg/mL; (G) *J. gendarussa* at 250 µg/mL; (H) *J. gendarussa* at 500 µg/mL; (I) *J. gendarussa* at 1000 µg/mLl.

The effect of *J. gendarussa* ethanolic extract on ALP activity was expressed in Figure 2. The result showed that ALP activity is significantly higher than control at the concentration of 37.5 µg/mL and 75 µg/mL. This suggests that at lower concentration (below than 100 µg/mL), this plant extract could stimulate the ALP activity in osteoblast cell. While, *J. gendarussa* ethanolic extract treated group at the concentration of 9.38 µg/mL, 18.75 µg/mL and 150 µg/mL showed not much difference in ALP activity when compared with control. This shows that at these concentrations there is no stimulation of ALP activity. The *J. gendarussa* ethanolic extract at the concentration of 150 µg/mL is above the IC_{50} value 89.1 µg/mL which that concentration has more than 50% of inhibited cells growth. The concentration of 150 µg/mL is chosen in this experiment to show that higher ALP is not detected when MC3T3-E1 cells growth are inhibited. As ALP is an early marker which used to detect osteoblast cell differentiation and the ALP activity is elevated when there is increased in osteoblast cell differentiation [34-35]. Differentiation of osteoblast cell to become osteocyte is the final phase of differention, where the osteocyte cells embedded in the mineralized bone matrix and forms bone [36]. From this, we can suggest that *J. gendarussa* can increase osteoblastic differentiation into osteocyte at the a specific concentration. Since early stage is a necessary step in bone mineralization, the enhancing effect of *J. gendarussa* may stimulate the bone fracture healing.

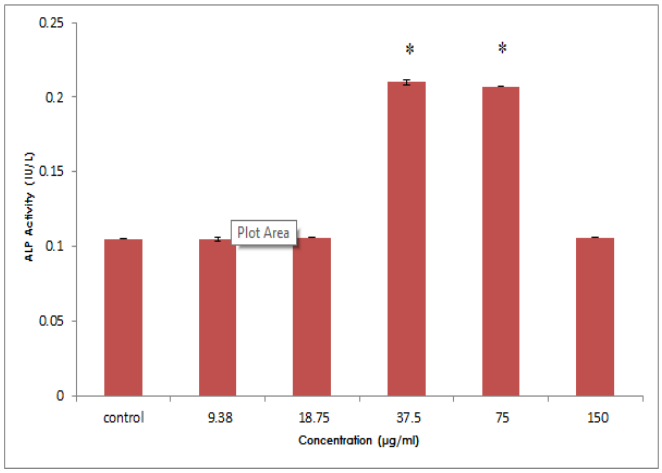

Figure 2 ALP activity of osteoblast cell line treated with different concentrations of *J. gendarussa* ethanolic leaves extract. Values are expressed as the mean ± SD for three replicates, *p<0.05 compared with control

4.0 CONCLUSION

As a conclusion, this study showed that *J.gendarussa* has potential in increasing the ALP activity in osteoblast cells. This suggested that *J. gendarussa* treatment can stimulate early stage of mineralization and thus, helps osteoblast cell differentiation. Therefore, *J. gendarussa* treatment may be favorable for the bone fracture healing, with a potential mechanism of stimulating the ALP activity in osteoblast cell. Further studies are needed to explore the exact mechanism of this plant acting on the osteoblast cells during the bone healing process.

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