

WOUND HEALING ACTIVITY OF PUNICALIN AND PUNICALAGIN ISOLATED FROM *Punica granatum* L.

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ABSTRACT

This study aims to carry out the extraction and isolation of bioactive compounds from the peel of *Punica Granatum* Linn and investigate the wound healing activity of isolated compounds. Soxhlet extraction of P. Granatum peel powder was done with ethanol as solvent. The isolation of phytochemical constituents, Punicalin and Punicalagin present in the obtained extract, was done with the help of column chromatography using n-butanol and ethyl acetate fractions. The column was packed with silica gel, and 100-200 mesh was used as a stationary phase separately at different stages of the isolation process. All fractions obtained from column chromatography were subjected to HPLC, LCMS, FTIR, ¹HNMR studies to identify and confirm the obtained isolated bioactive. The study was further extended to assess the wound healing activity of isolated punicalin and punicalagin by using *in vitro* excision and incision models. The activity of prepared alcoholic extract and isolated bioactive was compared to standard 10 % w/w povidone-iodine ointment. The highest wound healing strength observed was 201.83 ± 4.98 for isolated 10% w/w punicalagin ointment in the incision wound model. Also, in the excision wound model, the highest % reduction in the wound was observed on the 15th day as 88 ± 0.78 , which was optimum compared to the standard with a % reduction in the wound as 92 ± 0.91 . As a result, bioactive punicalagin and punicalin obtained from methanol extract of *Punica Granatum* peel powder can be used as potent phytoconstituents and wound healing agents for pharmaceutical preparations.

Keywords: *Punica Granatum*, Bioactives, Wound Healing, Phytochemical, Isolation, Extraction, Punicalin, Punicalagin.

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INTRODUCTION

Punica Granatum (Pomegranate) is a tree having 5-8 meters of height mainly found in the northern Himalayan region, Iran, China, and the United States.^{1,2} Pomegranate is considered a wonder fruit as it serves as a reservoir of a large number of bioactive that are responsible for a wide variety of pharmacological actions.^{3,4} The fruit of *Punica Granatum* is traditionally used for the treatment of different pathogenic diseases.^{5,6} *Punica granatum* contains seven highly active carbonic anhydrase inhibitors Punicalin, punicalagin, granatin B, gallagylidilactone, punicalagin, pedunculagin, and tellimagrandin.^{7,8} Gallic acid, granatin A, corilagin, and ellagic acid are four weakly active inhibitors with antimicrobial, antifungal, and antimutagenic activity.⁹ Different polyphenols present as phytoconstituent in pomegranate are also known for their property of precipitation of proteins of animal hide, which forms one of the supportive clues for the wound healing property of *Punica Granatum* bioactive.¹⁰ Many extracts obtained from different parts of *Punica Granatum* have also been reported to treat different skin ailments and also for inflammatory conditions.^{11,12} Punicalin and Punicalagin come in the category of ellagitannins.^{13,14} The ellagitannins such as punicalin and punicalagin present in pomegranate possess many pharmacological activities such as anti-oxidant, hepatoprotective activity, anti-inflammatory and also helps as a supplement in some cases of prostate cancer.^{15,16} Therefore, this study investigates the wound healing property of bioactive like punicalin and punicalagin, isolated from the prepared methanolic extract of *Punica Granatum* Peel.

EXPERIMENTAL

Sample Preparation and Chemical Analysis

Punica Granatum samples were collected from the campus of Noida Institute of Engineering and Technology; Greater Noida authenticated for further use from the Botanical Garden, Noida, Uttar Pradesh.

