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PHYTOCHEMICAL SCREENING AND *IN-VITRO* CORNEAL WOUND HEALING ACTIVITY OF THE LEAVES OF *MANILKARA ZAPOTA (L)* VAN ROYEN Var. PKM1 IN NEWER HERBAL DRUG DEVELOPMENT

Swarnakumari S.[1*], Periyanayagam K.[2], Ida Christi V. E.[1] and Mohan S.[3]

[1]Department of Pharmacognosy, Karpagam College of Pharmacy, Coimbatore, Tamil Nadu, India.

[2]Department of Pharmacognosy, Madurai Medical College, Madurai, Tamil Nadu, India and

[3]Department of Pharmaceutics, Karpagam College of Pharmacy, Coimbatore, Tamil Nadu, India.

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***Corresponding Author**
Swarnakumari S.
Department of Pharmacognosy, Karpagam College of Pharmacy, Coimbatore, Tamil Nadu, India.

ABSTRACT

Research on wound healing is a developing area in modern biomedical science. A large number of plant / plant extract / decoctions or paste are equally used by tribal and folklore traditions in India for treatment of cuts, wounds and burns. Preliminary phytochemical screening was carried out with solvents of different polarities reveals the presence of carbohydrates, proteins and amino acids, flavonoids, terpenoids, tannins and phytosterols. Ethanolic extract of leaves indicates the presence of flavonoids while in other extract it seems to be negative. Developing *in vitro* bovine model, a new avenue for the exploration of newer drug development in ophthalmic diseases has been furnished. This system seems to be very rapid, economic and reliable. Study aims to reveal the significant wound healing dose dependent corneal wound healing effect of ethanolic extract of the leaves of *Manilkara zapota (L)*. The mechanism of action may be due to the presence of bioflavonoid by its antioxidant property. In cornea, EGF epidermal growth factor is thought to be the main chemical mediator but in the skin no single factor has been identified as the major regulator. It is not clear whether upregulation of miotic activator (or) down regulation of a miotic inhibitor was responsible for the high level of cell division post wounding.

KEYWORDS: Bioflavonoids, *In-vitro*, Ophthalmic, Wound healing, Ethanolic extract, *M. zapota* Leaves.

INTRODUCTION

Health is Wealth – Herb is Health. Now–a–days Traditional medicine is fetching progressive attention towards the health care provision. Traditional use of medicines is acknowledged as a smart way to learn about potential future medicines. People use herbal medicines to try to sustain or meliorate their health since, many people believe that "natural" products are always harmless and good for them.[6] Plants have a very good prospective for producing new drugs for the welfare of mankind.[19] Phtyochemicals are nothing but secondary metabolites which are present in any parts of the plant but more accumulation was seen in leaves.[5]

Vision is probably most important of the senses. In which cornea, exposed area of the eye is an gateway for the external images and thus liable to suffer damages. Cornea is a clear, limpid, and elliptical a vascular structure with smooth shining surface, accounts for more than two-thirds of the total refractive power of the eye.[13] Corneal epithelium, the upper most layer of the cornea is composed of non keratinized, stratified squamous, and epithelial cells. They acts as protective barrier and also modulates fluid transport to maintain normal stromal hydration.[14] Continuous work of epithelium may be interrupted by various mechanical, thermal, chemical and biological insults, often results in wound formation.[11] Mechanical injury seems to be most prevailing one, which ranges from simple corneal excoriation to pervade refractive surgery.[1-2] Corneal wound healing is a prodigious problem and involves a number of conjunctive processes, including cell migration, proliferation, cell matrix adhesion, and tissue remodeling, which are impelled by growth factors and other factors released coordinately into the injured area by epithelial cells[15,24] and retrieve within a period of 7 days.[10] In this work attempts have been made to improve corneal wound healing, collaterally repress the time taken for wound healing.

Manilkara zapota commonly known as sapodilla belongs to family Sapotaceae, is a evergreen tree which extensively cultivated in coastal India for their edible fruit. Traditionally decoction from leaves were used to treat cough, cold, diarrhea, fever, haemorrhage, wounds and ulcers.[19] The ethanolic extract of Leaves of *M.zapota* seems to be potent drug for the treatment against cancer, tumor, oxidative stress and many infective diseases.[3,9] Since no elaborated scientific data were available concerning the wound-healing activity of *Manilkara zapota*, the present study was planned to explore the same.

MATERIALS AND METHODS

Collection of plant material

The leaves of the plant *Manilkara zapota*Van Royen var. PKM1 selected for our study was collected from The Department of Horticulture, Agriculture University, Madurai, Tamilnadu, India.

