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Anethum Graveolens Leaves Extract Accelerate Wound Healing *In vitro* and *In vivo*

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Wound healing is a curative process that starts with trauma and finishes with scar formation. Various plant extracts have been used for the treatment and controlling of wounds. In this study Anethum graveolens has been used to accelerate in vivo excision model of wound healing on Sprague Dawley rats and to proliferate the in vitro cell viability model using skin fibroblast cell line through the scratch assay. Results confirm that this plant extract decreases the wound area and increases itswound size reduction percentage, hydroxyproline and nitric oxide levels of the plant extract treated groups were near to the normal control group that indicated effective healing process. On the other hand, in vitro cytotoxicity results should that Anethum graveolens plant extract was safe on skin fibroblast cell lines and induced the normal proliferation and growth of these cells. The migration rate to heal the in vitro wound gaps was 89.1% which indicates a perfect wound size reduction. In conclusion, the results proved that the topical application of Anethum graveolens plant extract quicken the wound healing process.

Keywords: Anethum Graveolens; Hydroxyproline; Nitric Oxide; Wound Healing.

Wound healing process is a complex sequence of interacion between cells and inflammatory mediators like cytokines, proteases, kinins, nitric oxide, eicosanoids and the other cellular elements [1]. Each cell type activity through three phases: proliferation, migration, contraction and matrix synthesis also the growth factor and matrix signals are reported to be present at a wound site [2]. The natural healing reaction begins the minute the tissue is injured then blood elements leak into the site of injury. Platelets connect with collagen and other elements of the extracellular matrix then release the clotting factors, cytokines and the growth factors like platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-ß) afterwards, the neutrophil cells enter the wound location and begin the phagocytotic process to clean out the wound site. Fibroblasts transfer in and begin the proliferative phase later the collagen matrix undergoes the remodelling phase [3]. Tissue healing process depend on the balance between the deleterious effects of reactive oxygen species (ROS) which are a group of potent molecules that limits successful tissue regeneration [4].

Many medicinal plant extracts or decoctions had been used by traditions for the treatment of wounds, cuts and burns [5-8]. *Anethum graveolens*, a herb from the celery family Apiaceae. Commonly known as dill, however, in the Middle east region it is named as *Ain jaradeh* (Grasshopper's eye) while in Arab countries and Kurdistan region of Iraq it called *Shibit.* Fresh and dried dill leaves called "dill weed" to differentiate it from dill seed, both has been used widely

in traditional medicine for enclosing different activities such as antibacterial effect [9] diuretic, stomachic, appetite stimulant and it reported to stimulate milk flow in a lactating mother [10]. Also dill is used to reduce blood cholesterol and lipid levels, menstrual bleeding and dysmenorrhea [11]. Studies showed that a tea made from dill seed can be used in the initial stages of labour to reduce labour pain without any side effects on mother and fetus [12]. The induction of apoptosis of the fungus *"Candida albicans"* apoptosis was revealed by *Anethum graveolens* seed essential oils [13] its antifungal activity was reported as potential food preservative [14]. In addition, other studies reported different bioactivities of *Antheum graveolens* like anti-inflammatory, antimicrobial [15], antioxidant and free radical scavenging activities [16]. The current study aimed to test the in vitro wound healing activity of *Anethum graveolens* and it's effects on fibroblast cell viability along with the in vivo wound healing activity on *Sprague Dawley* rats.

MATERIALS AND METHODS

Plant Extraction

For the preparation of ethanol extract of *Anethum graveolens*, 100 grams of the dried weed part was soaked in 1000 milliters of ethanol for 72 hours. After that, the mixture was filtered by filter paper (Whatman No.1) and extracted under compact pressure in an Eyla rotatary evaporator (Sigma Aldrich, USA). Intrasite gel was used as the reference control (is a trademark for Smith and Nephew Healthcare Limited,UK). Acacia Arabic gum (Sigma Aldrich, USA) was used as the vehicle control following the method of Dhiyaaldeen et al.[17] One gram of gum acacia was dissolved in 100 ml of normal saline, from this, 10 ml of solution, which contains 100mg of gum acacia, was used for dissolving 100mg and 200mg of Plant extract each.

