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Rheum emodi (Rhubarb) root powder accelerates dermal wound healing by up regulating the expression of Ccr1, EGF, K6 and EGR-1

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Abstract
The present study was conducted to investigate the effect of *Rheum emodi* (Rhubarb) root powder on the expression of wound healing genes Ccr1, EGF, Keratin 6 and EGR-1 in rabbits of different age groups. The wounds treated with Rhubarb powder showed enhanced expression of Ccr1, EGF, K6 and EGR-1 by using reverse transcription polymerase chain reaction against normal saline treated wounds in all the groups. The expression was more pronounced in group A and B treated wounds as against group C treated wounds. The *Rheum emodi* (Rhubarb) root powder demonstrated wound healing activity by enhancing the expression of Ccr1, EGF, K6 and EGR-1thereby confirming the therapeutic potential of *Rheum emodi*.

Keywords: *Rheum emodi*, wound healing, Ccr1, EGF, K6, EGR-1

Introduction
Wound results from the disintegration of cellular and anatomical continuity of a tissue and may be produced by many factors such as physical, chemical, thermal, microbial or immunological injury to the tissue. These wounds if not properly managed may result in severe complications like septicemia and toxemia because of the secondary infection to wounds that will result in delayed wound healing. These complications in turn result in deterioration of health condition [1, 2]. So wound healing needs proper care and management to heal in time and without developing any complication. Thus wound healing has got high priority among body functions and plays a vital role in life for survival. During the process of wound healing a number of cellular and biochemical events are set in motion resulting into the re-establishment of structural and functional integrity of the injured tissue with regain of strength. The normal sequence of events in wound healing begins with an inflammatory response that initiates the migration and proliferation of fibroblasts [3]. The fibroblast migration and proliferation is responsible for wound healing and scar formation[4].Tissue damage also initiates the movement of inflammatory leukocytes to the site of wound that play a vital role in clearing the wound from the invading microbes and also release signals that may be essential for the repair of damaged tissue and lead to fibrosis.

More than 1000 genes have been reported to be differentially expressed post wounding [5]. Among these genes the expression of early growth response-1(*Egr-1*) *keratin 6(K6)*, chemokine receptor 1*(Ccr1)* and epidermal growth (*EGF)* factor increase significantly during the healing process.*Egr-1* is an immediate early gene whose expression is rapidly induced by many stimuli, including hypoxia, shear stress and injury. On activation it binds promoter regions of several growth factors, cytokines, receptors and adhesion molecules [6]. The knock down of*Egr-1*attenuates the pro angiogenic effect of fibroblast growth factor- 2 (*FGF-2*) and vascular endothelial growth factor (*VEGF*) on proliferation and differentiation of endothelial cells [7, 8]. The protective barrier of epidermisis determined by the presence and integrity of its keratin network and forms the intermediate filament cytoskeleton of epithelial cells[9, 10].Chemokines are chemotactic cytokines that are involved with the migration and activation of inflammatory cells. Originally studied for their role in inflammation, they are concerned with angiogenesis, homeostasis, development, migration of stem cells and wound healing [11, 12].Epidermal growth factor a potent mitogen for epithelial cells, endothelial cells and fibroblasts, enhancesfibronectin synthesis, angiogenesis, fibroplasias and collagenase activity [13].

The *Rheum emodi (Rhubarb)* plant is a perineal stout herb belonging to family polygonaceae and has been cultivated for over 5000 years for its medicinal properties. It is distributed in the

temperate and subtropical regions of the world. The main parts used as drug are roots and rhizomes. The major phytoconstituents reported to have been isolated from the roots and rhizomes of the plant are anthraquinone derivatives rhein, emodin, aloe-emodin, chrysophenol, physcion (emodinmonoethyl ether), chrysophanein [14], glycosides, flavonoids, tannins, terpenes, saponins [15]. Different derivatives of oxanthrone: oxanthrone ether (revandchinone-4), oxanthrone esters (revandchinone-1 and revandchinone-2) and revandchinone-3 [16, 17]. Compounds identified in *Rheum emodi* (Rhubarb) are reported to possess antioxidant [18], antimicrobial and antifungal [16, 19], anticancer [20], wound healing [21, 22] and immune enhancing activity [23]. The plant has been referred as a wondrous drug [24[. The present study was undertaken to investigate the effect of *Rheum emodi (Rhubarb)* root powder on the expression of genes involved in wound healing.

