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Influence of the Leaves of *Tectona grandis* L. (Verbenaceae) on *ex-vivo* Porcine Skin Wound Healing Model

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ABSTRACT

To pre-screen the *ex- vivo* wound healing activity of ethyl acetate extract of the leaves of *Tectona grandis* Linn. Family Verbenaceae along with phytochemical, EDS, HPTLC analysis to provide pharmacological validation to the traditional claim for this activity of *Tectona grandis* leaves. Total phenolic content by UV spectral methods and apigenin by HPTLC, trace elements by Energy Dispersive X-ray Spectrometer were determined. The wound healing effect was evaluated using *ex- vivo* porcine skin wound healing model Total phenolic content, HPTLC determination of apigenin content of TGEAE was found to be 22.2µg/g , 0.7% respectively. EDS study showed calcium (2.19%), potassium (9.24%), magnesium (0.62%), and sulphur(0.48%), phosphorous (0.97%). Histopathological evaluation showed all treated wounds were sound with no signs of apoptosis, necrosis or bacterial contamination and no toxicity of the tested concentrations. Morphology of the wound margins, epidermis and dermis layer were found to be normal. TGEAE (3%) promoted statistically significant wound healing effect. This study indicates that the ethyl acetate extract of the leaves of *T.grandis* possesses wound healing activity on *ex-vivo* porcine skin wound healing model. The activity may be due to its phenolic content, apigenin (flavonoids) and betulinic acid. Trace elements supports wound healing as they required for cellular growth and replication. This present investigation provides scientific evidence to ethno medical use of *T.grandis* leaves in wound healing. Therefore it can be safely used as auxiliary therapy in diabetic foot ulcers as the leaves possesses scientifically validated traditional use in diabetes.

Keywords: *Tectona grandis*, Verbenaceae, Epidermal migration, ex-vivo wound healing, Trace element, Energy Dispersive X-ray Spectrometer

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INTRODUCTION

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury an inflammatory response occurs and the cells below the dermis begins to increase collagen production. Later the epithelial tissue is regenerated [1]. Wound healing management is a complicate and expensive one. So that research on drug which enhances the wound healing process is a thrust area in drug research [2]. Present days, wound healing regimen mainly synthetic chemical moieties (mostly antibacterial) which possess a wide range of side effects. Therefore research needed on herbals with devoid of side effects which associated with synthetic one and wound healing potential of many of traditional medicinal plants remain unexplored [3]. So there is need of the hour to identify the various medicinal plants or their chemical constituents and formulated into convenient form for treatment and management of wounds. Medicinal plants have been reported to be very beneficial in wound care, promote the rate of wound healing with minimal scar [4]. The *T.grandis*(Teak tree) leaf is used for tuberculosis, various kinds of wounds especially burn wound, malaria, anaemia, leprosy, skin diseases, indolent ulcers, haemorrhages, menstrual disorder, bone joint disease, cooling, haemostatic, depurative, anti-inflammatory, diabetes II, constipation, blood dysentery, astringent, vermifuge, anti-cancer[5-17]. The present study investigate the wound healing effect of the TGEAE as it contains flavonoid fraction using *ex-vivo* porcine skin wound healing model (PSWHM). Further it was reported that the leaves of *T.grandis* contains interesting triterpenoid of nature, betulinic acid[13] as it possesses many beneficial effects like anti-cancer, anti-bacterial, anti-malarial, anti-HIV, anthelmintic, anti-inflammatory and anti-oxidant properties etc[18,19]. More over trace elements like Magnesium (Mg), Potassium (K), Calcium (Ca), Phosphorous (P) supports wound healing activity as essential trace mineral are required for cellular growth and replication. Survey of available literature showed that there was no report available on the trace element content of the leaves. So we have decided to estimate the trace element content of the leaves. Pig skin architecture (in both physiological and anatomical) is similar to human skin [20]. Further PSWHM is an excellent model system due to its high reproducibility, easy to handle, economical, without the need of ethical clearance[21]. Epidermal regeneration is an important part of cutaneous wound healing, causing permanent closure of wound and restoration of essential functions of the skin. This process involving the keratinocyte migration and proliferation at the margin of wound and variety of interaction with component of the dermis. Here we want to emphasise the traditional use of the leaves for the treatment of diabetes and the several supportive scientific research of