Preparation of plant extracts

Fresh plants were washed thoroughly 3-4 times with running tap water then finally with sterile water followed by shade drying at room temperature for 15-20 days. The dried plant material was made into coarse powder and passed through sieve and then used for crude extraction. Fine powder (5gm) was extracted in 100 ml of respective solvent at 50-55°C for 24 hours in rotary shaker at room temperature. The extract was filtered through Whatman filter paper No.1 and then concentrated by using a rotary evaporator at low temperature (40-50°C) and reduced pressure. Further, the dried residue was preserved in airtight container and kept at 4-5°C until further use.[17] The residue prevailed were used for the phytochemical screening.

Phytochemical screening

Thus different extracts obtained were qualitatively tested to determine the presence of various chemical constituents.[8, 12, 22]

In vitro Corneal wound healing activity

Culture Medium

All the medium and chemicals used were prevailed commercially and of analytical grade. Minimum essential medium (without sodium bicarbonate) powder of about 9.6g was suspended in 900 ml of sterile double distilled water with constant stirring, until medium was completely dissolved. (Heating avoided). About 2.2g of sodium bicarbonate powder for 1 litre of medium was added and stirred until dissolved. pH is adjusted to 0.2 – 0.3 pH using 1 N HCl or NaOH since pH tends to rise during filtration. Final volume is made up to 1000 ml with sterile water. Medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron or less. Packed into sterile container and the liquid medium were stored at 2 – 8^0C in dark until use.

Bovine eyes culture / organ culture

Normal Bovine eyes were obtained from a local abattoir immediately after death. In sterile environment, Saline used to remove adhering tissue and drops of Gentamycin eye drops were used to avoid unwanted infections. Eyes are suffused with culture medium and room temperature retained 35^0C, with humidification. The medium should cover upto limbal conjuctiva, whereas leaving the epithelium exposed to air. The intra ocular pressure was maintained about 18 mm Hg. Medium was suffused drop wise periodically over the surface of cornea to avoid dryness. [20, 21, 23]

Corneal Wounding

A circular trephine mark was made by disposable trephine in the center of the cornea to mark the wound area. Surgical scalpel blade was used to create a superficial epithelial wound. The epithelium within the trephine marked area were scraped away and corneal surface was rubbed with cotton tipped applicator so as to remove debris. 0.5% fluorescein was applied on the corneal wound for observing the extent of re-epithelization. After saline wash, the exposed basement membrane retains the fluorescein stains. Wounded area was viewed under 10x microscope and also under dissection microscope. Cornea without wound used as a positive control and untreated without wound considered as a negative control. The ethanolic extract of leaves of various concentrations was analysed.

Analysis of Wound Area

Rates of wound closure were monitored by staining with fluorescein and recorded the average radius of remaining wound. Corneas were allowed to heal for 48 hrs, until the fluorescein failed to stain the cornea, which was reported as the time of wound closure. Diameter of wound was measured using calibrated eye piece micrometer. Area of remaining wound and percentage of wound area healed were calculated. Statistical significance was calculated using student t - test

RESULTS AND DISCUSSION

The leaves powder with various extracts were subjected to preliminary phytochemical screening and presented in **Table 1.** The preliminary phytochemical screening reveals the presence of carbohydrates, proteins and amino acids, flavonoids, terpenoids, tannins and phytosterols. Alkaloids, glycosides, saponins, volatile oil, fixed oil were found to be absent.

Table 1: Results for the preliminary phytochemical screening of leaf extracts of *Manilkara zapota*.

Tests	Petroleum ether extract	Ethyl acetate extract	Ethanolic extract	Aqueous extract
Alkaloids	-	-	-	-
Carbohydrates	-	-	-	+
Glycosides	-	-	-	-
Sterols	+	+	+	-
Saponins	-	-	-	+
Tannins	-	-	-	+
Protein	-	-	+	-
Mucilages	-	-	-	-
Terpenoids	-	-	-	-
Flavonoids	-	-	+	-
Volatile oil	-	-	-	-
Fixed oil	-	-	-	-

An *in vitro* model of rabbit cornea has been used to evaluate epithelial wound healing over a period of 72 hours.[4, 20] Bovine corneas utilized in this work were selected on the basis of its inexpensiveness and readily accessible also to avoid the deliberate sacrifice of animals. Numerous scientific demonstrated that bovine cornea has similar structure and a biochemical makeup as that of human cornea.[23] In the present investigation, Minimum Essential Medium (MEM) as well as M-199 medium with 5% dextran both was selected. M-K medium was recommended by previous researches for rabbit eye.[16] M-K medium is the mixture of medium 199 with 5% dextran and 100 units per ml of streptomycin and penicillin mixture (with pH7.4).In this medium, the cornea swelled it may be due to swelling pressure of stromal polysaccharides. For improved corneal storage, MEM medium seems to be preferable, in which cornea was viable about 60 hours in our study.This model provides considerable contribution for producing *in vitro* model for cornea and also clears that considerable scope remaining for improvement.

