J.Natn.Sci.Foundation Sri Lanka 2023 51 (2): 327 - 340
DOI: http://dx.doi.org/10.4038/jnsfsr.v51i2.11232

RESEARCH ARTICLE

Natural Products

In-vitro wound healing potential of *Ziziphus oenoplia* (L.) Miller

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Submitted: 15 June 2022; Revised: 22 November 2022; Accepted: 27 January 2023

Abstract: Non-healing wounds are a global health problem. Substances that enhance cell proliferation, angiogenesis, and prevention of bacterial infections accelerate the wound healing process. In this study, the wound healing potential of *Ziziphus oenoplia*, is investigated for its ability in cell proliferation, angiogenesis, and antibacterial potential. The potential of cell proliferation enhancement (mean percent wound closure) and angiogenic response (mean vascular index) of hexanes, dichloromethane, ethyl acetate, and methanol extracts of leaf and bark of *Z. oenoplia* were evaluated by scratch wound assay (SWA) using Madin-Darby Canine Kidney (MDCK) cells and chick chorioallantoic membrane (CAM) assay, respectively. The antibacterial activity of these extracts was evaluated against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa* by disc diffusion method. Enhanced cell proliferation was shown by the hexanes, dichloromethane, and ethyl acetate extracts of leaf and the hexanes extract of bark. An enhanced angiogenic response was shown by the methanol and ethyl acetate extracts of leaves and the methanol and hexanes extracts of bark. Dichloromethane extract of both leaf and bark showed considerable antibacterial activity against *P. aeruginosa* which is less susceptible to common antibiotics. SWA-directed fractionation of the hexanes extract of the leaf has resulted in the isolation and identification of an active fraction showing mean percent wound closure of 86.4% (positive control 90.2%) and mean vascular index of 34.5 (positive control 48.6). This fraction contained lupeol, *α*-amyrin, *β*-amyrin, hexacosanol, and octacosanol. The present study provides supportive evidence for the potential of wound healing enhancement of leaf and bark extracts of *Z. oenoplia*.

Keywords: Antibacterial assay, chick chorioallantoic membrane (CAM) assay, Rhamnaceae, scratch wound assay (SWA), wound healing, *Ziziphus oenoplia*.

INTRODUCTION

Non-healing wounds are a major health concern in the human population. Impairment of the natural healing process of wounds can result in a serious threat to life. Wounds are physical, chemical, or thermal injuries that result in an opening or breaking in the integrity of the skin (Singh *et al*., 2006) or may also be defined as the disruption of the anatomical and functional integrity of living tissue (Agyare *et al*., 2016). Wound healing is a multifaceted process governed by sequential, yet overlapping phases including hemostasis, inflammation, proliferation, and remodelling (Lindley *et al*., 2016). Cell proliferation is a characteristic event during the proliferative phase (Heil *et al*., 2017) while angiogenesis is a critical process involved in wound healing in order to re-establish the nutrient supply to regenerating tissue (Gerritsen, 2008). The natural wound healing process could also be delayed by microbial infections (Serra *et al*., 2014). It is known that natural products can enhance the wound healing process through multiple cellular mechanisms (Thakur *et al*., 2011). The scratch wound assay (cell migration assay) is a classic and commonly used *in-vitro* method for studying cell proliferation (Yarrow *et al*., 2004). The chick embryo chorioallantoic membrane (CAM) assay is a method for evaluating angiogenic activity which can also be used as a wound healing model (Ribatt *et al*., 1996).

Sri Lanka has rich traditional systems of medicine which have been practiced over 3000 years (Weragoda, 1980). Plants and herbal preparations are most frequently employed as therapeutics in indigenous medicine in Sri Lanka, especially in Ayurveda for curing a wide variety of diseases (Weragoda, 1980). Even though a large number of plants are being used by people in many countries including Sri Lanka for the treatment of wounds and

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burns (Krishnappa *et al*., 2016) most of them are scientifically unexplored for their validity with pharmacological models and human subjects (Kumar *et al*., 2007).

In continuing our studies on plants used for the enhancement of wound healing, (Bopage *et al*., 2018) we initiated a chemical and biological investigation on *Ziziphus oenoplia* (L.) Miller (family: Rhamnaceae), commonly known as the 'jackal jujube' in English and '*Heen Eraminiya*' in Sinhala. Various parts of *Z. oenoplia* have been used as a remedy for an array of illnesses in Ayurveda and in indigenous medicine practiced in Sri Lanka. The leaf and bark of this plant have been used for the treatment of wounds, particularly for fresh cuts (Nadkarni, 1976) while the water boiled with a few medicinal plants including *Z. oenoplia* has been used to wash chronic wounds (Kumarasinghe, 1987). Although it is not reported in literature, rural communities in Sri Lanka use crushed fresh leaves of this plant on fresh cuts on skin as a home remedy. Members of *Z. oenoplia* (L.) Miller belonging to the family Rhamnaceae, and are often trailing or ascending shrubs with curved spines, solitary or in pairs. They are distributed in forests of dry regions in India, Pakistan, Malaysia, Australia, and Sri Lanka (Wadhwa, 1996). An array of biological activities such as analgesic and antinociceptive, anthelmintic, antibacterial, anti-denaturation, antioxidant, hepatoprotective, hypoglycaemic have been shown by different parts of this plant (Shukla *et al*., 2016; Rashmi *et al*., 2018). The wound-healing activity of aqueous and alcoholic extracts of the fruits of this plant has been evaluated by *in-vivo* methods (Kuppast & Kumar, 2012). The angiogenic potential of ethanol extract of root of *Z. oenoplia* has been evaluated using the chorioallantoic membrane (CAM) model (Mahapatra *et al*., 2011) while wound healing potential of several solvent extracts of root have been evaluated by a rat model (Majumder, 2012).

