PRELIMINARY INVESTIGATION OF WOUND-HEALING POTENTIAL OF THE LEAVES AND BARK OF *Ziziphus oenoplia* (L.) Miller

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INTRODUCTION

Plant preparations have been used to enhance wound healing from ancient times. *Ziziphus oenoplia* (L.) Miller (family: Rhamnaceae, 'heen-eraminiya' in Sinhala) has been used in Ayurveda and the indigenous systems of medicine for the treatment of wounds (Nadkarni, 1976). Traditionally, the leaves (Kuvar and Bapat, 2010), bark and root (Rashmi et al., 2018) are the mostly used plant parts in the treatment of wounds. The wound-healing activity of aqueous and alcoholic extracts of the fruits of this plant has been evaluated by *in-vivo* methods (Kuppast and Kumar, 2012). In the present study, the wound-healing potential of the leaf and bark of *Z. oenoplia* was evaluated using scratch wound assay to rationalise the scientific basis for the traditional use of this plant in wound healing.

MATERIALS AND METHODS

Plant material

Z. oenoplia stem bark and leaves were collected from the Dompe area in the Gampaha district (6°56'08.8"N 80°03'03.3"E) and identified by Dr. S. Somaratne. Voucher specimen (NSF/PSF/ICRP/2017/HS02/PT/03) was authenticated and deposited at the National herbarium, Department of Botanical gardens, Peradeniya. Each plant material was chopped and air dried at room temperature (30±2 ºC).

Extraction of plant material

The solvents used for the extraction were distilled at their boiling point at 1 atm. Dried and powdered bark and leaves of *Z. oenoplia* (each 100.0 g) were sequentially extracted with hexanes, dichloromethane, ethyl acetate and methanol (1.0 L each for 24 h × 3) in an orbital shaker (~90 rpm) at room temperature (30±2 °C). Solvents were removed under reduced pressure at 40 °C and each extract was dried in vacuum oven for 2 days and weighed.

Establishment of cell cultures

Madin-Darby Canine Kidney (MDCK: ATCC®CRL-2936) cell cultures were established in the laboratory using standard *in-vitro* procedures. Cells were grown in plastic tissue culture flasks (25 cm^2) in Dulbecco's Modified Eagles Medium (DMEM) containing 10% Fetal Bovine Serum [10% Growth medium (10% GM)] supplemented with antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin). Cultures were maintained at 37 °C in a 5% CO_2 humidified incubator until they reach the confluent stage of the growth.

Scratch wound assay (SWA)

Cells that grow at confluent stage were harvested and inoculated at a cell density of 2×10^4 cells per well in clear bottom 24 well plates in 10% growth medium. Plates were incubated at 37 °C for 24 h in a humidified incubator for the formation of monolayer. Scratch wound assay (SWA) was carried out in 20% DMEM solution, which was prepared in the following procedure. DMEM powder supplemented with high glucose and L-glutamine (Sigma-Aldrich, 2.680 g) was dissolved in 1 L of 2.25% $NaHCO_3$ solution. (This solution contains an amount of DMEM equivalent to 1/5th (20%) of a standard DMEM solution used for cell culturing). At this DMEM concentration,

untreated cells were found to be almost static for 24 h.

A scratch was performed on a monolayer of cells along the vertical axis of each well under the microscope using a sterile micropipette tip (0.5-10 µL). The monolayer with the wound was washed with phosphate buffered saline (PBS) (400 µL × 2). Each test well was filled with 495 µL of 20% DMEM and 5 µL of DMSO containing an appropriate amount of the test sample was added. Two potent wound healing compounds, lupeol (25 µL) and asiaticoside (25 µL) were used as positive controls, and 1% DMSO in 20% DMEM and 20% DMEM were used as negative controls in this experiment. The initial width of each wound was measured. Plates were incubated for 24 h at 37 °C with 5% CO_2. The width of each wound was measured after 24 h of incubation. The percentage wound closure was calculated, and the cell migration enhancement is presented as the percentage wound closure. The plant extracts/fractions, which have shown >75.0% mean wound closure at 24 hours, is considered as wound healing active extracts/fractions.