The peels were manually removed, sun-dried, and changed to powder form by grinding the peels of the same. Uniformity of the powder was then achieved by passing the powder through a sieve of 40-micron size. This uniform-sized pomegranate peel powder was stored in a dry and airtight plastic bottle for final use. The powder was extracted with a Soxhlet extractor using Methanol for 36 hours. The extract was then dried under controlled temperature and pressure by the use of a rotary evaporator. The obtained extract was checked for its polyphenol content by the Folin phenol reagent method. Column Chromatography was then used to isolate phytochemical constituents (Punicalin and Punicalagin) by using the n-butanol and ethyl acetate different fractions. The column was packed with Silica gel 100-200 mesh was used as a stationary phase separately at different stages of the isolation process. All fractions obtained from column chromatography were subjected to outsourced HPLC, LCMS, FTIR, ¹HNMR, and ¹³CNMR techniques for analysis and identification of isolated bioactive.¹⁷

Wound Healing Activity

For wound healing activity evaluation, the ointment base of the methanol extract obtained and also of isolated bioactive were prepared by using the ointment base by using the ingredients (wool fat 5g, hard paraffin 5g, cetostearyl alcohol 5g, soft white paraffin 85g) as per British Pharmacopoeia (1980) in a beaker at 65°C in a water bath. After cooling, the mixture was homogenized with a homogenizer at 1500 rpm for 10–15 minutes.¹⁸

Evaluation of Wound-healing Activity

The current study used albino rats of 150-180 gms weight. The animals were kept in a cross-ventilated animal house. The Institutional Animal Ethics Committee approved the study protocol (Approval Number 1845/PO/Re/S/16 CPCSEA) by the guidelines of the Committee for Control and Supervision of Experiments on Animals in India.

The wound-healing activity of methanol and isolated constituents obtained from the extract of Pomegranate fruit was assessed using excision incision models.¹⁹ The rats were divided into five groups for the excision and incision wound models, each with six animals. Group I animals was the control group, while group II animals received methanol extract. Groups IV and V received 10 % w/w isolated compound ointment. Group III has given a 10 % w/w povidone–iodine ointment.²⁰

Excision Wound

The animals were treated with ketamine hydrochloride (100 mg/kg body weight) before and after the experimental wounds. Rats are then released into the wild. Rats are then inflicted with excision wounds, as described by Morton and Malone. The animal's dorsal fur was shaved with a razor to 500 mm full-thickness excision wound, and an electric clipper toothed forceps, a surgical instrument, was used to create a line along the marking. Blade and scissor with a pointed tip the wound were left completely open. All of the animals were treated in the same way that men were treated mentioned earlier. Tracing the healing of a wound was used to assess its progress in 4 days intervals for 16 days.²¹ After this percentage, wound contraction was calculated after every 3-day interval by using the formula given below and shown in Table -1.

Table-1: Percentage Wound Contraction in Excision Wound Model given as mean $\pm$ STD

Group	% Wound Contraction			
	4 th day	8 th day	12 th day	16 th day
Group 1 (Control)	10 $\pm$ 0.81	45 $\pm$ 0.67	65 $\pm$ 2.54	75 $\pm$ 0.91
Group 2 (extract)	4 $\pm$ 0.19	28 $\pm$ 0.65	55 $\pm$ 1.18	80 $\pm$ 1.19
Group 3 (Standard)	15 $\pm$ 0.18	50 $\pm$ 0.88	75 $\pm$ 1.36	92 $\pm$ 0.91
Group 4 (10 % w/w Punicalin)	5 $\pm$ 0.86	15 $\pm$ 0.46	50 $\pm$ 2.01	84 $\pm$ 0.43

Ointment 1				
Group 5 (10% w/w Punicalagin)	4 ± 1.0	18 ± 0.81	48 ± 0.79	88 ± 0.78
Ointment 2				

Percentage Wound Contraction = (initial wound area-specific day wound area) / initial wound area $\times 100$

Incision Wound

Before and during the creation of experimental wounds, rats were anesthetized with ketamine hydrochloride (100mg/kg).²² The animals' dorsal fur was shaved. Two paravertebral long incisions and shaved with an electric clipper 6cm long were pierced through the skin at a distance of approximately 1.5 cm on each side of the depilated back of the head from the midline animals previously described.²¹ Following that, the parted skin was stitched together at intervals of a few millimeters after the incision. Using surgical thread (No.000) and a curved needle, make a centimeter (Number 11). After that, the wounds were left undressed.²³ Every group of animals was given the treatment described above. On the eighth post-wounding day, the sutures were removed, and the treatment continued. On the tenth day, the skin breaking strength of the healed wound was measured using Lee's method.²⁴ The breaking strength of a healing wound is determined by the force required to disrupt it, as shown in Table-2.