Excision Model Of *In Vivo* Wound Healing

Sprague Dawley (SD) adult rats weighing between (250 -300 g), were gained from the animal house unit of the Faculty of Medicine /University of Malaya/ Malaysia. They were maintained at 22 ± 3 °C in wire bottom cages under a 12 h–12 h light–dark cycle with 50% to 60 % humidity for at least one week former to the test. The study was approved by the ethics Committee for animal experimentation, Faculty of Medicine, University of Malaya, Ethic No. PM/27/07/2009/MAA. For wound healing experiment, the rats were divided randomly into four groups each with eight rats which housed individually. After anesthetizing the animals, the skin was shaved with an electrical shaver and disinfected with 70% alcohol. An identical wound area with 2cm diameter (Figure 1) was excised from the napes of the rats using a curved stamp, as described by Morton and Malone [18]. All rats were treated twice a day, as follows: the vehicle control group was treated with 0.2 ml gum acacia; the reference drug group was treated with 0.2 ml intrasite gel; one of the treated groups was topically administered 0.2 ml of the 100 mg/ml plant extract; and the other treated group was topically administered 0.2 ml of the 200 mg/ml plant extract.

Reduction of the wound area was measured on the day of surgery and then on days 5 and 10. An excision edge was traced after wound formation with transparent paper, and the wound area was measured using graphing paper. The tracing paper was placed on 1 mm^2 graph sheet and traced out. The squares were counted and the area was recorded. Then the percentage of wound size reduction was calculated following the formula:

Wound size reduction (%) = 1- (Ad/A0)*100 (A0 is wound area on day zero, Ad is wound area on corresponding day)[19] .Wound contraction was measured at 5-day-intervals. At day 10, the animals were anesthetized under a high dose then the skin from the restored wound area was excised;The skin tissue was homogenized and the supernatant was used to measure nitric oxide (NO) and hydroxyproline (HXP) determinations using the NO and HXP assay kit (Cayman Chemical Co., USA).

In Vitro Wound Healing

The MTT (3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide) assay (Merck, Germnay) was performed to study *Anethum graveolens* cytotoxicity. Cells were grown in DMEM medium (Dulbecco's Modified Eagle's) (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, USA) at 37°C under 5% CO2 in a humidified incubator (NUAIRE laboratory equipment supply, USA). Cells (0.5×10^5 cells/ml) were transmitted into a 96-well plate, and incubated for 48 hours then serial dilutions of

Anethum graveolens plant extract was prepared by using 0.25 % DMSO (Dimethyl Sulfoxide) as solvents to give final concentrations of (1.25, 2.5, 5, 10 and 20 mg/ml) then 10µl was injected to each well and incubated for further 48 hours. MTT test was performed following the method of Amin et al [8] then, the absorbance was read by the ELISA plate reader (PowerWave X340, BIO-TEK Instruments Ltd.) at 595 nm. The percentage of cell growth inhibition was calculated as:

Cell viability % = (abs of extract sample – abs of control/abs of control) × 100

Moreover, the cytoselect wound healing assay kit (Cell Biolabs, Inc., USA) was used to assess the *in vitro* wound healing assay in which specific inserts were used to make wound gaps (0.9 mm), then cells were cultured (0.5 x10^6 cells/ml) in media containing 10% fetal bovine serum (FBS) and incubated for 48 hours. The inserts detached and the cells were treated with the plant extract (200µg/ml), incubated for further 48 hours. The cells migration into the wound gaps was measured and the wound size reduction was calculated as:

Total surface area = 0.9 mm * length

Migrated cell surface area= length of cell migration (mm) * 2 * length

Percent wound size reduction (%) = migrated cell surface area / total surface area * 100 as mentioned by cytoselect wound healing assay kit (Cell Biolabs, Inc., USA)

Statistical Analysis

All data were expressed as Mean ± SEM. The statistical analysis was achieved using one-way ANOVA, Post Hoc Boneferoni test to compare the groups, a *p*-value d" 0.05 as considered significant.

