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The Effects of *In Vitro* Treatment of *Melastoma Malabathricum* on Fibroblast Proliferation

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Abstract. As the demands in wound care have increased, it is imperative to quickly address this issue at hand. *Melastoma malabathricum* has been claimed to have astounding treatment in wound repair. This study aims to determine its wound healing potentials by assessing and comparing the rate of proliferation of fibroblasts, before and after, treatment of *M. malabathricum'*s methanol and aqueous extracts. The study also aims to compare the potential proliferation of cells between different concentrations of extracts through Methylene Blue Assay (MBA). Results were analyzed through two-way ANOVA using SPSS. The results showed a marked proliferation of cells where 39.3 % and 20.7 % were seen at concentrations A3 and A4 respectively. Concentrations A3 and A4 showed a decrease to 13.2 % and 16.4 % at 48 hours to a negligible proliferation at 72 hours, which may indicate probable impairment in the wound treatment. The methanol extracts revealed a gradual dose-response inhibitory effect throughout all the concentrations, accounting for its toxicity effect on cells. This depicts a time and dose-dependent proliferative effect of *M. malabathricum'*s aqueous extract on fibroblasts, while its methanol extract had a toxic effect on the cells.

Keywords: *Melastoma malabathricum*, Wound healing, Fibroblast

1. Introduction

There has been an increasing usage and acceptance of natural medicinal products worldwide, especially those of developing countries, including Malaysia. Thousands of plant samples representing various species are screened each year for its medicinal properties. *M. malabathricum* are also undergoing scientific analysis in order to identify their potential medicinal properties. *M. malabathricum* is a wildly dispersed shrub with an average height of 1 meter tall that is found mostly in tropical and temperate regions. Various parts of the plant have been used in Malay, Indian, Chinese and Indonesian folklore as medical treatments for many illnesses, including stomachache, wounds and many more [1, 2] but in this study, emphases shall be made in its wound healing properties. Recent findings have shown that the plants possess peripheral and central antinociceptive, wound-healing, anti-inflammatory, cytotoxic, antipyretic, antioxidant and antimicrobial activities [2]. Tripenes, amides and flavanoids have also been found to be present in various parts of the plant [3]. This study is designed in order to analyze the wound healing activities of *M. malabathricum*'s aqueous and methanol extracts through *in-vitro* treatment with fibroblast cells. Serial dilutions of both extracts were treated on fibroblast cell cultures and its rate of proliferation before and after treatment were compared. The aims of the study is to examine the wound healing properties of this plant, in the hope that it will help to further enhance the treatment of wounds in healthcare.

2. Research Methothodology

2.1. Materials and Samples

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M. malabathricum were collected from within International Islamic University Malaysia, Kuantan, Pahang, Malaysia. The samples were dried at 45 °C before extraction. The materials used are as follows: Methanol (Merck, Germany), Dimethyl sulfoxide (DMSO) (Santa Cruz, CA), Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA), Fetal Bovine Serum (FBS) (Invitrogen, USA), penicillin-streptomycin (PenStrep) (GIBCO, USA), Phosphate Buffer Saline (PBS) – magnesium and calcium free (Fisher Scientific; New Hampshire, USA), HEPES solution (GIBCO, USA), sodium pyruvate (Invitrogen, USA), Methylene blue (Fisher Scientific, USA), Hydrochloric acid (HCl) (Fisher Scientific, USA).

2.2. Aqueous and Ethanol Extraction

In aqueous extraction, the leaves were weighted and placed into a beaker filled with distilled water before being heated at 80 °C for 2 hours. The extracts were then stored in -80 °C before being freeze dried. The resulting extracts were diluted with distilled water according to the concentrations needed [100 µg/mL (A1), 50 µg/mL (A2), 25 µg/mL (A3), 12.5 µg/mL (A4)], and kept at 4 °C. For methanol extraction, the grinded leaves were extracted using Soxhlet extractor through methanol extraction process. The resultant solution was then concentrated to dryness by using the rotavaporator at a reduced pressure of 40. Crude extract was then diluted with 1 % DMSO according to the concentration required [100 µg/mL (M1), 50 µg/mL (M2), 25 µg/mL (M3), 12.5 µg/mL (M4)], and stored at 4 °C.

2.3. Cell Culture

L929 mouse fibroblast cells were retrieved from University Sains Malaysia. Cells were kept in T-flask containing DMEM (supplemented with sodium bicarbonate, HEPES buffer, PenStrep, FBS) and incubated at 37 °C in 5 % CO_2 atmosphere. To seed the cells, they were first trypsinized before being transferred into test tubes for centrifugation at 1500 rpm for 15 minutes. The standard seeding density (1.0×10^5 cells/cm^2) was established via cell counting. Cells were then seeded onto the 96-well plates.

