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Research Article

WOUND HEALING ACTIVITY OF THE LEAVES OF *Artocarpus heterophyllus* Lam. (Moraceae) ON *ex-vivo* PORCINE SKIN WOUND HEALING MODEL

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ABSTRACT

Objective: To prescreen the *ex-vivo* wound healing activity of flavonoid rich fraction of ethyl acetate extract of the leaves of *Artocarpus heterophyllus* Lam. Family Moraceae using porcine skin wound healing model (PSWHM) along with phytochemical, XRF, HPTLC analysis. The aim of this present study is to provide pharmacological validation to the traditional claim for wound healing activity of *Artocarpus heterophyllus* leaves.

Method: Total phenolic content by UV spectral methods and ursolic acid content by HPTLC, trace elements by X-ray fluorescence were determined. The wound healing effect of the ethyl acetate extract of the leaves of *A.heterophyllus* (EAAH) was evaluated using *ex- vivo* porcine skin wound healing model - a novel organ culture model system for evaluation of drugs in cell-cell junction in the wound healing process.

Results: Total phenolic content by UV method, HPTLC determination of ursolic acid content of EAAH was found to be 376.5mg/g GAE, 134mg/g respectively. XRF study showed the presence of calcium (39.4%), potassium (29.6%), magnesium (2.06%), Iron (0.99%), sulphur (1.83%), Zinc (0.083%), strontium (0.23%), Manganese (0.13%) and Aluminium (0.005%). 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 EAAH 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 EAAH (1.5%) promoted statistically significant (Anova $p<0.05$) dose dependent wound healing effect which is comparable to the standard drug Mupirocin.

Conclusion: This study indicates that the ethyl acetate extract of the leaves of *A.heterophyllus* possesses potential wound healing activity on *ex-vivo* porcine skin wound healing model. Wound healing activity of EAAH (leaves) may be due to its phenolic content (flavonoids), triterpenoids constituents especially ursolic acid. Both of them known to have astringent property which is responsible for wound contraction and increased rate of epithelialisation along with the supportive anti-microbial activity. More over trace elements like Zinc (Zn), Copper (Cu), Manganese (Mn), Iron (Fe) supports wound healing property as essential trace mineral are required for cellular growth and replication. This present investigation provides scientific evidence to ethnomedical use of *A.heterophyllus* leaves in wound healing activity. 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 as the leaves possesses scientifically validated traditional use in diabetes.

Keywords: *Artocarpus heterophyllus*, Moraceae, Epidermal migration, *ex-vivo* wound healing, Trace element, X- Ray Fluorescence (XRF) Spectrometer, HPTLC

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 posses 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 *A.heterophyllus* (Jackfruit) leaf is used for asthma, wound healing, ring worm infestation, gallstones, abscesses, antishyphillic, anthelmintic, lactogogue, ear ache, antiulcer, anticariogenic, adsorbent, antibacterial, anti-inflammatory, anemia, dermatitis, cough, diarrhea, fever, sedative, digestive [5, 6].

The present study investigate the wound healing effect of the EAAH as it contains flavonoid fraction using *ex-vivo* porcine skin wound healing model (PSWHM). Further it was reported that the roots of *A.heterophyllus* contains interesting triterpenoid of nature, ursolic acid as it possesses many beneficial effects like anti-inflammatory, hepatoprotective, antibacterial, antiulcer etc [7]. It prompted us to identify and determine the presence of ursolic acid in the leaves also. More over trace elements like Zinc (Zn), Copper (Cu), Manganese (Mn), Iron (Fe) supports wound healing property 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 [8]. Further PSWHM is an excellent model system due to its high reproducibility, easy to handle, economical, without the need of ethical clearance [9]. 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 emphasize the traditional use of the leaves for the treatment of diabetes and the several supportive scientific research of this claim as anti-diabetic [10, 11]. 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 PSWHMs – a novel *ex-vivo* wound healing model. In this model epidermal or keratinocyte migration was measured.

MATERIALS & METHODS

Pig ears (6month old), Biopsy punch (6mm, 3mm); Phosphate Buffer Saline (PBS), 70% ethanol, Hemotoxylin/eosin, Ethyl acetate extract of *A.heterophyllus* leaves (EAAH), Mupirocin ointment. All chemicals used are Sd fine chemicals. For the determination of trace element by X-ray fluorescence Bruker S-4 pioneer and CAMAG HPTLC with win CATS 1.4.3 software, Densitometry TLC scanner (520nm) for HPTLC analysis and CO_2 incubator were used in this experiment.