Materials and methods

Animals: 36 clinically healthy *Rabbits* in the age group of 3-21 months were used as experimental animals. These were divided in three groups viz. A, B & C. Each group had twelve animals. The group A had the animals in the age group of 3-9 months; the group B in the age group of 9-15 months while group C in the age group of 15-21 months. All the animals were housed in the cages had access to fresh water at a room temperature of 22 ± 2ºC. A balanced feed was used throughout the period of study. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee. All the animals were acclimatized for a period of 7 days prior to the commencement of the experiments.

Wound creation: The animals were anesthetised locally with injection of xylocaine (10 mg/kg) and Ketamine (40 mg/kg). The anesthetised animals were shaved on the dorsal aspect of the animal just distal to scapula. The shaved area was swabbed with alcohol and an area of 3 x 3 cm^2 (300mm^2) was demarcated with a self-designed stamp on the midline of the shaved area. Two wounds were created on the midline of the back wherein one acted as a control.The marked skin was excised with the help of a scalpel and scissors to the depth of loose subcutaneous tissue. Care was taken to restrict the haemorrhage to bare minimum. Animals after recovery from anaesthesia were housed individually in properly disinfected cages. Swab was collected at 0hr, 1hr, 3hr, 6hr, 24hr and a piece of tissue of appropriate quantity was excised from the edge of each wound at a gap of 2days up to 14th day.

Preparation of *Drug*: The *R. emodi* was collected from the upper reaches of Sonamarag in the month of May-June at an altitude of 3000m and identified at the centre of plant taxonomy. The roots of *R. emodi* were dried properly under shade and then grinded to fine powder. The powder was stored in plastic jars for use. *Rheum emodi* (Rhubarb) powder was applied on the wounds everyday with normal saline as control.

RNA extraction &RT-PCR analysis. Total RNA was extracted from swab and tissue cells with Trizol method, adding DNase I treatment to prevent cellular DNA contamination in the PCR as per the instructions [25]. cDNA synthesis was carried from the mRNA present in total RNA with the First Strand cDNA synthesis kit (MBI Fermentas, Heidelberg, Germany) with a random hexamer primer and the Moloney murine leukemia virus reverse transcriptase as per the instructions of manufacturer.

Primers used

S. No,	Gene	Primer Sequences Annealing temperature	
1	GAPDH	F 5' TCACCATCTTCCAGGAGCGA3'	
		R 5' CACAATGCCGAAGTGGTCGT 3'	53°C
2	CcR1	F 5' ATCGTCACCAGCATCGTCA 3'	
		R 5' CAGCAGGGGCAGAACAAG 3'	51.5°C
3	EGF	F 5' TATGTCTGCCGGTGCTCAGAA 3'	
		R 5' AGCGTGGCGCAGTTCCCACCA 3'	53.4°C
4	105	F 5' TCTGTCTCTGTGTCCCGCT 3'	
		R 5' TGTCGATGAAGGAGGCGAAC 3'	53°C
5	EGR1	F 5' AGACCAGTTACCCCAGCCAAAC 3'	
		R 5' AAAATGTCAGTGTTCGGCGTG 3'	54°C

Semi-quantitative PCR: The cDNA preparations were checked for quality by performing PCR with standard GAPDH primers. The polymerase chain reaction was standardized for each gene using CDNA from skin tissue. The reaction was carried out at different annealing temperatures viz., 50°C, 52°C, 54°C, 56°C, and 60°C and also with different $MgCl_2$, cDNA and primer concentrations. The semi-quantitative PCR was standardized by dilution of cDNA as 1, 1:10 and 1:100 and using these dilutions as template for amplification of *GAPDH*. The intensity of the PCR products in the gels was observed and the dilutions with same intensity were used for the amplification of other genes.