Compounds belonging to different chemical classes have been identified from *Z. oenoplia*. Of these, the cyclopeptide alkaloids belonging to the ziziphine series, having 13–15 membered rings, are the most abundant chemical class. The chemistry of the cyclopeptide alkaloids of genus *Ziziphus* has been reviewed (Tuenter *et al*., 2017). Apart from several ziziphine alkaloids, betulinic acid (Nahar *et al*., 1997), zizyotin, a terpenoid saponin (Maurya *et al*., 1995), *β*-sitosterol, *β*-sitosteryl-*β*-D-glucoside, luteolin, and quercetin have been identified from bark (Singh & Singh, 2012). Several antiplasmodial ziziphine alkaloids (Suksamrarn *et al*., 2005), an antibacterial hydroxy carboxylic acid (Prabhavathi & Vijayalakshmi, 2015), and an aliphatic hydroxy ether (Prabhavathi & Vijayalakshmi, 2016) have been reported from the root of *Z. oenoplia*. Chemical work on leaf of *Z. oenoplia* is scarce. Presence of some volatile constituents from the ethanol extract of leaf of *Z. oenoplia* has been reported (Shyamala & Manikandan, 2019).

Herein we report the *in-vitro* wound healing activity of leaf and bark extracts of *Z. oenoplia* with respect to the results of scratch wound assay (SWA), chick chorioallantoic membrane (CAM) assay and the antibacterial activity of these extracts against Gram positive bacteria, *B. subtilis* and *S. aureus* and Gram negative bacteria, *E. coli* and *P. aeruginosa* using agar disc diffusion method and the identification of a fraction exhibiting cell proliferation by SWA and proangiogenic activity by CAM assay. This fraction was found to be an inseparable mixture consisting of lupeol (**1**), *α*-amyrin (**2**), *β*-amyrin (**3**), hexacosanol (**4**) and octacosanol (**5**) (Figure 1). Their identities were confirmed by GC-MS analysis and ^{1}H-NMR and ^{13}C-NMR spectroscopic techniques. It is noteworthy that this constitutes the first report of compounds **2**–**5** from *Z. oenoplia*.

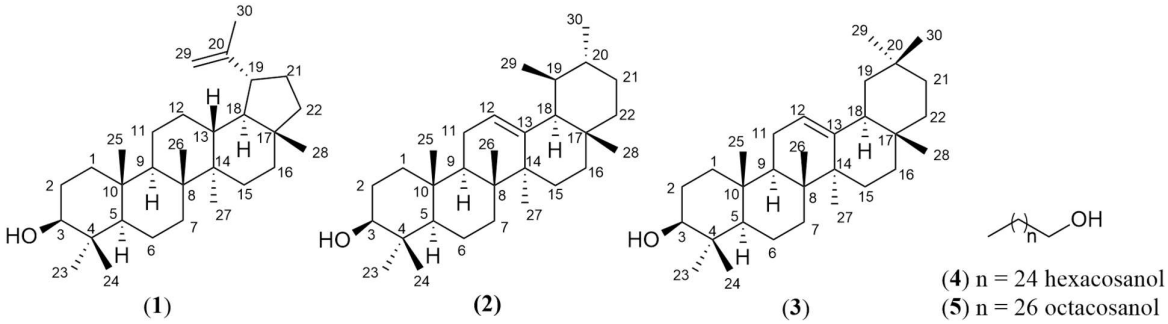

Figure 1. Chemical structures of lupeol **(1)**, *α*-amyrin **(2)**, *β*-amyrin **(3)**, hexacosanol **(4)** and octacosanol **(5)**

MATERIALS AND METHODS

General

Chemicals and reagents were purchased from Merck, Sigma-Aldrich, Fluka, Himedia and Invitrogen. Silica gel 60 G (Fluka−60741) was used as solid phase, for gravity columns. Analytical Thin Layer Chromatography (TLC) was performed on pre-coated 0.25 mm thick plates of silica gel 60 F_{254} (Merck). Preparative TLCs were carried out on glass plates (20 × 20 cm) coated with a 1:1 mixture of silica gel 60 G (Fluka 60760) and silica gel 60 GF_{254} (Fluka 60765), 0.5 mm thickness. TLC plates were visualized by UV-illumination (254 nm and 365 nm) and spraying with anisaldehyde-sulfuric acid reagent which was made by mixing anisaldehyde (0.5 mL) with glacial acetic acid (10 mL), followed by 85 mL of methanol and concentrated sulfuric acid (5 mL). Column fractions having similar TLC patterns were combined and evaporated under reduced pressure. 1D and 2D NMR spectra were recorded in $CDCl_3$ with a Bruker Ascend 400 spectrometer at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR using residual $CHCl_3$ as the internal reference. GC-MS analysis was carried out using an Agilent 7890 A GC system equipped with 5975C inert XL MSD Triple-Axis Detector, and HP-5 MS fused silica capillary column with (5% Phenyl)-methylpolysiloxane stationary phase (30 m × 0.25 mm Id, × 0.25 µm film thickness). Helium (99.999%) was used as the carrier gas. Mass spectra were acquired in the EI mode at 70 eV. MDCK cell line was purchased from American Type Culture Collection (ATCC®), Manassas, Virginia, USA. Fertilized chicken eggs were purchased from Three Acre Farms PLC, Colombo 15.

Plant material

Fresh leaf and bark of *Ziziphus oenoplia* (L.) Miller was collected from Dompe area in Gampaha District (Sri Lanka, 6°56'08.8"N 80°03'03.3"E) and identified by one of the authors, Dr S. Somaratna. The voucher specimen, NSF/PSF/ICRP/2017/HS02/PT/03 was authenticated and deposited at the National Herbarium, Department of National Botanical Gardens, Peradeniya, Sri Lanka. The plant materials were chopped, and air dried at room temperature (30 °C) for the extraction.

Extraction

Both leaf (LF) and bark (BK) of *Z. oenoplia* (100 g each) were extracted sequentially with hexanes, dichloromethane, ethyl acetate, and methanol (1.0 L each for 24 h × 3) in an orbital shaker (~90 rpm) at 30±2 °C. The solvents were removed under reduced pressure at < 35 °C using a rotary evaporator and each extract was dried in a vacuum oven at 30±2 °C for 2 days and weighed.