Collection and authentication of the leaves of *A.heterophyllus*:

The leaves of the healthy *A.heterophyllus* selected for our study was collected from Suchindram, Kanyakumari (Dt), Tamil Nadu. It was identified, and authenticated by Prof. Dr. P. Jayaraman, Director of Plant Anatomy Research Institute, Tambaram, Chennai, Tamil Nadu, India and Dr. Stephen, Taxonomist, Dept. of Botany, The American College, Madurai. A voucher specimen was deposited at the herbarium of Dept. of Pharmacognosy, Madurai Medical College, Madurai, Tamil Nadu, India (PCG-276).

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, evaporated under vacuum (Rotavapor RII, Buchi). The pale green residue obtained (EAAH) was stored in the refrigerator until further use.

Preparation of EAAH ointment: 0.5, 1, 1.5% EAAH ointment was prepared by using simple ointment base IP.

Preliminary phytochemical analysis of EAAH

Preliminary phytochemical screening of EAAH was carried out to identify the presence of various phytoconstituents like flavonoids, sterols, carbohydrates, proteins, tannins, phenolic compounds, alkaloids, volatile & fixed oils, glycosides such as anthroquinone, cardiac, cyanogenetic and isothiocyanate [12-15].

Determination of Total Phenolic Content

The total phenolic content of extracts was determined by Folin-Ciocalteau method [16]. 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 25-50 mg/L) was expressed as mg GA equivalent/L of extract.

Elemental analysis by XRF Spectrometer:

We have quantitatively determined the trace elements present in the *A.heterophyllus* leaves by X-Ray fluorescence spectrometer (XRF) which has the advantage generally being non-destructive, multi elemental, fast & cost effective [17, 18].

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 EAAH

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 Win CATS 1.4.3 was used.

Sample

The EAAH was dissolved in ethyl acetate to get a concentration of 2mg/ml and 2µl of this solution was used for taking HPTLC fingerprint.

Stationary Phase

Aluminium sheets pre-coated with silica gel Merck G F_{254}, 0.2mm layer thickness were used as the stationary phase.

Mobile phase

Toluene: Ethyl acetate: Methanol (7:2:1) 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 520nm in Densitometry TLC scanner 3. The TLC visualization, 3D display of the finger print profile and peak display at 520nm.

Effect of EAAH leaves on *ex-vivo* Porcine skin wound healing model [19]

Wound healing evaluated by *ex-vivo* porcine skin wound healing model (PSWHM). Porcine (6 months old) ears were 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 PSWHMs were divided into five groups (n=6). 0.5%, 1%, 1.5% test drug (EAAH) ointment, standard drug (Mupirocin) 2% ointment treated and immersed in PBS along with control in triplicate. The PSWHMs 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 value 0.01 was considered to be statistically significant.

RESULT

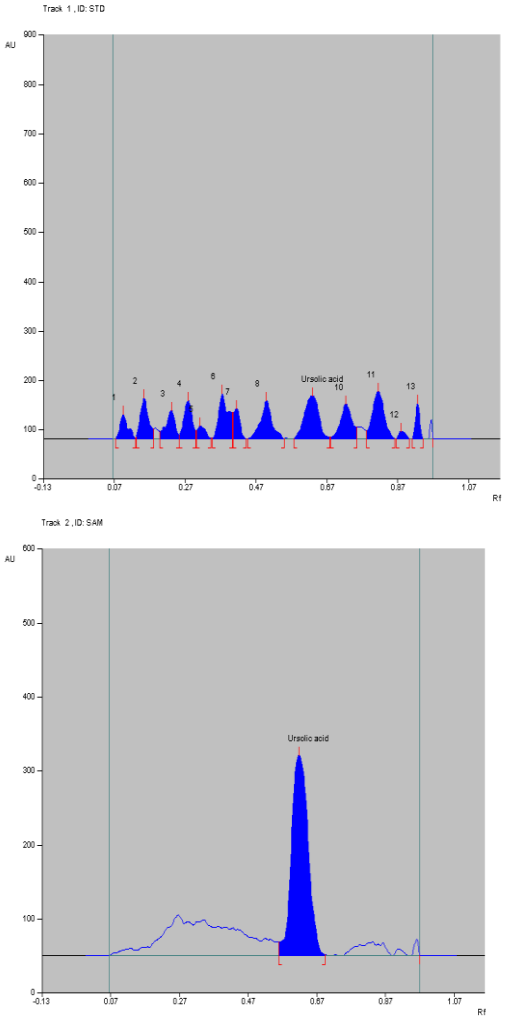
Preliminary phytochemical screening of EAAH leaves showed the presence of flavonoids, sterols, carbohydrates, proteins, tannins, phenolic compounds and absence of alkaloids, volatile & fixed oils, glycosides like anthroquinone, cardiac, cyanogenetic and isothiocyanate.