this claim as anti-diabetic [7, 22, 23]. The common complication of diabetic patient is wound as an adverse effect which is an enormous burden on the health care system, both in terms of cost and intensity of care required. Hence this prompted us to investigate the effect of the flavonoid rich on PSWHM – a novel *ex-vivo* wound healing model. In this model epidermal or keratinocyte migration was measured.

MATERIALS AND METHODS

Pig ears (6month old), Biopsy punch (6mm, 3mm); Phosphate Buffer Solution (PBS), 70% ethanol, Hemotoxylin/eosin, Ethyl acetate extract of *T. grandis* leaves (TGEAE), Mupirocin ointment. All chemicals used are SD fine chemicals. For the determination of trace element by Energy Dispersive X-ray Spectrometer and CAMAG HPTLC with win CATS 1.4.3 software, densitometry TLC scanner (520nm) was used for HPTLC analysis, Rotavapor RII, Buchi, CO_2 incubator.

Collection and authentication of the leaves of *T.grandis:*

The leaves of the healthy *T.grandis* selected for our study was collected from Karungal, Kanyakumari (Dt), Tamil nadu. It was identified, and authenticated by Dr. Stephen, Taxonomist, Dept. of Botany, The American College, Madurai and Dr. Sasikala, Director of Siddha Research Centre, Arunbakkam, Chennai, Tamil nadu, India and. A voucher specimen was deposited at the herbarium of Dept. of Pharmacognosy, Madurai Medical College, Madurai, Tamil nadu, India (PCG-278).

Preparation of extract:

The leaves were dried at room temperature under shade and powdered, sieved (60mesh) and stored in a well closed container. Extracted with ethyl acetate and filtered (TGEAE), evaporated under vacuum The pale green residue obtained was stored in the refrigerator until further use.

Preparation of TGEAE ointment:

1%, 2%, 3%TGEAE ointment was prepared by using simple ointment base IP.

Preliminary phytochemical screening:

Preliminary phytochemical screening was carried out to find out the presence of various phytoconstituents using standard procedure[24-27].

Determination of Total Phenolic Content:

The total phenolic content of extracts was determined by Folin- Ciocalteau method [28,29]. The extracts were oxidized with Folin- Ciocalteau reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting solution was measured at 760 nm after 20min.

Using gallic acid as standard total phenolic content (standard curve was prepared using concentrations 2,4,6,8,10 µg/ml) was expressed as mg GA equivalent/ gram of extract.

Elemental Analysis by EDS:

Scanning Electron Microscope model JSM-5610 LV with an EDS microanalysis attachment was used to study the microscopical characters and elements present [30].

Preparation of solid sample:

Mix equal volume of powder and binder pressed up to 30 ton made into pellet. The binder must be free from contaminant element and low absorption. It must stable under vacuum and irradiation conditions.

HPTLC profile of TGEAE:

Development of HPTLC Fingerprint

Instrument

CAMAG TLC Scanner 3 "Scanner3-070408"S/N 070408(1.41.21) was used for detection and CAMAG Linomat 5 sample applicator was used for the application of the track. Twin trough plate development chamber was used for development of chromatogram. Software was used Win CATS 1.43 at 254nm.

Sample

The TGEAE was dissolved in ethyl acetate to get a concentration of 4,8 µl of this solution was used for taking HPTLC fingerprint.

Stationary Phase

Aluminium sheets pre-coated with silica gel 60 GF254 HPTLC plates were used as a stationary phase.