Cell culture

Madin-Darby Canine Kidney (MDCK: ATCC®CRL-2936) cell cultures were established in the laboratory using standard *in-vitro* methods. The 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) supplemented with antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin). MDCK cell culture was maintained at 37 °C in a 5% CO_2 humidified incubator until they reached the confluent stage of the growth.

Scratch wound assay (SWA)

The cells which grow at confluent stage were harvested and inoculated in clear bottom 24 well plates at a cell density of 2×10^4 cells/well in 10% growth medium. The well 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 (amount of DMEM in this solution is equivalent to 1/5th of a standard DMEM solution) which was prepared by 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. 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 (LABOMED TCM400™) using a sterile micropipette tip. The monolayer of cells with the wound (scratch) 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 such that its final concentration is 20 mg/L for the extracts and 10 or 5 mg/L for the fractions. A potent wound healing compound, asiaticoside (25 µM) was used as the positive control (Lee *et al*., 2012), and 1% DMSO in 20% DMEM was used as the negative control in this experiment. Initial width of each wound was measured. The well plates were incubated for 24 h at 37 °C with 5% CO_2. Width of each wound was measured after 24 h of incubation period. Both stages of each wound were photographed under inverted tissue culture microscope equipped with a digital camera (SONY CORP.DSC-W210). Percent wound closure was calculated, and the cell proliferation enhancement is presented as the mean percent wound closure. Plant extracts which have shown >75.0% mean wound closures at 24 h are considered as wound healing active extracts.

Chick chorioallantoic membrane (CAM) assay

The chick embryo chorioallantoic membrane (CAM) assay was performed according to Talekar and co-workers (Talekar *et al*., 2017) with a few modifications as described below. The surface of freshly laid fertilized chicken eggs was wiped with 70% ethanol and incubated at 37 °C in 60% humidity in an egg incubator for 9 days with continuous monitoring. On the 9th day, vascularized regions were identified using an egg Candler (Life Basis, LSEC001BK). A small window (1 cm^2) was made in the shell above the vascularized region. Each of the plant extracts (50 µg in 10 µL DMSO) and active fractions in SWA (10 µg in 10 µL DMSO) was introduced into a sterile Whatman® No. 01 filter paper disc (diameter, 6 mm) and placed on the chicken chorioallantoic membrane in each egg. Filter paper disc containing *β*-sitosterol (10 µg in 10 µL DMSO) served as the positive control while filter paper disc containing 10 µL of DMSO served as the negative control (Moon *et al*., 1999). The windows were sealed with parafilm® and incubated for further 72 h. On the 12th day of incubation, the windows were opened and the surrounding CAM area with the paper disc was photographed with a digital camera (SONY CORP.DSC-W210). The CAM assay for each plant extract and for the controls was performed in 05 replicates. Vascular index of each CAM for different extracts and controls was determined and the angiogenic response is expressed as the mean vascular index (Barnhill & Ryan, 1983).

Antibacterial assay: Kirby-Bauer disk diffusion method

The anti-bacterial activity of each plant extract was tested against two Gram positive bacterial strains; *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923), and two Gram negative bacterial strains, *Escherichia coli*, (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) (Bacterial culture collection, Department of Microbiology, Faculty of Medicine, University of Colombo, Sri Lanka) using disk diffusion method in Petri plates (diameter, 9 cm). The pure bacterial cultures of each bacterial strain were grown on nutrient agar (NA) (Himedia- M001) plates and incubated at 37 °C during the study period.

Each culture was made into a suspension in sterilized distilled water up to a certain density, which was measured as a predetermined optical density (OD value); 0.05 OD at the wavelength (λ) = 500 nm for *Bacillus subtilis*, 0.1 OD at λ = 500 nm for *E. coli, S. aureus and P. aeruginosa.* From each suspension 100 µL aliquots were transferred to different nutrient agar plates and spread using a sterilized glass spreader to prepare a uniform lawn of each bacterial species under study.

The discs containing plant extracts for the antibacterial assay were prepared by impregnating 500 µg of each plant extract dissolved in 10 µL DMSO into sterile Whatman® No. 3 filter paper discs (diameter, 6 mm). The discs containing 10 µg of Amoxycillin (Himedia) served as the positive control (Pommerville & Alcamo, 2004) while sterile Whatman® No. 3 filter paper discs (diameter, 6 mm) treated with 10 µL of DMSO served as the negative control in this experiment. Two discs containing same plant extract, a disc of positive control and a disc of negative control were placed on each nutrient agar plate and were incubated at 37 °C for 24 h. Experiments were performed in triplicate for each extract. The diameter of the inhibition zone was measured, and the results are expressed as the mean diameter of inhibition zone with the standard deviation.

Bioactivity directed chemical investigation of hexanes extract of leaf of *Z. oenoplia*

Hexanes extract of leaf 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. The column was eluted with hexanes containing increasing amounts 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 (HF_1–HF_6). Each fraction was subjected to scratch wound assay at a concentration of 10 mg/L. The fraction HF_3 (220 mg) which showed the highest mean wound closure (75.8%) in SWA, was subjected to further fractionation over a column of silica gel (8.0 g; Fluka, 220–440 mesh) made up in hexanes. The column was eluted with hexanes containing increasing amounts of dichloromethane and, finally, washed with methanol. Of the 92 fractions (7 mL each) collected, those having similar TLC patterns were combined to yield 4 major fractions (HF_{3A}–HF_{3D}). Each fraction was subjected to SWA at a concentration of 5 mg/L. The fraction HF_{3C} (42 mg) which showed the highest mean wound closure (75.8%) was subjected to preparative TLC (20 × 20 cm^2, thickness 0.50 mm, eluent: CH_2Cl_2) and the prominent band appeared on TLC plate was scraped off and eluted with 10% MeOH in CH_2Cl_2 to yield of HF_{3CA} (24.6 mg).

Statistical analysis

Results of all experiments are presented as mean ± standard deviation. The mean comparisons were performed using ANOVA and the Least Significant Difference (LSD) with significance level of $p \leq 0.05$. All statistical analyses were carried out using the SAS® Ver. 9.00 (2002) (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Dry weights of extracts with their percentage yields, are given in Table 1.