Table-2: Tensile Strength in Incision Wound Model expressed as mean $\pm$ STD

Groups	Wound Breaking Strength
Group 1 (Control)	167.35 ± 3.51
Group 2 (extract)	190.35 ± 6.40
Group 3 (Standard)	209.76 ± 6.64
Group 4 (10 % w/w Punicalin) ointment 1	195.35 ± 5.40
Group 5 (10% w/w Punicalagin) ointment 2	201.83 ± 4.98

RESULTS AND DISCUSSION

All fractions obtained from column chromatography were subjected to outsourced HPLC, LCMS, FTIR, ¹HNMR and ¹³CNMR for analysis and identification of isolated bioactive.

In a High-performance liquid chromatography graph of phytoconstituents, retention time for punicalagin and punicalin were obtained as 17.4 and 14.81 min shown in Fig-1 and 2 compared with standard punicalagin and punicalin, which was obtained as 18 min and 15.12 min respectively.

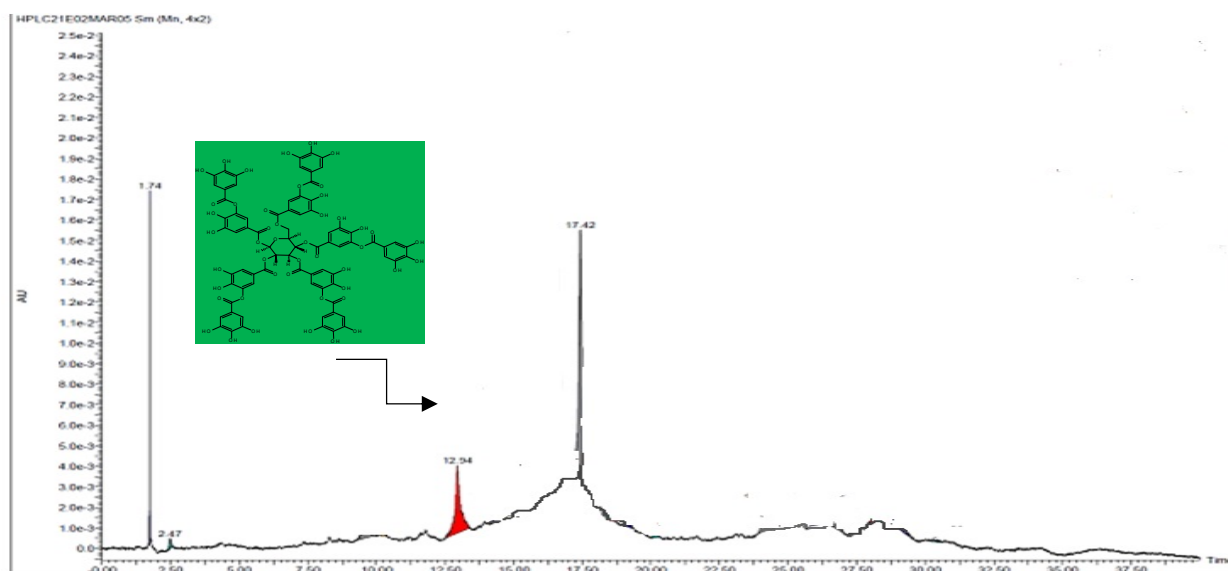


Fig-1: HPLC Graph of Isolated Punicalagin

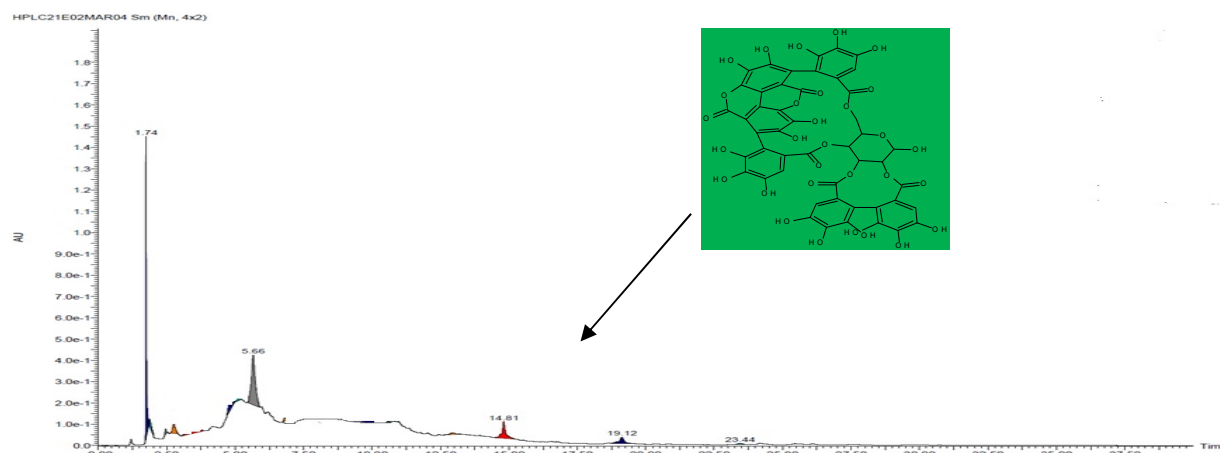


Fig.- 2: HPLC Graph of Punicalin

In FTIR of both isolated phytoconstituents, different peaks of functional groups were obtained, which justified the chemical structure of both isolated bioactive and given in Fig.-3 and 4.