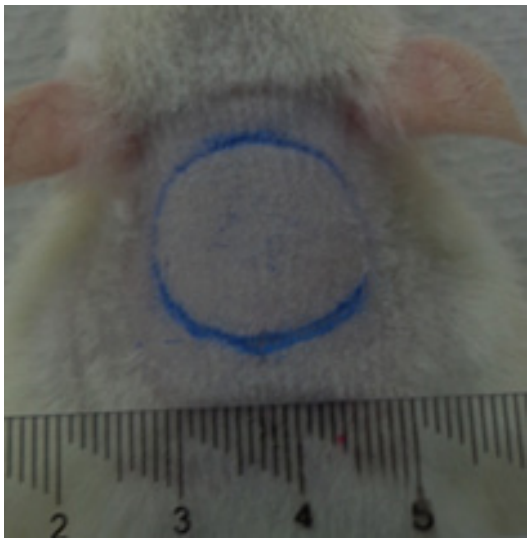

Fig. 1. 2cm diameter excision skin wound on the day of surgery

RESULTS

Excision Model Of *In Vivo* Wound Healing

Table (1) shows the effects of *Anethum graveolens* ethanol extract on the percentage of wound healed on days post-surgery. Throughout the experiment, the percentage of healing in the vehicle control group gross wounds was significantly lower than those of *Anethum graveolens* extract treated groups and intrasite gel control groups (Figure 1). On day 10 post-surgery showed that wound dressed with *Anethum graveolens* extract showed comparatively less scar width at wound size reduction compared to the vehicle -treated group.

The hydroxyproline parameter result (HXP) is shown in Figure 3. Generally, the rats dressed with gum accacia showed significant (5ØCÜ< 0.05) decreased levels of HXP compared to the treatment groups. Notably, the animals dressed topically with 200mg/kg *Anethum graveolens* plant extract showed significantly

Table 1. Effect of *Anethum graveolens* ethanol extract on percentage wound healing in *Sprague Dawley* rats

Groups	Doses Twice daily	Day 0 Wound area Mm²	Day 7 Wound area Mm²	Day 7 Wound size Reduction %	Day 14 Wound area Mm²	Day 14 Wound size Reduction %
Gum acacia (n = 6)	0.2 ml/kg	312.5±0.03	198.3±1.01	36.6[a]	77.5±1.01	75.2[a]
Anethum graveolens (n = 6)	100 mg/kg	313.7±0.09	173.3±0.19	44.9[b]	46.7±0.09	85.3[b]
Anethum graveolens (n = 6)	200 mg/kg	313.3±0.51	148.1±0.07	52.8[c]	34.3±0.04	89.1[c]
Intrasite gel (n = 6)	0.2 ml/kg	312.9±0.01	132.1±0.03	57.8[c]	32.3±0.11	90.1[c]

Data expressed as Mean ± SEM, Mean values followed by different letters (a, b, and c) in a column are significantly different as compared to Gum acacia group (p< 0.05).

increased levels of HXP in wound homogenate compared to the animals treated with the gum accacia.

On the other hand, the nitric oxide (NO) results were higher in rats dressed with *Anethum graveolens* plant extract and Intrasite gel compared to gum accacia group (Figure 4). Rats dressed with Intrasite gel had the highest NO levels. Dressing the animals with high dose of *Anethum graveolens* significantly ($p< 0.05$) increased NO levels.

***In Vitro* Fibroblast Cell Proliferation**

The Effect of *Anethum graveolens* extracton human fibroblast cells of the skin showed initially a significant cell viability and proliferation as presented in Figure5. This indicates that the plant extract is safe and non- toxic to normal skin cells. Furthermore, *Anethum graveolens* ethanol extract showed an increase fibroblast migration rate toward closing the wound gap which assessed *in vitro,* with a percent wound size reduction of the wound around (89.1%).

Dýscussýon

During the wound healing procedure, risk factors should be reduced that prevent the wound from healing and causes infections [20]. Indeed, plant extracts are inexpensive, affordable, and safe with no or rare side effects [21]. The results of the present study showed effective activity of *Anethum graveolens* in wounds induced *in vivo* on *Sprague dawely* rats. The healing process was successful as represented by decreased wound area measurements and increased wound size reduction percentage of the rats treated with the *Anethum graveolens* ethanol extract as shown in Table 1 and Figure2. There are plenty of evidences that proposes that increased production of lipid peroxidation; reactive oxygen species and ineffective scavenging activity play a vital role in most skin lesions and