Agarose gel electrophoresis: DNA amplified by PCR was subjected to agarose gel electrophoresisas. Agarose (2 %) in 20 ml 0.5XTAE buffer was dissolved by heating and allowed to cool to 50ºC. To this, 1 µlethidium bromide (final concentration 0.5 µg/ml) was added and mixed thoroughly. The mixture was poured into a gel-casting tray fitted with acrylic comb and allowed to solidify. Once the gel had formed, a few ml of TAE was added near the comb, which was later removed carefully and the gel was immersed in an electrophoresis tank containing 1X TAE buffer. 5 µl of 6X DNA loading buffer was mixed with 5 µl of DNA samples and loaded into the wells. Electrophoresis was carried out at 50 volts until the tracking dye (bromophenol blue) reached

the end of the gel. The PCR products were visualized as bands under UV-illumination and compared with a standard marker (100-1000 bp).

Results and discussion

The relative expression of *GAPDH* used as standard in response to *Rheum emodi* powder has been shown in fig. 1. The excised tissue were analysed for expression studies at 0 hr, 1 hr, 3 hr, 6 hr, 24hr and then at a gap of two days up to day 14 after creation of the wound. The level of expression of *GAPDH* was uniform in all the samples.

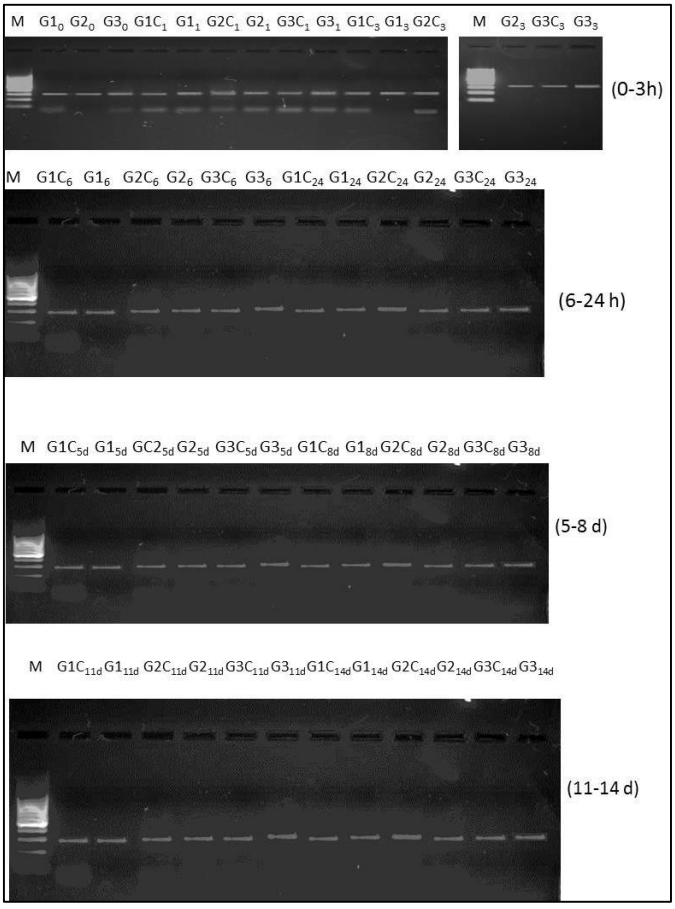

Fig 1: expression of Gapdh M rna (293bp) in wound tissue of treated

The relative expression of *Ccr1* in response to *Rheum emodi* powder is shown in fig. 2. *Rheum emodi* treatment resulted in increased *Ccr1* expression at 1 hr after creation of the wound as compared to control in all age groups. This trend continued up to 8 days after creation of wound in group A treated animals, group B treated animals showed increased expression of *Ccr1* up to 11 days after creation of wound while group C had increased expression of *Ccr1* up to 5 days after creation of wound when compared to their controls. The group C treated animals marginally showed lower expression of *Ccr1* up to 24 hr after creation of the wound as compared to group A and group B treated animals but on day 5 the expression of *Ccr1* was at par in all the treated groups. The expression of *Ccr1* were down regulated on day 11 in group A, on day 14 in group B and on day 8 in group C after creation of the wound and were at par with respective control.