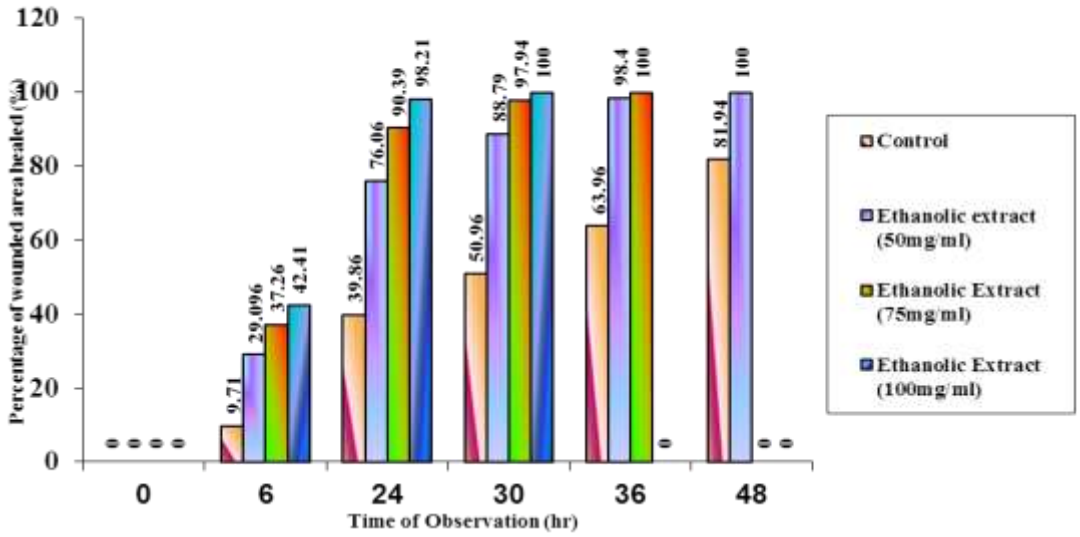
Corneal wound has been made and ascertained that enhancement of re-epithelialization takes place after fetching proper care to stroma and endothelial layer.[7] The percentage of wound healing compared to untreated wound was determined. Interestingly, the rate of wound healing was remarkable and dose dependent. The response of corneal epithelium to injury by migrating as a sheet to cover the defect was rapid, apparently and noted in table 2 and based on that charted in fig.1.

Table 2: Effect of various concentrations of Ethanolic extract of leaves on Corneal Wound Healing.

Drug	Concentration (mg/ml)	Time of observation (hours)	Area of wound (sq.mm)*	Percentage of wounded area healed (%)
Control	-	0	12.572	0
		6	11.3514(±0.4875)	9.71
		24	7.5563(±0.3978)	39.86
		30	6.1655(±0.3593)	50.96
		36	4.5311(±0.3079)	63.96
		48	2.2708(±0.2182)	81.94
Ethanolic extract	50	0	12.572	0
		6	8.9180(±0.6552)	29.096
		24	3.2607(±0.4034)	76.06
		30	1.4090(±0.2649)	88.79
		36	0.2016(±0.0642)	98.4
		48	0	100
Ethanolic extract	75	0	12.572	0
		6	7.888(±0.6132)	37.26
		24	1.208(±0.2465)	90.39
		30	0.259(±0.0891)	97.94
		48	0	100
Ethanolic extract	100	0	12.572	0
		6	7.241(±0.5993)	42.41
		24	0.225(±0.0405)	98.21
		30	**0**	**100**

** values ± SEM (P<0.05)*

Fig - 1
Effect of various concentrations of EEL and corneal wound healing



Bioflavonoids possess anti allergic, anti-inflammatory anti-viral, anti- carcinogenic and potent anti-oxidant activities and have influence on mast cell, basophils, neutrophils, eosinophils, T & B lymphocytes, macrophages, platelets, smooth muscle, hepatocytes and others on balance.[18]

The mechanism of action may be due to presence of bioflavonoid especially quercetin by its anti-oxidant action. Wound healing response of corneal epithelium is significantly different from that of other epithelia because of the location of stem cells to the limbus. It was already reported that in cornea, EGF epidermal growth factor is thought to be the main chemical mediator but in the skin no single factor has been identified as the major regulator. Indeed it is not clear whether up regulation of a miotic activator (or) down regulation of a miotic inhibitor was responsible for the high level of cell division post wounding.

CONCLUSION

The preliminary phytochemical screening indicates the presence of flavonoids which seems to be responsible for the activity. This model provides considerable contribution for producing *in vitro* model for cornea and also clears that considerable scope remaining for improvement. The above findings required the detailed investigation for its exact mechanism of action to provide a suitable drug candidate and to deduce a definite conclusion, as there is no treatment method is available for corneal wounds.

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