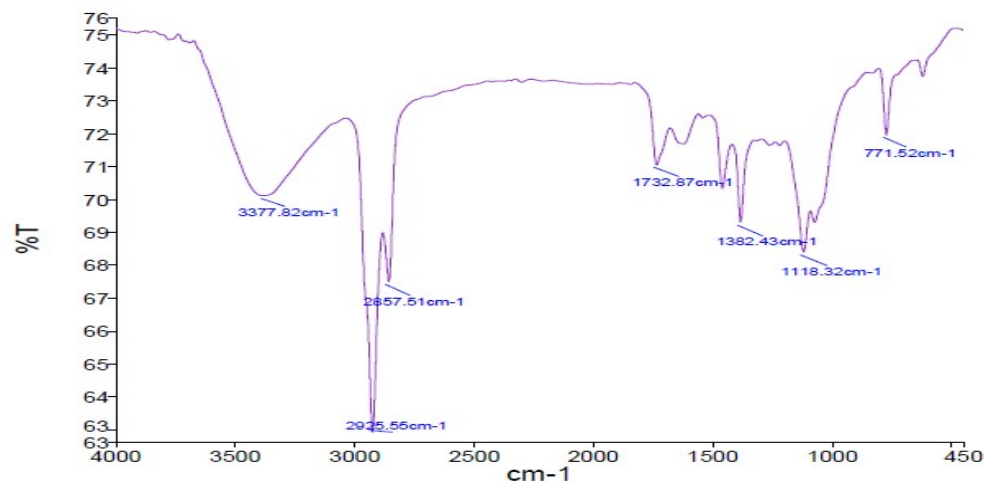


Fig.-3: FTIR Analysis of Punicalagin

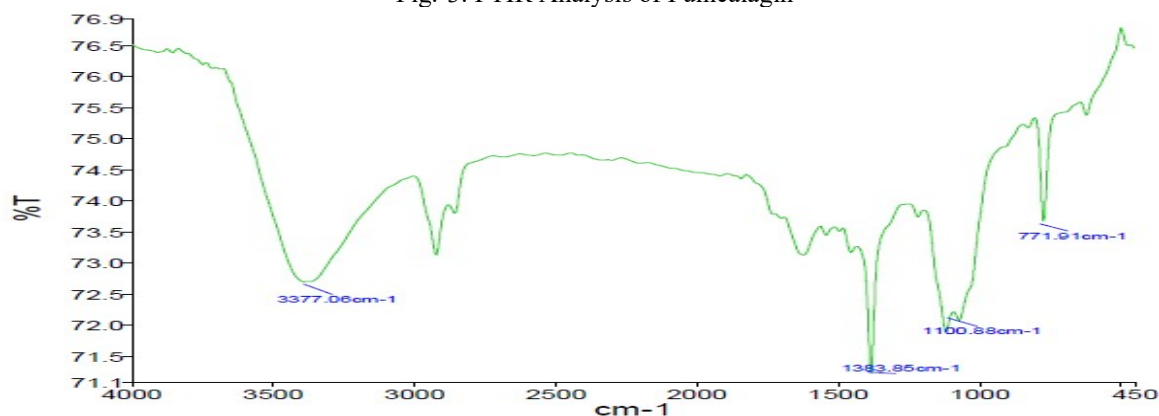


Fig.- 4: FTIR Analysis of Punicalin

LCMS analysis of isolated phytochemicals shown in Fig.-5 and 6 also showed their M^+ peak at 783.7 and 1083.9.

Sample Report (continued):