Table 1: Dry weights and percentage yields of extracts of *Z. oenoplia*

Plant part	Weight /g (% yield) of solvent extract			
	Hexane	Dichloromethane	Ethyl acetate	Methanol
Leaf	0.935 (0.935)	1.009 (1.009)	0.743 (0.743)	2.786 (2.786)
Bark	0.321 (0.321)	0.876 (0.876)	0.524 (0.524)	3.567 (3.567)

Scratch wound assay (SWA)

The scratch wound assay (SWA) has been established as a simple and low-cost tool to obtain first insight into whether plant preparations or their secondary metabolites can enhance the formation of new tissue by unidirectional cell migration (Liang *et al*., 2007). During the scratch wound assay, scratched cell monolayer responds to the disturbance of cell-cell contacts by increasing the concentration of growth factors and cytokines at the wound edge (Lipton *et al*., 1971).

Microscopic images (×400) of scratched wounds of monolayer of MDCK cells treated with extracts of *Z. oenoplia*, asiaticoside (25 μM) (positive control), and 1% DMSO in 20% DMEM (negative control) at the beginning and after 24 h of the experiment are shown in Figure 2.

The wound healing activity of *Z. oenoplia* extracts is expressed in terms of mean percent wound closure within 24 h of incubation period. It is evident that the hexanes, dichloromethane and ethyl acetate extracts of leaf and the hexanes extract of bark have shown a significantly high wound closure within the specified time (Figure 3 and Supplementary Table S1). The wound healing activity shown by these extracts is almost comparable with that of positive control, a well-established wound healing compound. Because, all extracts, except the methanol extract, are highly active in SWA, it is suggested that in terms of cell proliferation, the leaf of *Z. oenoplia* consists of an array of secondary metabolites with varying polarities which assist wound healing.

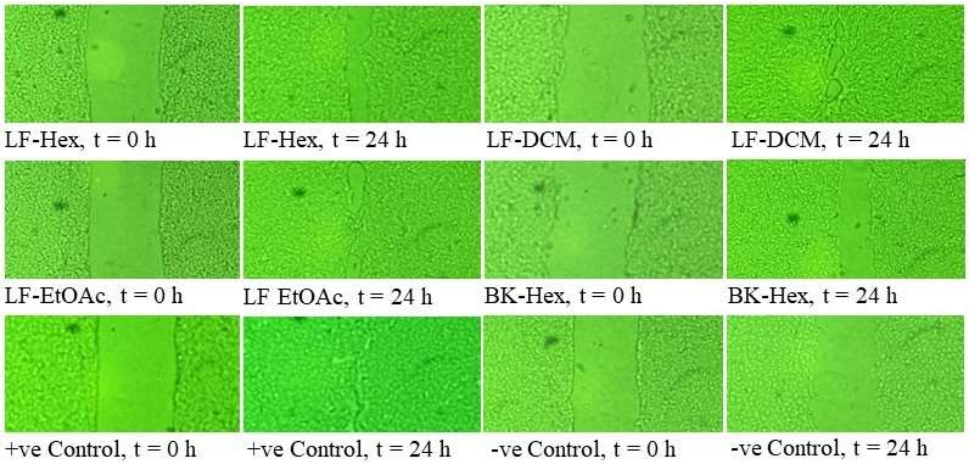

Figure 2. Microscopic images (×400) of scratched wounds of monolayer of MDCK cells at the beginning (t = 0 hours) and after 24 hours (t = 24 hours) in SWA of *Z. oenoplia* extracts, positive and negative controls. (LF = leaf, BK = bark, Hex = hexanes extract, DCM = dichloromethane extract, EtOAc = ethyl acetate extract)

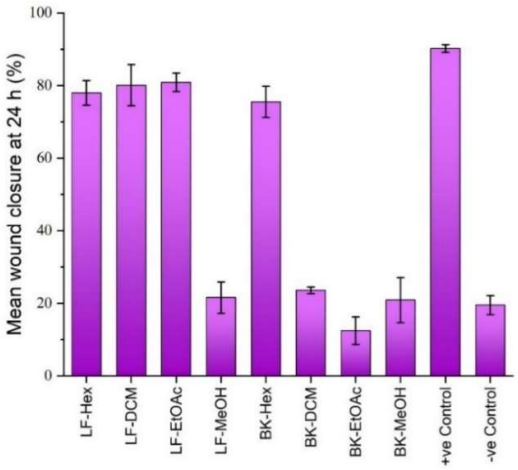

Figure 3: Mean percentage wound closure (±SD) at 24 hours of leaf and bark extracts of *Z. oenoplia* with positive and negative controls in SWA. (LF = leaf, BK = bark, Hex = hexanes extract, DCM = dichloromethane extract, EtOAc = ethyl acetate extract, MeOH = methanol extract)

The proliferative phase of the wound healing process is characterized by several cellular activities of keratinocytes, fibroblasts, macrophages, and endothelial cells regulated by multiple growth factors and cytokines (Werner & Grose, 2003). The proliferation and migration of epithelial cells from the cutaneous wound edges to close the wound area is termed as re-epithelization (Beldon, 2010; Rousselle *et al*., 2019). Thus, the enhanced proliferation of MDCK cells observed in SWA resembles the re-epithelization of the wound healing process. Therefore, it is possible that the leaf and bark extractives of *Z. oenoplia* enhance the wound healing process by accelerating the re-epithelization during the proliferative phase.

Chick chorioallantoic membrane (CAM) assay

The chick chorioallantoic membrane (CAM) assay is a method to study the process of blood vessel sprouting during the wound healing in response to the angiogenic agents (Surekha *et al*., 2013). The angiogenic response of CAM assay is generally expressed as the vascular index. It is obtained by counting all the noticeable blood vessels

such as capillaries, arterioles and venules traversing a 1 mm annulus around the filter disc provided they form an angle less than 45° with a line radiating from the centre (Barnhill & Ryan, 1983).