Mobile Phase

Toluene: Ethyl acetate: Formic acid: Methanol (3:6:1.6:0.4) was used as the mobile phase for development of chromatogram. The mobile phase was taken in a CAMAG twin trough glass chamber.

Detection Wavelength

The developed plates were examined at wavelength 254 nm in CAMAG TLC scanner 3. The TLC visualization, 3D display of the finger print profile and peak display at 254 nm.

Effect of TGEAE leaves on *Ex-Vivo* Porcine Skin Wound Healing Model

Wound healing evaluated by *ex-vivo* porcine skin wound healing model (PSWHM). Porcine (6 months old) ears obtained from the local slaughter house were washed with PBS and disinfected with 70% ethanol. Circular porcine skin (6mm diameter) taken out from the inner side of the ear

by using sterile circular biopsy punch. Subsequently on the excised portion small circular wound (3mm diameter) was made by using sterile circular biopsy punch. Epidermis and upper dermis was removed from the centre for making the wound under sterile conditions. The PSWHM were divided into five groups (n=6). 1%, 2%, 3% test drug (TGEAE) ointment, standard drug (Mupirocin) 2% ointment treated and immersed in PBS along with control in triplicate. The PSWHM kept in CO_2 incubator at 37°C for 2 days. Histopathological evaluation was done after staining with hematoxylin / Eosin. The migration was normalized with the PBS group and expressed as mean % ± SE. statistical analysis was performed using one way analysis of variance (ANOVA). $p<0.01$ was considered to be statistically significant [31].

RESULTS AND DISCUSSION:

Preliminary phytochemical screening showed the presence of flavonoids, steroids, tannins,anthraquinone glycosides,carbohydrates,saponins,terpenoids, proteins and aminoacids.The total phenolic content of TGEAE in terms of gallic acid was found to be 22.2µg/g. The leaves were also subjected to elemental analysis by Energy Dispersive X-ray Spectrometer (EDS or EDAX) which was in connection to the SEM.EDS results showed K (9.24%), Ca (2.19%) S(0.48%), P (0.97%), Mg (0.62%).Determination by HPTLC analysis of TGEAE showed 0.7%apigenin(Figure 1&2, Table 1).

Standard Apigenin **Sample TGEAE**

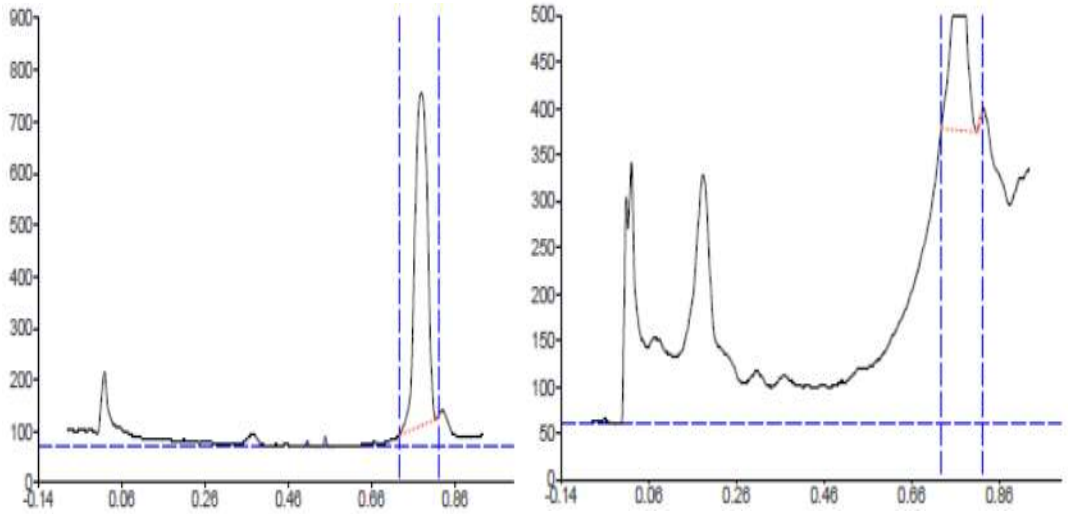

Figure 1 HPTLC peak display

Table-1 Rf value of TGEAE

SI NO	Samples	Start Rf	Max Rf	End Rf	Area	Area %
1	Standard Apigenin	0.73	0.78	0.81	15860.2	100.00
2	Sample TGEAE	0.73	0.77	0.81	5696.2	100.00