The total phenolic content of EAAH was found to be 376.5mg/g.

Trace element content by XRF analysis showed the presence of Calcium (39.4%), Potassium (29.6%), Magnesium (2.06%), Iron (0.99%), Sulphur (1.83%), Zinc (0.083%), Strontium (0.23%), Manganese (0.13%) and Aluminium (0.005%).

HPTLC analysis of EAAH contains 134mg/g of ursolic acid (Figure 1&2, Table 1).

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 EAAH 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%. Measurement of epidermal migration distances from the wound showed EAAH (1.5%) statistically significant (Anova, $p<0.05$) dose dependent wound healing effect which is comparable to the standard drug Mupirocin (Figure 3 & 4).



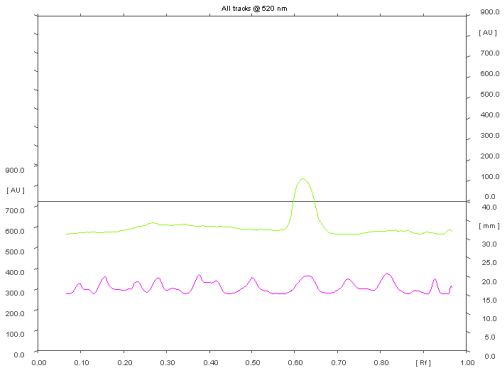


Figure 1: HPTLC peak display

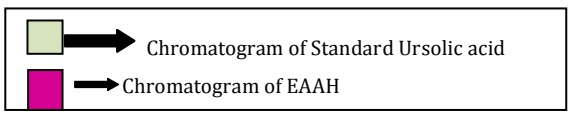


Figure 2: Co-HPTLC profile of EAAH showing the presence of Ursolic acid 3D display

Table 1: R_f value and area of separated compounds

S. No	@520nm			
	R_fValue		AREA (AU)	
	TRACK			
	STD	Extract	STD	Extract
1		0.10		781.2
2		0.15		1369.7
3		0.23		1063.7
4		0.28		1286.5
5		0.31		428.3
6		0.38		1813.0
7		0.42		801.7
		0.50		1748.4
	0.62	**0.63**	**9451.7**	**2534.1**
		0.72		1649.0
		0.82		2308.4
		0.88		211.7
		0.93		612.9

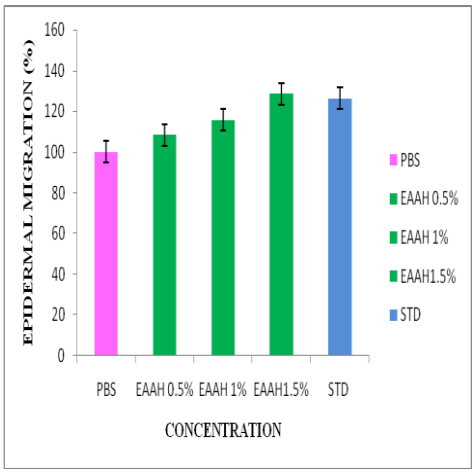
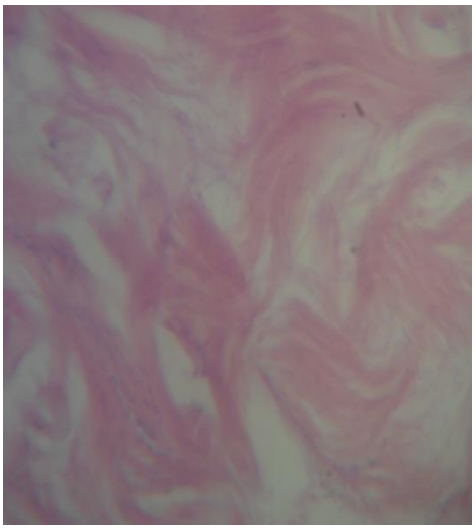