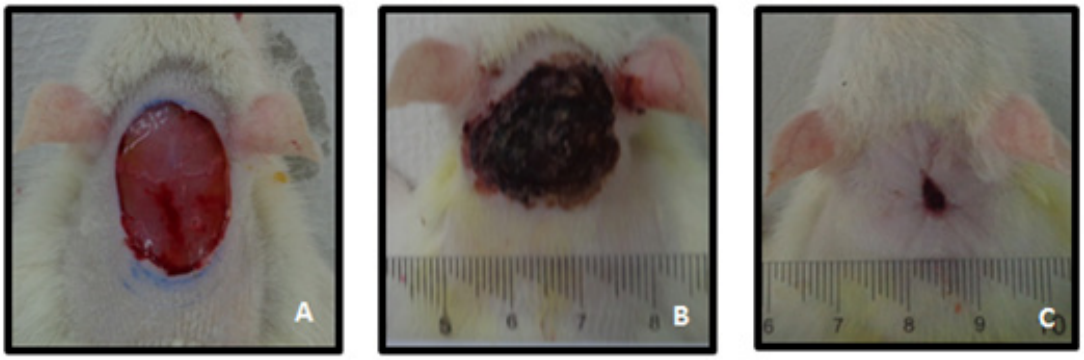

Fig. 2. Excisional wound appearance after treatment with 200mg/kg *Anethum graveolens* ethanol extract: (A) at day 0 of treatment, (B) at day 7 of treatment and (C) at day 14 of treatment

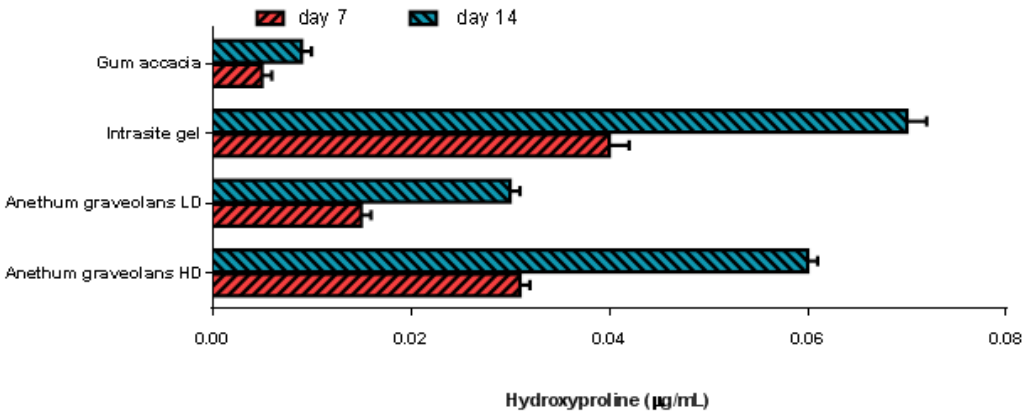

Fig. 3. Hydroxyproline (HXP) levels in healed skin homogenates treated with 2% gum accacia, Intrasite gel, *Anethum graveolens* (100 mg/kg), *Anethum graveolens* (200 mg/kg)

in modulation of fibroblast proliferation [22]. In the present investigation, *Anethum graveolens* was found to treat wounds through restoring the levels of hydroxyproline and nitric oxide when compared with control (Figures 3 and 4). Nitric oxide (NO) is an essential regulatory molecule in several developmental and physiological processes. It has been demonstrated that NO participates in the wound-healing response [23]. It is a small radical, formed from the amino acid L-arginine by three distinct isoforms of nitric oxide synthase. The inducible isoform (iNOS) is synthesized in the early phase of wound healing by inflammatory cells, mainly macrophages [24]. However, many cells participate in NO synthesis during the proliferative phase after wounding. NO released through iNOS regulates collagen formation, cell proliferation, and wound contraction in distinct ways in animal models of wound healing [25]. Furthermore, there are numerous cells in the wound area that can

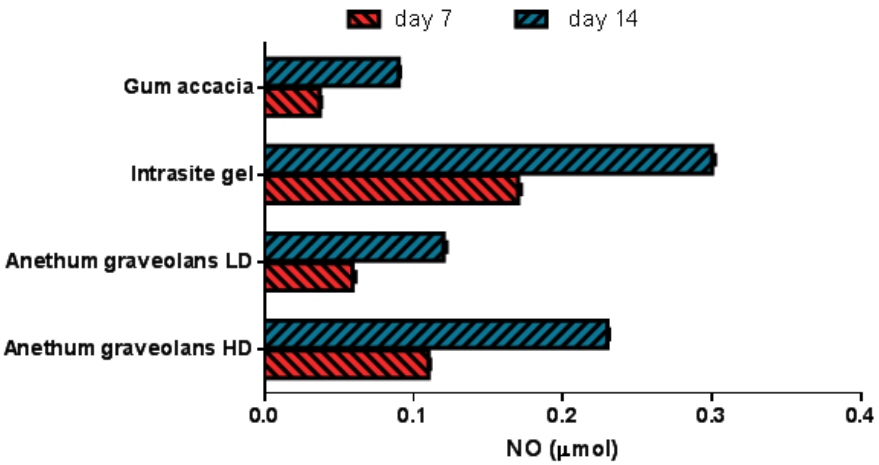

Fig. 4. Nitric oxide (NO) levels in healed skin homogenates treated with 2% gum accacia, Intrasite gel, *Anethum graveolens* (100 mg/kg), *Anethum graveolens* (200 mg/kg)