Images of harvested chorioallantoic membranes treated with methanol and ethyl acetate extracts of leaf, and methanol and hexane extracts of bark and positive and negative controls are shown in Figure 4.

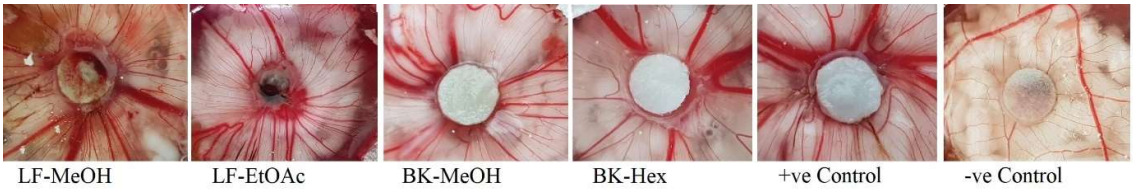

Figure 4: Images of CAM vascularization after 72 hours of incubation, treated with methanol and ethyl acetate extracts of leaf, methanol and hexane extracts of bark, *β*-sitosterol (positive control) (10 µg/disc), and DMSO (negative control) (10 µL/disc). (LF = leaf, BK = bark, Hex = hexanes extract, EtOAc = ethyl acetate extract, MeOH = methanol extract)

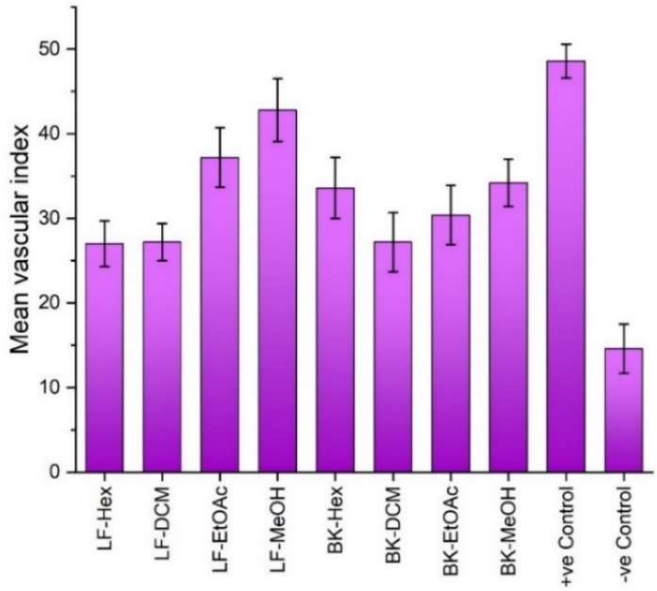

Figure 5: Mean vascular indices (±SD) of leaf and bark extracts of *Z. oenoplia* with controls in the CAM assay (LF = leaf, BK = bark, Hex = hexane extract, DCM = dichloromethane extract, EtOAc = ethyl acetate extract, MeOH = methanol extract)

Mean vascular indices of different extracts of *Z. oenoplia* suggest that the ethyl acetate and methanol extracts of leaf and the hexane and methanol extracts of bark has exhibited a higher angiogenic activity in comparison with rest of the extracts (Figure 5 and Supplementary Table S1).

New blood vessel formation (angiogenesis) during the proliferative stage of wound healing process is a significant event in which, replacement of damaged capillaries, reestablishment of oxygen and nutrient supply, and dilatation of vascularization occur (Bauer *et al*., 2005). Enhanced vascularization observed in the CAM assay is a result of higher fibroblastic activity, which is necessary for the enhanced wound healing process (Theoret, 2017). Since different extracts of *Z. oenoplia* leaf and bark exhibit varying degrees of increased angiogenic response, it shows that the chemical constituents having diverse structural motifs present in these extracts of *Z. oenoplia* may be acting as proangiogenic substances.

Antibacterial assay

Results of the antibacterial assay of *Z. oenoplia* plant extracts against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa* showed that other than the dichloromethane extract against *P. aeruginosa*, they are either much less active than Amoxicillin (positive control), or inactive (Supplementary Table S2). Dichloromethane extracts of both leaf and bark showed mild antibacterial activity against all four tested bacterial strains indicating that both Gram positive (*B. subtilis*, *S. aureus*) and Gram negative (*E. coli* and *P. aeruginosa*) bacteria are sensitive to these two extracts. It is noteworthy that the activities shown by dichloromethane extracts are somewhat comparable across all four bacteria strains while the activity of Amoxicillin (positive control) against *P. aeruginosa* is distinctly less when compared with the remaining bacterial strains (Figure 6).

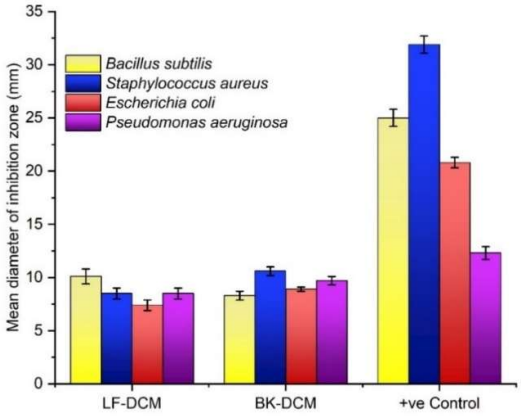

Figure 6: Antibacterial activity of dichloromethane extracts of leaf and bark of *Z. oenoplia* and the Positive control (amoxicillin) against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*

In the treatment of wounds, acceleration of the healing process and protecting the wound from bacterial infections are the two main objectives. The open blood vessels and tissues in a wound area are a favourable place for bacterial growth. Bacterial infections delay the wound healing process. As such, in addition to enhancing cell proliferation and angiogenesis, prevention of bacterial invasion of the wound is also an essential requirement in wound healing (Rodeheaver *et al*., 1980). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most common bacteria isolated from chronic wounds. Hence, the compounds present in dichloromethane extracts of both leaf and bark of *Z. oenoplia* which showed relatively enhanced antibacterial activity against *P. aeruginosa* can play a vital role during the wound healing.