Fractionation of hexanes extract of Leaf

Hexanes extract of leaves of *Z. oenoplia* (1.00 g) was subjected to column chromatography over a column of silica gel (35.0 g; Merck, 230 - 400 mesh) made up in hexanes and eluted with hexanes containing increasing amount of ethyl acetate and, finally, washed with methanol. Of the 99 fractions (18 mL each) collected, those having similar TLC patterns were combined to give 6 major fractions (F_1–F_6). These 6 fractions were subjected to scratch wound assay at a concentration of 10 mg/L. Fraction F_3 (220 mg) was subjected to column chromatography over a column of silica gel (8.0 g; Fluka, 220 - 440 mesh) made up in hexanes and eluted with hexanes containing increasing amount of dichloromethane and, finally, washed with methanol. Of the 92 fractions (7 mL each) collected, those having similar TLC patterns were combined to give 4 major fractions F_{3A}–F_{3D}. These fractions were subjected to SWA at a concentration of 5 mg/L.

Statistical analysis

The mean percentage wound-closure and the standard deviation were calculated. Mean comparisons were performed using ANOVA and the least significant test (LSD) with significance level of $p \leq 0.05$. All statistical analyses were carried out using the SAS Ver. 9 (1989).

RESULTS AND DISCUSSION

All eight plant extracts were subjected to SWA at a concentration of 20 mg/L and the experiments were carried out in three replicates, and three measurements were taken for each wound. The dry weights of each extract of bark and leaves of *Z. oenoplia* and mean percentage wound closure with standard deviation of those extracts with two negative controls (20% DMEM and 1% DMSO in 20% DMEM) and two positive controls (lupeol: 25 µM and asiaticoside: 25 µM) are shown in **Table 1**.

Table 1: Dry weights of extracts of *Z. oenoplia* and mean percentage of wound closure at 24 hours of those extracts of concentration 20 mg/L, with negative and positive controls

Plant Part	Extract	Dry Weight (g)	Mean % Wound Closure (±SD)*
Bark	Hexanes	0.321	**75.5 (4.3)**^{C}
	CH_2Cl_2	0.876	23.6 (0.9)^{E}
	EtOAc	0.524	12.5 (3.8)^{F}
	MeOH	3.567	20.9 (6.2)^{E}
Leaves	Hexanes	0.935	**78.0 (3.4)**^{C}
	CH_2Cl_2	1.009	**80.1 (5.7)**^{C}
	EtOAc	0.743	**80.9 (2.5)**^{C}
	MeOH	2.786	21.6 (4.3)^{E}

Control Experiments	Mean % Wound Closure (±SD)*
20% DMEM	19.6 (3.0)^{E}
1% DMSO in 20% DMEM	19.5 (2.6)^{E}
Lupeol (25 µM)	86.0 (0.9)^{B}
Asiaticoside (25 µM)	90.2 (1.1)^{A}

* Different letters along the column indicate significant difference at t=1.98 and $p \leq 0.05$ level.

Microscopic images of scratched wounds of monolayer of MDCK Cells at the beginning of and after 24 h of scratch wound assay of active extracts of *Z. oenoplia* and positive and negative controls are shown in **Figure 1**.

Results (**Table 1**) indicated that hexanes, dichloromethane and ethyl acetate extracts of leaves and hexanes extract of the bark of *Z. oenoplia* have a statistically significant effect on enhancing wound healing as the percentage wound closure of MDCK cells upon wound healing assay (WHA) has been increased (> 75.0 % mean wound closure at 24 hours).