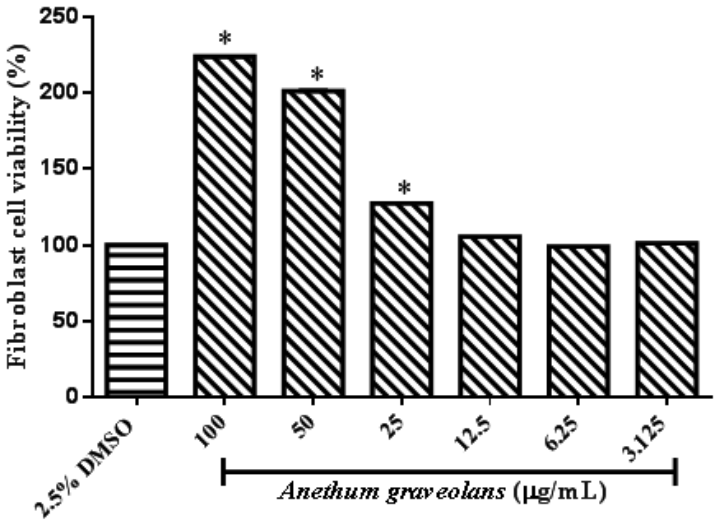

Fig. 5. The effect of *Anethum graveolens* on fibroblast cell viability

produce NO including platelets, macrophages, fibroblasts, endothelial cells and keratinocytes [26]. In the chronic wound cases, upregulated expression of NO and oxidants will result in constant production of constituents of oxidative and nitroxidative stress [27]. The favorable effects of NO on wound repair may be accredited to its functional influences on inflammation, angiogenesis, cell proliferation, remodelling and matrix deposition. These evidences support that NO plays a major role in the wound healing process [26]. On the other hand, the protein collagen contains the amino acid (hydroxyproline) which is the major element of extra cellular tissue, it provides support and strength. Breakdown of collagen releases free hydroxyproline and its peptides. Measurement of the hydroxyproline could be used as an index for collagen turnover[28]. As the Proliferative phase develops, the chief cell in the wound site is the fibroblast. It is responsible for producing the new matrix needed to restore structure and function to the injured tissue. During this process, there is an important step involving hydroxylation of proline deposits. Hydroxyproline in collagen is important because it gives the molecule its stable helical conformation [3]. Hydroxyproline content was found to be significantly increased in the skin burns, wounds and many other skin disorders[29]. The data of hydroxyproline content (Figure 3) showed that the hydroxyproline content of the homogenate tissue of the animals treated with ethanol extract of *Anethum graveolens* was significantly increased when compared to the control. Furthermore, the wound re-epithelialization potential of many crude plant extracts, isolated compounds or pharmaceutical preparations were investigated. Studies have proved various herbs contributed significantly to the stimulation of fibroblast proliferation and their migration to reduce the wound gap *in vitro* [30]. The same results were seen in this study where *Anethum graveolens* ethanol extract showed no cytotoxicity against these cells and oppositely it increased the viability rate and increased the wound closure induced by the scratch assay *in vitro*. These results are supported by the in vitro studies of some plant extracts which demonstrated that these extracts enhanced proliferation of endothelial cells, fibroblastsand keratinocytes also stimulated keratinocyte migration in vitro in a wound assay and inhibited collagen matrix contraction by fibroblasts [31]. Previous studies on dill seed extract showed increased expression of Lysyl oxidase–like enzyme (an extracellular enzyme that catalyzes the cross-linking between micro-fibrils and tropo-elastin) in cultured dermal fibroblasts [32]. The cytotoxicity of *Anethum graveolens* is most likely due to its main constituents: carvone and limonene which has been shown toincrease production of reactive oxygen species (ROS) and decrease mitochondria membrane potential in HL60 cells thus inducing apoptosis [33].

CONCLUSION

The results demonstrate that the ethanol extract of *Anethum graveolens* is capable of promoting wound-healing activity. However, it needs further evaluation in clinical settings before consideration for the treatment of wounds. More efforts should be made toward the characterization and isolation of the active constituents of *Anethum graveolens* and the relation between their activity and structure should be elucidated. The combination of modern and traditional knowledge can yield better medications for wound healing with fewer side effects.

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