2.4. Treatment of Plant Extract

After incubation, the treated plates were washed with PBS before adding PBS (with 2.5% gluteraldehyde) into each well and left for 15 minutes. The solution was then removed and 0.05% methylene blue solution was added and left for 15 minutes. Afterwards, the wells were washed with 0.15M NaCl and 0.33M HCl was added and left for an hour. In order to determine the number of living cells in each well, a microplate reader was used at 650 nm. The percentage cell viability was analyzed using Microsoft Professional Excel 2010. Statistical analysis was performed using two-way ANOVA in SPSS to analyze and compare the data.

3. Results and Discussion

3.1. Proliferation of Fibroblast

Fibroblast proliferation activity was analyzed through Methylene Blue Assay. The percentage of cell viability was also calculated. Results were then compared using two-way ANOVA in SPSS, for significant differences and pair-wise comparisons were generated between groups and between control and concentrations (Table 1 and 2).

Table 1: Light absorbance readings of viable cells in MBA Assay at 24 hrs, 48 hrs and 72 hrs after treatment with different concentrations of *M. malabathricum* methanol and aqueous extract.

Light Absorption of Viable Cells via MBA Assay						
	Mean cell viability ± S.D. (n=3)					
	Methanol Extracts			Aqueous Extract		
	24 Hrs	48 Hrs	72 Hrs	24 Hrs	48 Hrs	72 Hrs
100 µg/mL	0.303 ± 0.047	0.116 ± 0.006	0.420 ± 0.061	0.671 ± 0.036	1.156 ± 0.080	1.047 ± 0.072
50 µg/mL	0.303 ± 0.067	0.176 ± 0.005	0.507 ± 0.068	0.691 ± 0.050	1.188 ± 0.050	1.061 ± 0.026
25 µg/mL	0.544 ± 0.032	0.478 ± 0.065	0.716 ± 0.043	0.731 ± 0.020	1.101 ± 0.014	1.501 ± 0.116
12.5 µg/mL	0.614 ± 0.032	0.581 ± 0.024	0.755 ± 0.070	0.697 ± 0.066	1.056 ± 0.027	1.302 ± 0.051
Control	0.692 ± 0.035	1.021 ± 0.086	1.077 ± 0.070	0.692 ± 0.035	1.021 ± 0.086	1.077 ± 0.070

Note: S.D. = Standard Deviation; Control = Absorbance of cells without treatment.

Table 2: Percentage cell viability of cells at 24 hrs, 48 hrs and 72 hrs after treatment with different concentrations of *M. malabathricum* methanol and aqueous extract.

Percentage of Viable Cells via MBA Assay (%)						
	% Mean cell viability ± S.D. (n=3)					
	Methanol Extract			Aqueous Extract		
	24 Hrs	48 Hrs	72 Hrs	24 Hrs	48 Hrs	72 Hrs
100 µg/mL	-56.4 ± 4.61*	-88.5 ± 1.37*	-61.1 ± 4.17*	2.70 ± 5.18	13.2 ± 7.85*	-2.75 ± 6.73
50 µg/mL	-56.0 ± 10.7*	-83.0 ± 1.29*	-53.1 ± 3.34*	-0.34 ± 7.29	16.4 ± 4.93*	-1.45 ± 2.40
25 µg/mL	-21.2 ± 8.60*	-53.0 ± 7.52*	-33.2 ± 8.09*	0.72 ± 2.94	7.84 ± 1.41	39.3 ± 10.7*
12.5 µg/mL	-11.1 ± 7.79	-42.9 ± 3.98*	-29.5 ± 10.2*	3.08 ± 9.54	3.40 ± 2.69	20.7 ± 4.69*

Note: Methanol extract: the mean differences of comparisons between concentrations are significant at 0.05 level, except for conc. between 12.5µg/mL and 25µg/mL (*P* value=0.09) and 50µg/mL and 100µg/mL (*P* value=0.45). Aqueous extract: the mean difference of 2.5mg/mL in comparison with all other concentrations is significant at the 0.05 level.

*The mean difference of comparison with control (0%) is significant at the 0.05 level.

3.2. Inhibitory Effects of Methanol Extract

In Table 2, it was shown that the methanol extracts did not only inhibit cell proliferation, it also killed 11.1 % to more than 88 % of the cells. Generally, the cells death were relative to the concentration of the extracts, with M4 being the least toxic at -11.1 %, -42.9 % and -29.5 % at 24, 48 and 72 hrs respectively, to -56.4 %, -88.5 % and -61.1 % for M1 ($P < 0.05$). Throughout the 72 hrs time of treatment, it was presented that the toxicity of the extract was short-lived, where after killing 88.5 % of cells at 48 hrs, the cells were 13 to 27 % revived at 72 hrs. The extract was found to be most toxic at 48 hrs after treatment with M1 concentration of extracts (Fig. 1 (A) and Fig. 2 (A)).