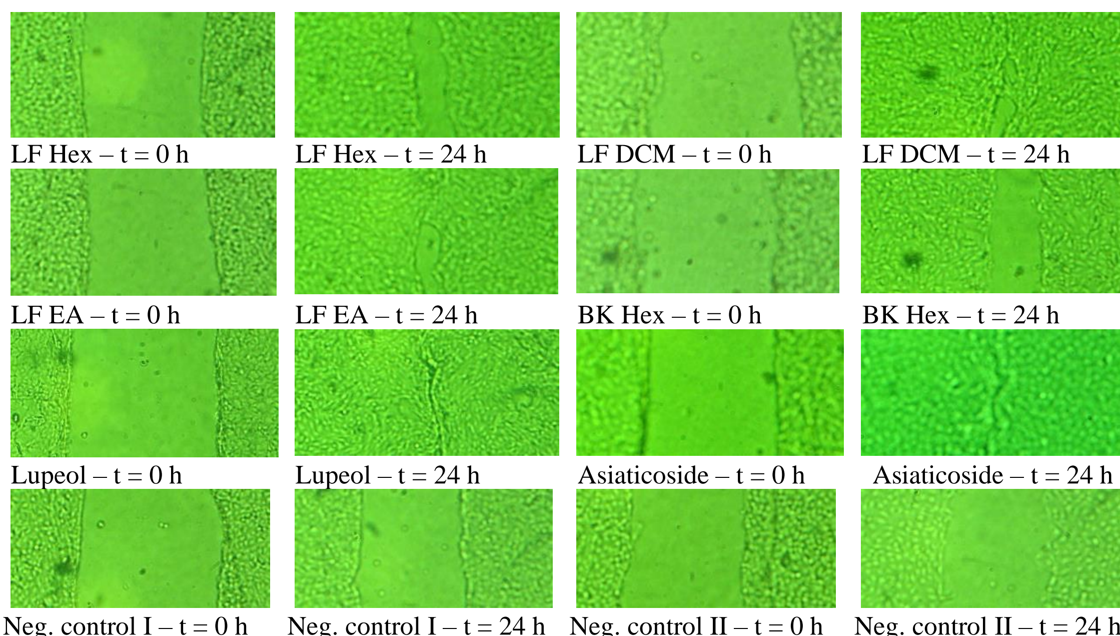

Figure 1: Microscopic images of the scratched wounds of monolayer of MDCK Cells at the beginning of (t = 0 h) and after 24 h (t = 24 h) of scratch wound assay of active extracts of *Z. oenoplia*, and positive and negative controls. LF = leaf, BK = bark, Hex = hexanes extract, DCM = CH_2Cl_2 extract, EA = ethyl acetate extract (×400)

The hexanes extract of leaves of *Z. oenoplia* was subjected to further investigation via bioactivity directed fractionation. The Silica gel column chromatographic separation of hexanes extract gave 6 major fractions that were subjected to SWA at a concentration of 10 mg/L. The experiments were carried out in three replicates, and five measurements were taken for each wound. The yields and percentage wound closure of these fractions, along with the percentage wound closure of positive and negative controls, are given in **Table 2**.

Table 2: Dry weights of column fractions of the hexane extract of leaves of *Z. oenoplia* and the mean percentage of wound closure at 24 hours of those extracts of concentration 10 mg/L, with negative and positive controls.

Fraction No.	Dry Weight (g)	Mean % Wound Closure (±SD)*
F_1	80.0	49.1 (0.6)^F
F_2	274.7	72.4 (1.0)^D
F_3	**220.0**	**75.8 (0.8)**^{C}
F_4	80.1	62.3 (0.5)^E
F_5	55.4	36.4 (1.2)^G
F_6	92.3	49.1 (2.8)^F

Control Experiments	Mean % Wound Closure (±SD)*
20% DMEM	26.1 (1.0)^H
1% DMSO in 20% DMEM	25.9 (1.6)^H
Lupeol (25 µM)	85.9 (1.5)^B
Asiaticoside (25 µM)	89.5 (0.4)^A

* Different letters along the column indicate significant difference at t =1.98 and p ≤ 0.05 level.

Results indicated that the fraction F_3 to be the most active fraction (75.8% wound closure). Hence, further fractionation of fraction F_3 was carried out. The Silica gel column chromatographic separation of fraction F_3 yielded 4 major fractions. They were subjected to SWA at a concentration of 5 mg/L. All the experiments were carried out in three replicates and five measurements were taken for each wound. The yields and percentage wound closure of these fractions, along with the percentage wound closure of positive and negative controls, are given in **Table 3**.

Table 3: Dry weights of column fractions of Fraction F_3 and the mean percentage of wound closure at 24 hours of those extracts of 5 mg/L concentration, with negative and positive controls.

Fraction No.	Dry Weight (g)	Mean % Wound Closure (±SD)*
F_{3A}	53.3	56.9 (1.5)^E
F_{3B}	34.2	73.1 (1.2)^C
F_{3C}	**42.7**	**86.4 (0.9)^B**
F_{3D}	55.7	68.4 (0.6)^D

Control Experiments	Mean % Wound Closure (±SD)*
20% DMEM	19.6 (0.3)^F
1% DMSO in 20% DMEM	20.1 (1.5)^F
Lupeol (25 µM)	90.4 (0.9)^A

* Different letters along the column indicate significant difference at t = 1.98 and $p \leq 0.05$ level.

The results indicated that the fraction F_{3C} was the most active fraction (86.4% wound closure) and it was found to contain lupeol as the major compound by TLC and Co-TLC.

CONCLUSIONS

It is evident that the hexane extracts of bark, and the hexanes, dichloromethane and ethyl acetate extracts of leaves of *Z. oenoplia*, exhibit wound healing potential. The wound healing potential of the hexanes extract of leaves may be mainly due to lupeol, which is to be confirmed. This study provides scientific proof of the traditional knowledge of using the leaf of *Z. oenoplia* for wound healing. Further investigation of these extracts to identify wound healing active constituents is underway.

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