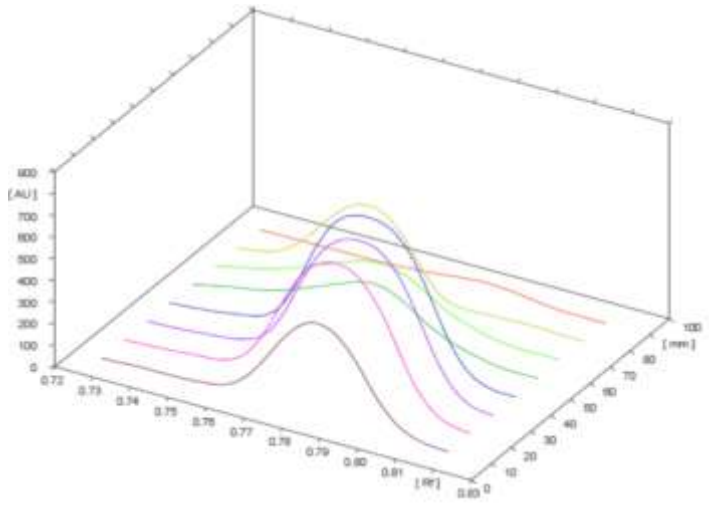

Figure 2: CO-HPTLC profile of TGEAE showing the presence of Apigenin3D display

Histopathological evaluation showed all treated wounds were sound with no signs of apoptosis, necrosis or bacterial contamination and no toxicity of the tested concentrations of TGEAE of the leaves. Morphology of the wound margins, epidermis and dermis layer were found to be normal. Epidermal migration or keratinocyte migration distances from the edges of each wound were measured, normalized with the PBS control group and expressed as mean%. The result clearly showed TGEAE (3%) statistically significant wound healing effect which is comparable to the standard drug Mupirocin (Figure 3&4).