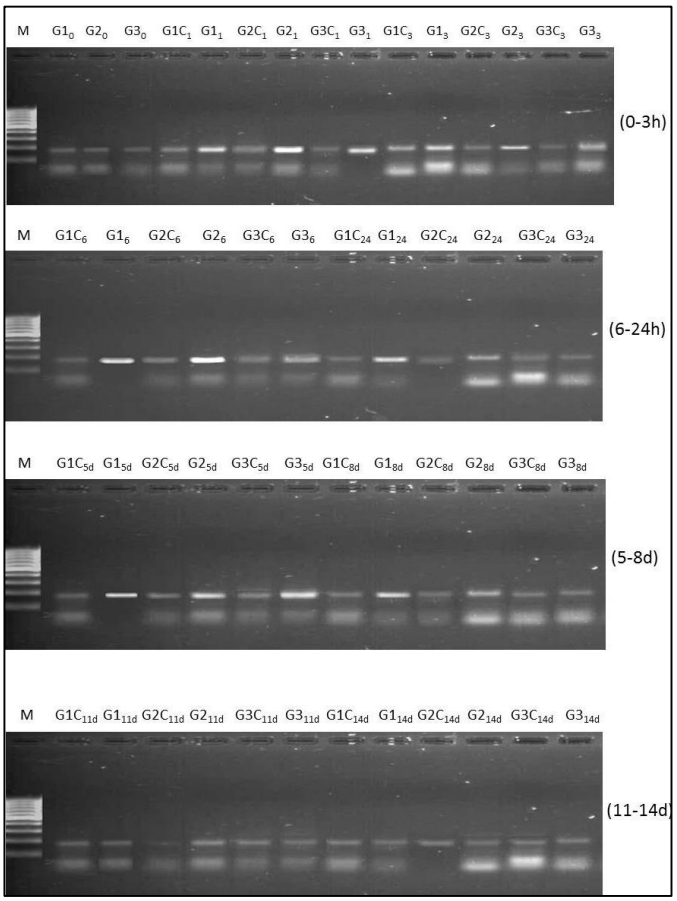

Fig 2: expression of Ccr! mRNa (189 bp) in wound tissue of treated and control animal

The *Rheum emodi* treated wounds showed strong up regulation of *Ccr1*, which promotes migration and proliferation of keratinocytes at the wound site and accelrates epithelialization and differentiation by formation of basal layer, spinous layer, granular layer and stratum corneum in order to facilitate wound healing [26, 27]. Metabolites obtained from plants have been attributed with a large number of biological activities which can affect biological processes. In the present study *Rheum emodi* treated wounds showed up regulation of Ccr 1 expression that enhanced the wound closure which may be due to increased my of broblast activity as flavonoids have been shown to increase myofibroblast activity and enhance epithelial cell growth [28].

Rheum emodi enhanced the expression of *EGF* at 1 hr after creation of the wound in all age groups when compared to respective controls Fig. 3. This trend continued up to day 11 in group A and group B animals while in group C animals *Rheum emodi* enhanced the expression of *EGF*up to day 8 after creation of the wound when compared to their controls. The group A and group B *Rheum emodi* treated animals had enhanced expression of *EGF* at 3 hr, 6 hr, 24 hr, 5 days and 8 days after creation of the wound when compared to group C treated animals. The expression of *EGF* in group A and group B treated animals were down regulated on day 14 after creation of the wound and were at par with respective controls while group C treated animals showed a down regulation in the expression of *EGF* from day 11 and were at par with control. The dermal repair requires the production of extra

cellular matrix (ECM) by fibroblasts and this ECM production by fibroblast regulated by growth factors of which members of *EGF* are key players for dermal wound healing. Emodin one of the main phytoconstituent of *Rheum emodi* have been reported to enhance wound healing by up regulation of transforming growth factor (TGF-β) one of the constituent of EGF family and Smad 2 and 3 [29, 30].