Peak ID Time
 10 4.69
 10: (Time: 4.69) Combine (153:189-(142:144+193:195))

2:MS ES-
 1.6e+005

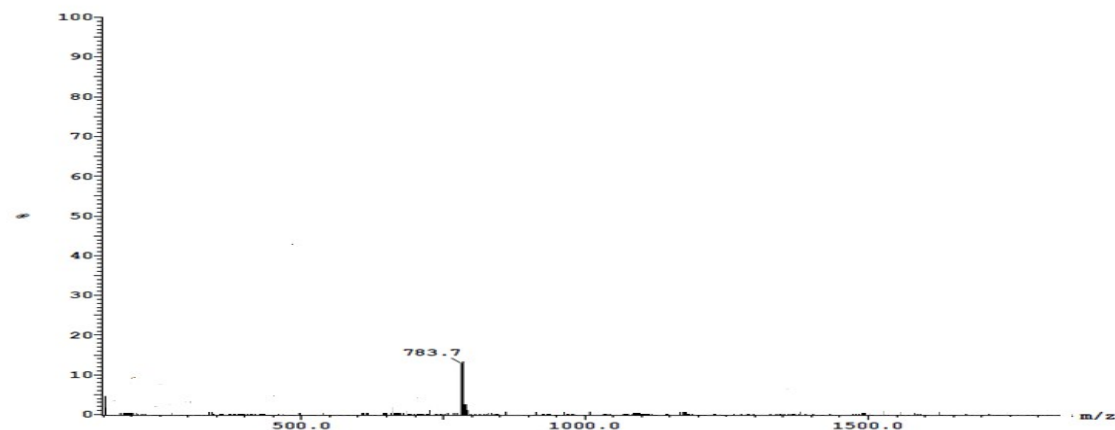


Fig.-5: LCMS Spectrum of Punicalin

Sample Report (continued):

Peak ID Time
 10 4.69
 10: (Time: 4.69) Combine (153:189-(142:144+193:195))