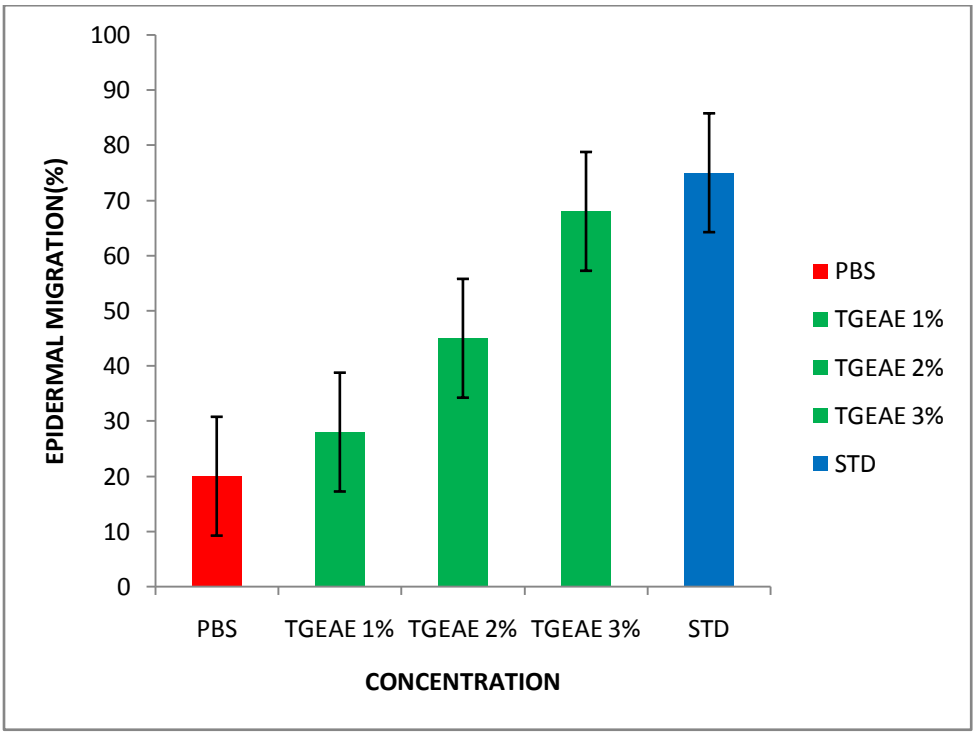

Figure 3: Effect of the TGEAE leaves on *ex-vivo* porcine skin wound healing model