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

Fraction no.	Weight (g)	Mean % wound closure (±SD)*
HF_1	80.0	49.1 (0.6) [e]
HF_2	274.7	72.4 (1.0) [c]
HF_3	220.0	75.8 (0.8) [b]
HF_4	80.1	62.3 (0.5) [d]
HF_5	55.4	36.4 (1.2) [f]
HF_6	92.3	49.1 (2.8) [e]
Positive control†		89.5 (0.4) [a]
Negative control ‡		20.9 (1.6) [g]

* Different letters in the same column indicate significant differences at t = 1.98 and p ≤ 0.05 level. †Asiaticoside (25 µM), ‡1% DMSO in 20% DMEM

Bioactivity directed chemical investigation of hexanes extract of leaf of *Z. oenoplia*

Chemical investigation of *Z. oenoplia* extracts was initiated with the hexanes extract which showed 78.0% wound closure in SWA. Since the SWA is relatively fast and cheap, it was selected as the guiding bioassay for the chemical investigation. The 6 major fractions obtained from the silica gel column chromatographic separation of the hexanes extract of leaf of *Z. oenoplia* were subjected to SWA at a concentration of 10 mg/L. The yields and mean percent wound closure of each fraction along with the percentage wound closure of positive and negative controls are given in Table 2.

Results indicated that the fraction HF_3 to be the most active fraction (75.8% wound closure). Hence, further fractionation of fraction HF_3 was carried out. The silica gel column chromatographic separation of fraction HF_3 yielded 4 major fractions. They were subjected to SWA at a concentration of 5 mg/L. The yields and percent wound closure of these fractions, along with the mean percent wound closure of positive and negative controls are given in Table 3.

Table 3: Dry weights of column fractions of Fraction HF_3 and the mean percentage of wound closure at 24 h of those extracts of 5 mg/L concentration, with negative and positive controls.

Fraction no.	Weight (g)	Mean % wound closure (±SD)*
HF_{3A}	53.3	56.9 (1.5) [e]
HF_{3B}	34.2	73.1 (1.2) [c]
HF_{3C}	42.7	86.4 (0.9) [b]
HF_{3D}	55.7	68.4 (0.6) [d]
Positive control†		90.4 (0.9) [a]
Negative control ‡		20.1 (1.5) [f]

***** Different letters in the same column indicate significant difference at t = 1.98 and $p \leq 0.05$ level. †Asiaticoside (25 μM), ‡1% DMSO in 20% DMEM

The results indicated that the fraction HF_{3C} was the most active fraction (86.4% wound closure) containing one major spot on TLC. A large portion of the fraction HF_{3C} (42 mg) was subjected to preparative TLC (20 × 20 cm, 0.50mm, eluent: CH_2Cl_2) and the prominent band that appeared on TLC plate was scraped off and eluted with 10% MeOH in CH_2Cl_2 to yield the 24.6 mg of HF_{3CA}. NMR spectroscopic analysis of the isolate HF_{3CA} was found to contain an inseparable mixture containing lupeol (**1**), [δ_C 150.3 (20-C), δ_H 4.68 (1H, br), 4.56 (1H, br), and 109.3, (29-CH_2), δ_H 3.18 (1H, dd, *J*=5.1 & 11.1 Hz), δ_C 79.0 (3-CH)], *α*-amyrin (**2**), [δ_C 139.6 (C-13), δ_H 5.12 (1H, t, *J*=3.4 Hz) and δ_C 124.4 (12-CH), δ_H 3.22 (1H, dd, *J*=4.8, 10.9 Hz) and δ_C 79.0 (3-CH)], *β*-amyrin (**3**), [δ_C 145.2 (C-13), δ_H 5.18 (1H, t, *J*=3.4 Hz), and δ_C 121.7 (12-CH), δ_H 3.22 (1H, dd, *J*=4.8 & 10.9 Hz, and δ_C 79.0 (3-CH)] and a fatty alcohol, [δ_H 3.60 (t, *J*=6.4 Hz) and δ_C 63.1 (1-CH_2), δ_C 29.3–29.7 $(CH_2)_n$, δ_C 14.1 (CH_3)] (See Supplementary material and Figures S3–S10). GC-MS analysis of this mixture showed 4 peaks in the total ion chromatogram (t_R = 13.955, 15.345, 17.720, and 18.211 min.) (Supplementary Figure S11) of which the first two at t_R = 13.955 and 15.345 min were identified as fatty alcohols, hexacosanol (**4**) and octacosanol (**5**) respectively. It is observed that ^{1}H-NMR and ^{13}C-NMR signals of these two compounds appear at the same δ values (Hamill *et al*., 2003; Park *et al*., 2008; Yun-Choi *et al*., 2003). The third peak in the GC-MS was identified as *β*-amyrin (**3**). Careful analysis of the remaining peak revealed that both lupeol (**1**) and *α*-amyrin (**2**), have been co-eluted during the GC-MS analysis. The identities of **1**, **2** and **3** were confirmed by the comparison of observed NMR data with those reported (Mahato & Kundu 1994; Burns *et al*., 2000; Viet *et al*., 2021). The molar ratio of lupeol (**1**), *α*-amyrin (**2**), *β*-amyrin (**3**), and the two alkanols has been determined as 1:2:2:3 by ^{1}H-NMR using the signals at δ 4.68, 1H (for lupeol), δ 5.12, 1H (for *α*-amyrin), δ 5.18, 1H (for *β*-amyrin), and δ 3.63, 2H (for two alkanols) and the ratio of their relative intensities (0.5: 1: 1: 3 respectively) (Supplementary Figures S4 and S5). The mean percent wound closure of the fraction, HF_{3CA} in SWA was found to be 87.5 % at 24 h at a concentration of 5 mg/L. Since the hexane extract of leaf of *Z. oenoplia* showed relatively moderate proangiogenic response in CAM assay, the fraction HF_{3CA} was subjected to CAM assay at a concentration of 10 μg/disc and mean vascular index was found to be 34.5.