2:MS ES-
 1.6e+005

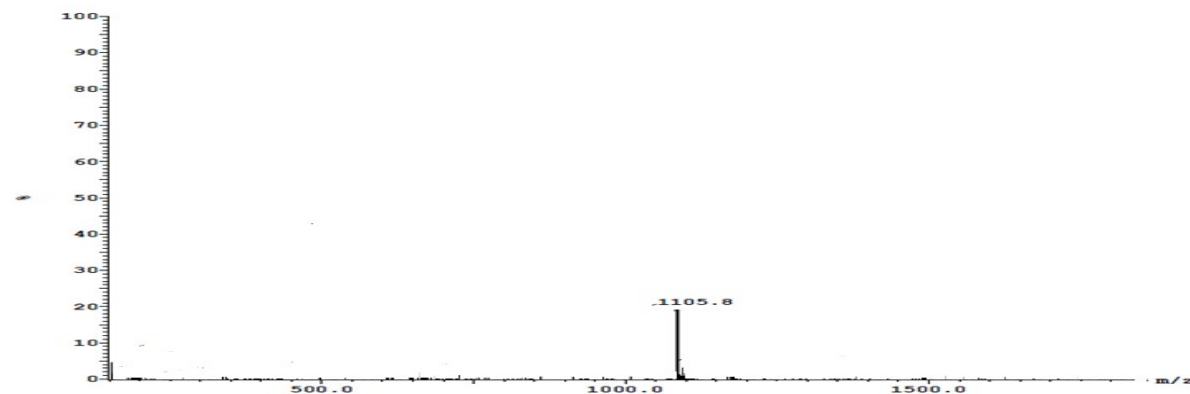
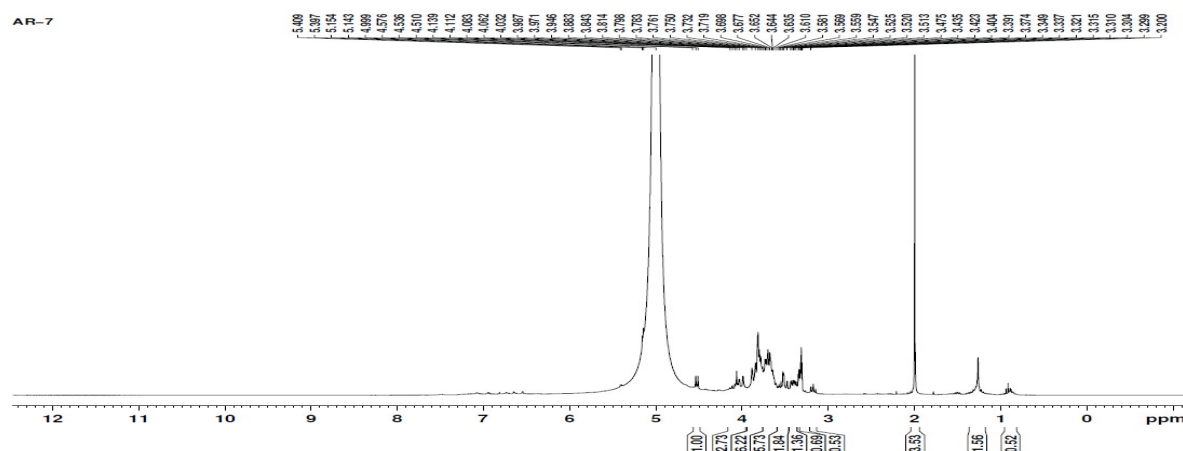
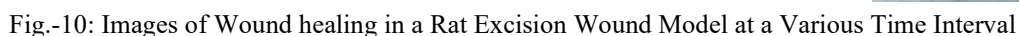


Fig.-6: LCMS Spectrum of Punicalagin

In ^1H NMR analysis obtained as Fig.-7 and 8 of both punicalin and punicalagin total of 22 hydrogens and 28 hydrogens are interpreted after studying the obtained graph, which satisfies the molecular formula of punicalin as $\text{C}_{34}\text{H}_{22}\text{O}_{22}$ and punicalagin as $\text{C}_{48}\text{H}_{28}\text{O}_{30}$.

Fig.-7: ^1H NMR Analysis of Punicalagin

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