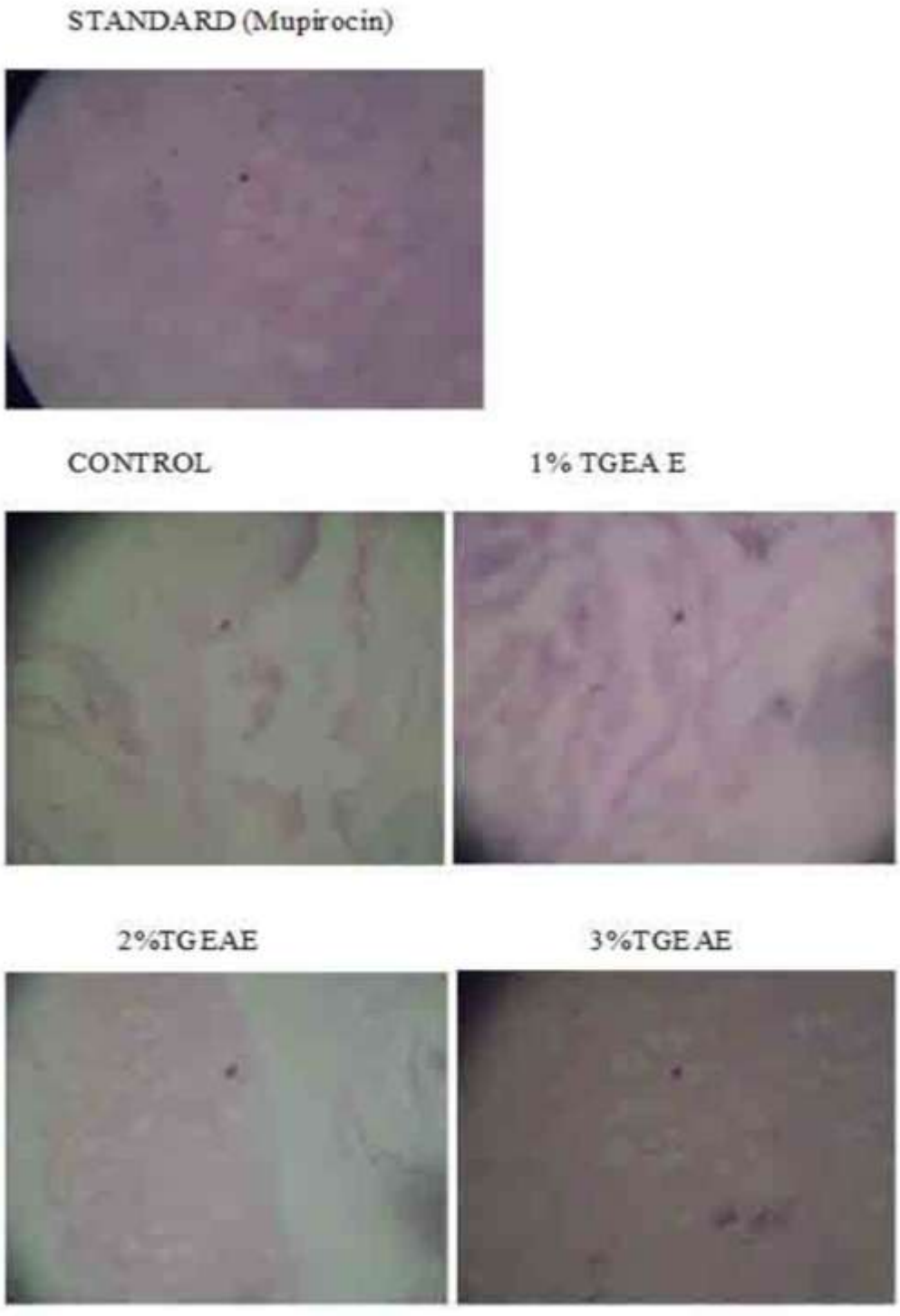

Figure 4: Histology showing epidermal layer migration of PSWHM

Wound healing is a dynamic and complex process in which the tissue layer of damaged tissue & cellular structure are restored into its normal state as closely as possible [32]. Basically healing is the natural body process of regenerating dermal and epidermal tissue [33]. So rapid healing of wound needed to provide suitable conditions that can regenerate the damaged tissue [34]. In recent

years, phytochemical constituents of plants with varied pharmacological, physiological and biochemical activities have received attention. Studies have shown that *T.grandis* contains many classes of compounds such as flavonoids, steroids, tannins,anthraquinone glycosides,carbohydrates,saponins,terpenoids, proteins and aminoacids. Significant anti diabetic[22,35], antimicrobial[36-38] antioxidant[36,39], analgesic and anti-inflammatory[40,41], anti-hypertensive activity[42],wound healing activity[11,12,43-46] have been reported. Plant phenolics act as primary anti-oxidants or free radical scavangers[47]. Lipid peroxidation is an important process in burns, wounds and skin ulcers. Collagen fibrils viability increases by inhibiting lipid peroxidation which cause increases the strength of collagen fibres. Finally prevents cell damage and promotes DNA synthesis[48,49]. Therapeutic potential phenolic compounds like anti-infective, anti-inflammatory as well as wound healing by decreasing lipid peroxidation which improve vascularity, increase collagen synthesis and promotes cross linking of collagen[50]. In our study total phenolic and apigenin content of TGEAE was found to be 22.2µg/g GAE 0.7% respectively. EDS study showed the presence of calcium (2.19%), potassium (9.24%), magnesium (0.62%), sulphur (0.48%), phosphorous (0.97%). Flavanoids, triterpenoids (apigenin) known to have astringent property which is responsible for wound contraction and increased rate of epithelialization along with the supportive anti-microbial activity . Trace elements are considered the "inorganic switches" in various medicinal systems. This concept has gained ground in Ayurveda and the traditional medicine. From the reports it is assumed that the higher trace elements content reported in EDS analysis might have also enhance the wound healing property. It is assumed that this effect may be due to the phenolic content, apigenin and the influence of Magnesium, Phosphorous, Sulphur, Potassium, Calcium content and antioxidant activity. The present finding provides scientific evidence to ethnomedical properties of *T.grandis* leaves in wound healing property. Here we want to emphasise the traditional use of the leaves for the treatment of diabetes and the several supportive scientific research of this claim as anti-diabetic [22, 35]. The common complication of diabetic patient is wound as an adverse effect which is an enormous burden on the health care system, both in terms of cost and intensity of care required.

CONCLUSION

Our study showed significant enhancement of wound repair and therefore can be beneficially, safely used as auxiliary therapy in diabetic patient with foot ulcers in addition to the other available treatment. Further investigations are needed with purified constituents to understand

the complete mechanism of wound healing process.

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