Figure 3: Effect of the EAAH leaves on *ex-vivo* porcine skin wound healing model



STANDARD

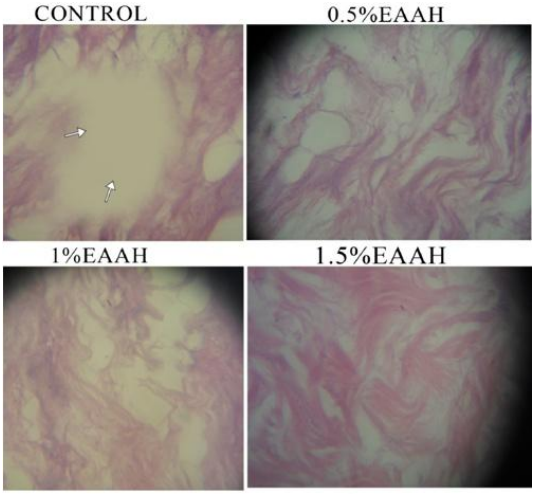


Figure 4: Histology showing epidermal layer migration of PSWHMS

DISCUSSION

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 [20]. Basically healing is the natural body process of regenerating dermal and epidermal tissue [21]. So rapid healing of wound needed to provide suitable conditions that can regenerate the damaged tissue [22]. In recent years, phytochemical constituents of plants with varied pharmacological, physiological and biochemical activities have received attention. Studies have shown that *A.heterophyllus* contains many classes of compounds such as flavonoids, volatile acids, sterols and tannins [6]. Antibacterial activity [23], anti cariogenic [24], hypoglycaemic [10, 11], anti cancer, α-amylase inhibitory, anti asthmatic, anti syphilic, vermifuge, lactogogue, analgesic, anti ulcer activities of leaves of *A.heterophyllus* have also been reported[25]. Plant phenolics act as primary anti-oxidants or free radical scavangers [26]. 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 [27, 28]. 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 [29]. In our study it was found out that EAAH contains 376.5mg/g phenolic content. It was observed that the root of *A.heterophyllus* contains beta sitosterol, ursolic acid, betulinic acid, cycloartenone and artoflavanone [5]. Ursolic acid and oleanolic acid are pentacyclic triterpenoids that are present in many medicinal herbs and other plants. It was reported that they are anti-inflammatory, hepatoprotective, analgesic, cardiotonic, etc. [7]. It prompted us to find out the presence of ursolic acid in the leaf of *A.heterophyllus*. In our investigation ursolic acid was found out that EAAH contains 134 mg/g of ursolic acid. Flavanoids, triterpenoids (ursolic acid) known to have astringent property which is responsible for wound contraction and increased rate of epithelialisation along with the supportive anti-microbial activity [30]. Mineral contents of various medicinal plants correlated with their therapeutic action by numerous studies [31, 32]. Trace elements are considered the "inorganic switches" in various medicinal systems. This concept has gained ground in Ayurveda and the traditional Indian medicinal systems [33]. More over trace elements like Zinc (Zn), Copper (Cu), Manganese (Mn), Iron (Fe) supports wound healing property as they are required for cellular growth and replication [34]. Zinc plays essential role in protein and collagen synthesis, tissue growth and healing [35]. Iron plays a role in collagen production by providing oxygen to the site of the wound [36]. Copper accelerates wound closure with more hyper proliferative epithelial tissue and density of the cells in the granulation layer is high through Vascular Endothelial Growth Factor (VEGF) expression [37]. From the reports it is assumed that the higher trace elements content reported in XRF analysis might have also enhance the wound healing property. It is assumed that this effect may be due to the phenolic content, ursolic acid and the influence of Zinc, iron, copper, manganese content and antioxidant activity. The present finding provides scientific evidence to ethnomedical properties of *A.heterophyllus* leaves used in wound healing. Here we want to emphasize the traditional use of the leaves for the treatment of diabetes and the several supportive scientific research of this claim as antidiabetic [10, 11]. 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.

Conflict of interest statement:
We do not have any conflict of interest.

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