Moreover, all three graphs for each time frame showed a similar pattern of regressive dose-dependent response curve, where the percentage cell proliferation generally decrease, or cell death increases with increasing dosage of its methanol extract (Fig. 1 (A) and Fig. 2 (A)). At 24 hrs and 72 hrs, they had similar toxicity level for the percentage cell death values were close, whereas at 48 hrs, the percentage cell deaths were almost 1.5 times more than the other two time frames, for it was most toxic at 48 hrs after treatment.

From the results obtained, methanol extract has shown to generally inhibit cell proliferation. This inhibitory effect follows a dose-response relationship as concentration M1 was found to be most toxic. This could be due to the methanol solvent itself. A previous study reported that methanol extract of *Centaurea Solstitialis L.* on fibroblast cells were effective in killing the cells [4], showing probable toxicity effects of methanol on fibroblast cells. It may also be inferred that the DMSO solvent used to dissolve the extract may intensify its toxic property. Studies have shown varying synergistic effects of DMSO as both inhibitory and stimulating agents [5]. This diverse effect of DMSO proves that it could in fact enhance the inhibitory effects of *M. malabathricum*'s methanol extracts, resulting in an enhanced level of toxicity. Nevertheless, cell inhibition was much reduced from 48 hrs to 72 hrs. This could be due to the acute toxicity of DMSO, as its half-life is between 60 to 96 hours after treatment. This supports the reduction in cell death at 72 hours, where there is an overall increase in cell proliferation, thus reducing the inhibitory effect of methanol extract throughout all the concentrations.

3.3. Proliferative Effects of Aqueous Extract

Overall proliferation of cells was around 40 % of cell growth after 72 hrs of treatment ($P = 0.046$). The first 24 hrs did not exhibit any significant increase in cell proliferation across all concentrations. At 48 hrs after treatment, there was considerable rise for A1 and A2 of aqueous extracts with 13.2% and 16.4% respectively ($P = 0.007$ and 0.026), while at A3 and A4 concentration, cell growth was not statistically significant ($P > 0.05$). For A3 and A4, proliferation increased after 72 hrs of treatment with 39.3% ($P < 0.05$) and 20.7% ($P = 0.021$) respectively. However, for A1 and A2, proliferation had atypically decreased from 13.2 % to -2.75 % and 16.4 % to -1.45 % respectively. There were no statistically significant differences for the concentrations A1 and A2 in comparison with control samples ($P = 0.983$ and 0.998 respectively). As a result, the negative values were not large enough to demonstrate cell inhibition (Fig. 1 (B) and Fig. 2 (B)).

Furthermore, the effects of aqueous extract at different time intervals showed that they had dissimilar pattern and graph shape (Fig. 2 (B)). At 24 hrs, the percentage cell proliferation was almost constant

throughout the concentrations. At 48 hrs, it was presented similar with that of a log-linear curve. The graph at 72 hrs had an initial drastic increase to 40 % cell proliferation at 24 µg/mL, before it dropped to -5 % at 65 µg/mL and were generally constant up to 100 µg/mL.

The results were found to be both dose and time-dependent. For A3 and A4, a gradual increase in the proliferation of cells was observed from 24 hrs to 48 hrs, and significantly increased at 72 hrs. This is relevant with the normal wound healing mechanism, as fibroblasts start to migrate and proliferate from day 3 after injury and reach optimum level on day 7 [6]. Treatment at A3 resulted in a 2-fold increase at 72 hrs, indicating a strong agent for cell proliferation, possibly making it a potential treatment for wound healing. Treatments at concentrations A1 and A2 showed an increase in cell viability from 24 hrs to 48 hrs and then decreased to having negligible cell proliferation at 72 hrs. This could imply that higher concentrations of aqueous extracts triggered impairment in the wound healing process. However, it is difficult to pin-point the exact problem that arose due to the overlapping intricacy of the wound repair system [6, 7].

It is presumed that cell proliferation increased in between 48 to 72 hrs at A1 and A2 caused an increase in thrombin and/or fibrinogen synthesis. In order to counteract the over-formation of thrombin, anti-thrombin (AT) was triggered. Other than neutralizing the enzymes in the clotting cascade, AT also stimulate apoptotic cell death of fibroblasts [7], thus demonstrating a reduction in viability of cells at 72 hrs. There is also the apoptotic effect of the protease inhibitor on fibroblasts. Fibrinogen inhibitors may also contribute in the cell death of fibroblasts, as it serves to block fibrinopeptide release and fibrin monomer polymerization [7]. A report shows of this correlation where three different chains of fibrinogen induce the replication and proliferation of fibroblasts. This proliferative effect was much enhanced when treated together with thrombin [9]. Therefore with the inhibition of fibrinogen, the proliferation of fibroblasts would also be inhibited [7].