Lupeol (**1**), α-amyrin (**2**), and β-amyrin (**3**) are pentacyclic triterpenoids found in many medicinal plants. Previous studies have reported that these individual compounds have numerous biological activities. Although the wound healing potential of **1** was previously reported (Beserra *et al*., 2018; Bopage *et al*., 2018) such activity for compounds **2**–**5** have not been reported. Therefore, investigating the effect of these individual compounds and their involvement in wound healing as constituents in a mixture would be important to identify their contribution to wound healing.

CONCLUSION

Cell proliferation and angiogenesis are key events in the proliferative stage of wound healing process. Enhanced cell proliferation in SWA and higher angiogenic response in CAM assay exhibited by the different extracts of *Z. oenoplia* leaf and bark during this study suggest that these extracts are capable of accelerating the wound healing process. In addition to the acceleration of wound healing process, it is shown that these extracts are capable of prevention of bacterial infections of wounds, especially the infections caused by *P. aeruginosa*, thus facilitating the wound healing process. Bioactivity guided chemical investigation of the hexane extract of leaf of *Z. oenoplia* led to the identification of a fraction exhibiting both cell migration and angiogenic potential that consists of lupeol (**1**), α-amyrin (**2**), β-amyrin (**3**) and two alkanols hexacosanol (**4**) and octacosanol (**5**). This is the first report of the presence of compounds **2**–**5** from *Z. oenoplia*. Chemical investigation of the other active extracts is underway to identify chemical constituents present in those extracts that have wound healing potential.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

Financial assistance from the National Science Foundation (NSF) of Sri Lanka through the research grant No: NSF/PSF/ICRP/2017/HS/02 is gratefully acknowledged. We are thankful to Dr Muhammad Yar, Associate Professor, IRCBM, COMSATS University Islamabad, Lahore Campus, Pakistan, for the support of establishing CAM assay in our laboratory, Dr Nuwan Dileepa De Silva, Sri Lanka Institute of Nano Technology (SLINTEC) and Dr T.M.S.G. Tennakoon, Link Natural Products Ltd., for providing NMR data and GC-MS data, respectively, and Mr. S.B.R. Chinthaka Samarakoon, Link Natural Products Ltd. for the assistance in obtaining GC-MS data. Ms. D.U. Dhanapala, Technical officer, Department of Chemistry, The Open University of Sri Lanka, for the technical support provided at the initial stages of CAM assay and Mr. K.C.K. Deraniyagala, Senior Staff Technical officer, Department of Mechanical Engineering, The Open University of Sri Lanka, for the technical support provided for maintenance of some equipment are also gratefully acknowledged.

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In-vitro wound healing potential of leaf and bark of *Ziziphus oenoplia* (L.) Miller
Supplementary material

S1. Mean percentages of wound closure at 24 h in SWA at a concentration of 20 mg/Land mean vascular indices in CAM assay at a dose of 10 µg of extracts of *Z. oenoplia* with positive and negative controls.

Plant part	Extract	Mean % wound Closure (±SD)*	Mean vascular index (±SD)*
Leaf	Hexanes	78.0 (3.4) ^{c}	27.0 (2.7) ^{e}
	Dichloromethane	80.1 (5.7) ^{c}	27.2 (2.2) ^{e}
	Ethyl acetate	80.9 (2.5) ^{c}	37.2 (3.5) ^{c}
	Methanol	21.6 (4.3) ^{e}	42.8 (3.7) ^{b}
Bark	Hexanes	75.5 (4.3) ^{c}	33.6 (3.6) ^{cd}
	Dichloromethane	23.6 (0.9) ^{e}	27.2 (3.5) ^{e}
	Ethyl acetate	12.5 (3.8) ^{f}	30.4 (3.5) ^{de}
	Methanol	20.9 (6.2) ^{e}	34.2 (2.8) ^{cd}
Positive control†		90.2 (1.1) ^{a}	48.6 (2.0) ^{a}
Negative control‡		19.5 (2.6) ^{e}	14.6 (2.9) ^{f}

* Different letters in the same column indicate significant differences at t=1.98 and $p \leq 0.05$ level.

† Asiaticoside (25 µM) was used in the SWA; *β*-sitosterol (10 µg) was used in the CAM assay.

‡1% DMSO in 20% DMEM was used in the SWA; DMSO was used in the CAM assay.

S2. Mean diameters of inhibition zones developed against different extracts of *Z. oenoplia* and the positive control (Amoxicillin, 10 µg) with *B. subtilis, S. aureus, E. coli,* and *P. aeruginosa at* 500 µg/disc.

Plant part	Extract	Mean diameter of inhibition zone (mm) (±SD)*			
		B. Subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
Leaf	Hexanes	9.4 (0.5) ^{d}	NA	NA	NA
	Dichloromethane	10.1 (0.7) ^{c}	8.5 (0.5) ^{d}	7.4 (0.5) ^{c}	8.5 (0.5) ^{c}
	Ethyl acetate	12.3 (0.4) ^{b}	9.5 (0.6) ^{c}	NA	NA
	Methanol	7.1 (0.3) ^{f}	9.3 (0.4) ^{c}	NA	NA
Bark	Hexanes	NA	NA	8.7 (0.4) ^{b}	NA
	Dichloromethane	8.3 (0.4) ^{e}	10.6 (0.4) ^{b}	8.9 (0.2) ^{b}	9.7 (0.4) ^{b}
	Ethyl acetate	10.2 (0.4) ^{c}	7.3 (0.4) ^{e}	7.5 (0.5) ^{c}	NA
	Methanol	NA	NA	NA	NA
Amoxicillin (10 μg)		25.0 (0.8) ^{a}	31.9 (0.8) ^{a}	20.8 (0.5) ^{a}	12.3 (0.6) ^{a}

* Different letters in the same column indicate significant differences at t=1.98 and $p \leq 0.05$ level.

NA = Not active; Clear zone not observed.

S3. Spectroscopic data of lupeol (**1**), α-amyrin (**2**), β-amyrin (**3**), hexacosanol (**4**) and octacosanol (**5**).