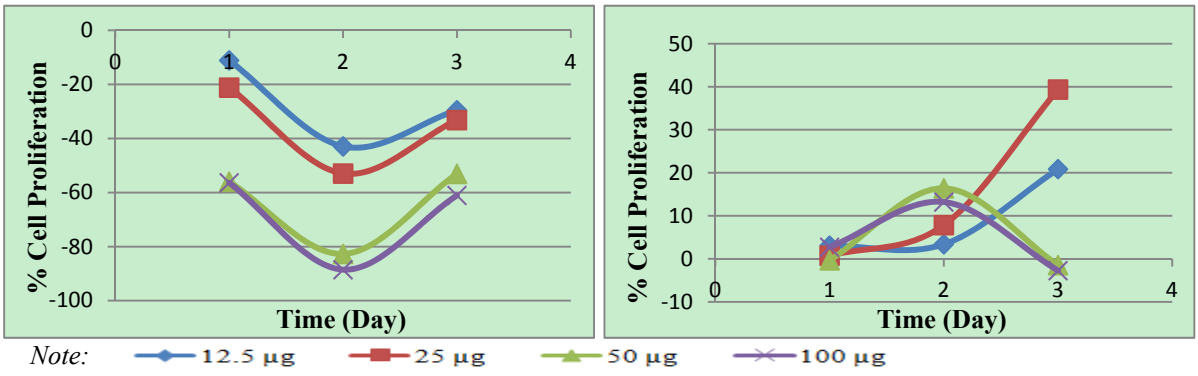

Fig. 1: (A) Methanol extract; and (B) Aqueous extract percentage of cell proliferation with respect to time.

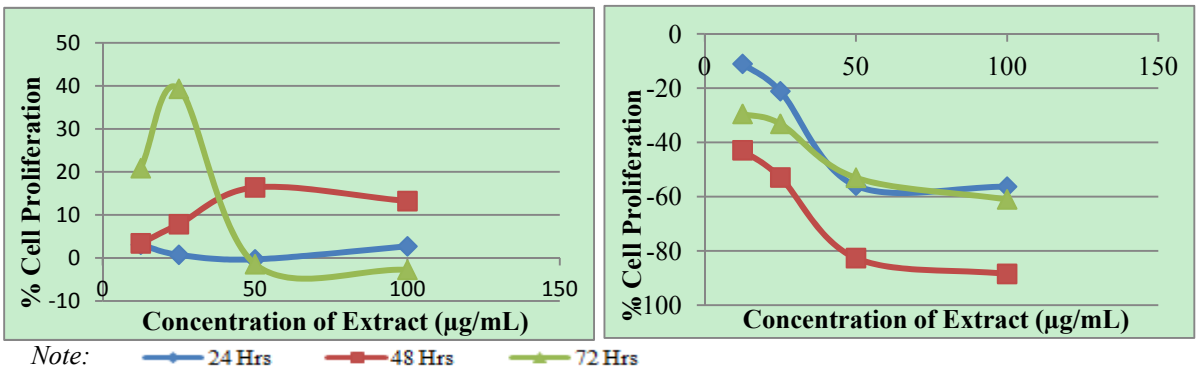

Fig. 2: (A) Methanol extract; and (B) Aqueous extract percentage of cell proliferation with respect to concentration

3.4. Comparison with Other Studies

There has been an extensively increasing research on this plant. However, not much had been conducted on its wound healing potential. In a study where 10-fold dilutions of *M. malabathricum's* aqueous extract were treated on fibroblasts cells, it was revealed that $1.0x10^{-6}$ to $1.0x10^{0}$ µg/mL have a constant proliferative effect of fibroblasts, while at $1.0x10^{1}$ to $1.0x10^{3}$ µg/mL, gradual increase in cell toxicity was observed [10].

This study shows *M. malabathricum's* proliferative effects on fibroblasts. Another study reported a significant response of its crude extract on its wound healing activity via animal testing, and was closely comparable with the standard drug [11]. These findings support wound healing activity of *M. malabathricum* as it has been stated to have higher original tissue regeneration as compared to standard drugs.

4. Conclusion

Potential implications of *M. malabathricum's* wound healing properties were addressed via *in vitro* testing of fibroblast cells with its aqueous and methanol extracts. The results showed that the two extracts have differing outcomes. Aqueous extract was shown to notably stimulate the proliferation of fibroblasts at lower concentrations while methanol extract had an inhibitory effect in a dose-response manner. However, high concentrations of aqueous extract showed an abnormal pattern of cell growth, which might be the resultant of a type of wound healing impairment. Hence, traditional folklores may hold some truth in wound healing activity of *M. malabathricum*, thus further studies need to be conducted for a deeper understanding.

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6. References

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