Lupeol (**1**) – ^{1}H NMR (400 MHz,CDCl3) δ (ppm): 4.68 (1H, br H-29b), 4.56 (1H, br, H-29a), 3.18 (1H, br dd, J = 5.1, 11.1 Hz, H-3), 2.37 (1H, m, H-19); ^{13}C NMR (100 MHz, CDCl3) δ (ppm): 150.9 (C-20), 109.3 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 48.3 (C-18), 48.0 (C-19), 43.0 (C-17), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.8 (s, C-4), 38.6 (C-1), 38.0 (C-13), 37.1 (C-10), 35.6 (C-16), 34.3 (t, C-7), 29.8 (C-21), 28.0 (C-23), 27.5 (C-15), 27.4 (C-2), 25.1 (C-12), 20.9 (C-11), 19.3 (C-30), 18.3 /(C-6), 18.0 (C-28), 16.1 (C-25), 15.9 (C-26), 15.4 (C-24), 14.5 (C-27).

GC-MS, Retention time (t_R): 18.211 min.; *m/z* (rel. int., %): 426 [M+] (13.7), 339.3 (1.6), 370.2 (0.9), 344.2 (0.4), 315.3 (4.3), 286.2 (1.3), 257.1(4.7), 218.2 (100.0), 207.15 (19.6), 203.2 (29.0), 189.2 (36.3), 175.1 (13.0), 161.15 (16.1), 147.0 (16.8), 143.0 (2.0), 119.1 (20.9), 109.1 (22.8), 107.1 (24.7), 95.1 (26.7), 81.1 (22.4), 67.1 (14.7), 57.1 (7.4), 41.1 (10.8).

α-Amyrin (**2**) – ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.12 (1H, t, J = 3.4 Hz, H-12), 3.22 (1H, dd, J = 4.8, 10.9 Hz, H-3); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 139.6 (C-13), 124.4 (C-12), 79.0 (C-3), 59.0 (C-18), 55.2 (C-5), 47.7 (C-9), 42.1

(C-14), 41.5 (C-22), 40.0 (C-8), 39.8 (C-19), 39.6 (C-20), 38.8 (C-1 and C-4), 36.9 (C-10), 33.7 (C-17), 32.9 (C-7), 31.3 (C-21), 28.7 (C-15), 28.1 (C-23 and C-28), 27.3 (C-2), 26.6 (C-15), 23.4 (C-11), 23.3 (C-27), 21.4 (C-30), 18.4 (C-6),17.5 (C-29), 16.8 (C-26), 15.7 (C-25), 15.6 (C-24).

GC-MS, t_R: 18.211 min.; *m*/*z*: 426.4 [M+];426.4 (12.2), 411.4 (4.7), 393.4 (1.4) , 366.3 (0.2), 341.1 (2.0), 315.3 (3.1), 281.1 (6.9), 253.0 (2.9), 218.2 (100.0), 218.2 (100), 203.2 (26.2), 189.2 (30.1), 179.1 (1.9), 161.2 (13.8), 147.1 (16.6), 135.1 (25.3), 121.1 (19.3), 81.1 (18.9), 69.1 (16.3), 55.1 (14.8), 41.1 (9.4).

β-Amyrin (**3**); ^{1}H-NMR (400 MHz, $CDCI_3$) δ (ppm): 5.18 (1H, t, J = 3.4 Hz, H-12), 3.22 (1H, dd, J = 4.8, 10.9 Hz, H-3); ^{13}C-NMR (100 MHz, $CDCI_3$) δ (ppm): 145.2 (C-13), 121.7 (C-12), 79.0 (C-3), 55.2 (C-5), 47.6 (C-9), 47.2 (C-18), 46.8 (C-19), 41.7 (C-14), 38.9 (C-4), 38.8 (C-8), 38.7 (C-1), 37.2 (C-22), 37.0 (C-10), 34.7 (C-21), 33.3 (C-29), 32.6 (C-7), 32.5 (C-17), 31.1 (C-20), 28.4 (C-28), 28.1 (C-23), 27.2 (C-2), 27.0 (C-16), 26.2 (C-15), 26.0 (C-27), 23.7 (C-30), 23.5 (C-11), 18.4 (C-6), 16.8 (C-26), 15.6 (C-25), 15.5 (C-24).

GC-MS, t_R: 17.720 min.; *m*/*z* (rel. int., %): 426.4 [M]+ (4.4), 408.4 (0.2), 393.4 (0.7), 281.1 (4.2), 218.2 (100), 203.2 (49.3), 189.2 (14.9), 161.2 (5.0), 135.1 (10.3), 95.1 (11.8), 69.1 (10.9), 43.1 (6.1)

Hexacosanol (**4**) and Octcosanol (**5**); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.60 (t, *J*=6.4 Hz, C*H*$_2$OH), ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 63.1 (C-1), 32.8 (C-2), 31.9 [C-24 (**4**) and C-26 (**5**)], 29.3 – 29.7 [C-4 to C-23 (**4**) and C-4 to C-25 (**5**)], 25.7 (C-3), 22.7 [C-25 (**4**) and C-27 (**5**), 14.1 [C-26 (**4**) and C-28 (**5**)].

Hexacosanol (**4**); GC-MS, t_R: 13.955 min.; *m*/*z* (rel. int., %): 364.4 $[M\text{-}H_2O]^+$ (2.3), 336.3 (2.5), 181.1 (3.2), 135.1 (0.6), 125.1 (29.3), 111.1(55.1), 97.1 (100), 83.1 (96.9), 57.1 (90.5), 41.1 (36.5)

Octcosanol (**5**); GC-MS, t_R: 15.345 min.; *m*/*z* (rel. int., %): 392.4 $[M\text{-}H_2O]^+$ (2.4), 364.4 (2.6), 336.4 (0.6), 181.1 (3.6), 153.1 (8.2), 125.1 (31.0), 111.1(57.0), 97.1 (100), 83.1 (95.2), 69.1 (74.3), 57.1 (90), 55.1 (68.9), 43.1 (63.4), 41.1 (32.5).