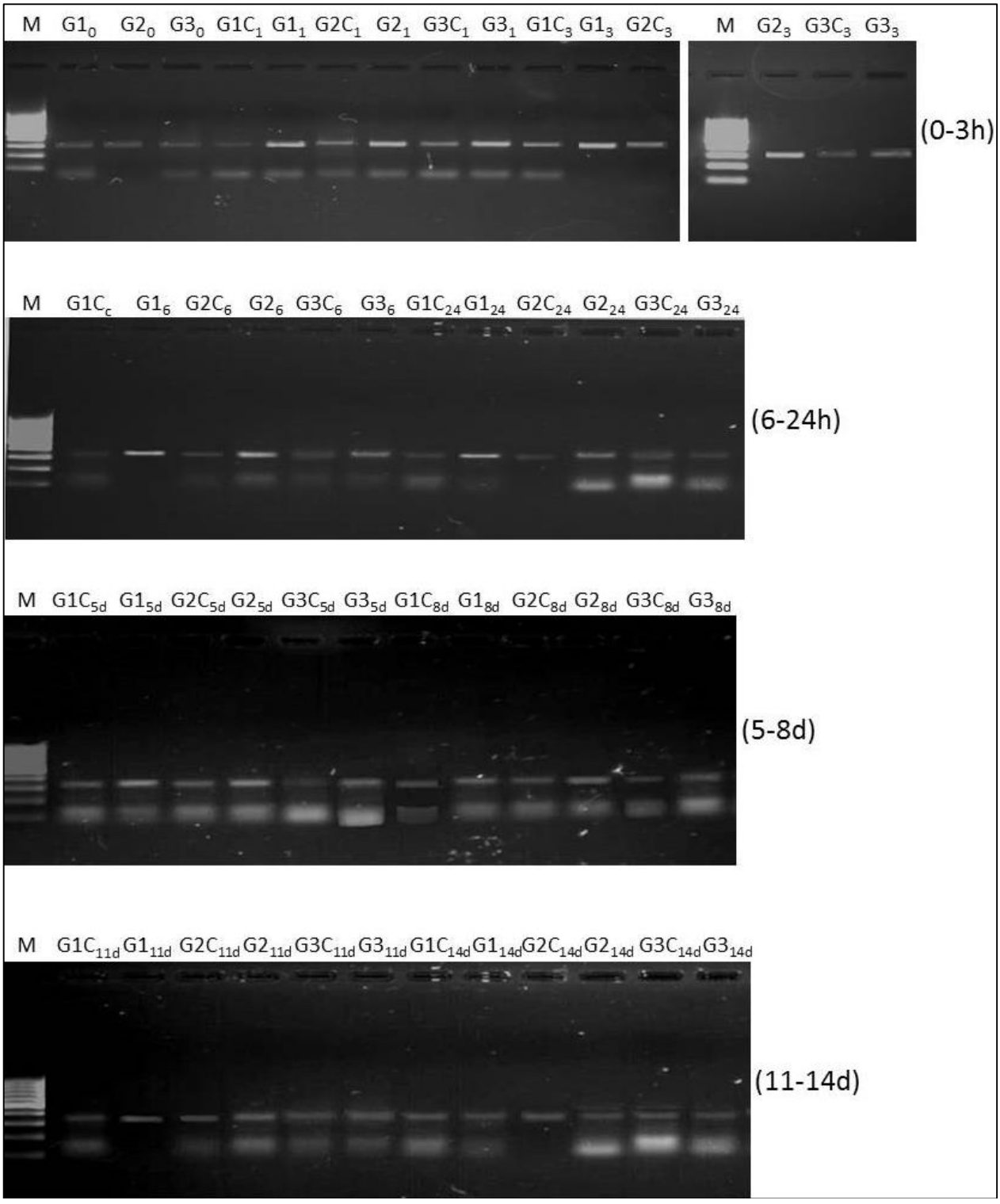

Fig 3: expression of EGF mRNA (394 bp) in wound tissue of treated and control animals.

The *Rheum emodi* treated wounds showed enhanced expression of *K6* Fig. 4. The expression like *Ccr1* and *EGF* was profound in group A and group B treated wounds than group C treated wounds. After a wound K6 facilitates the migration of keratinocytes from the wound periphery and epidermal appendages to the wound bed [31, 32], thus provides the provisional matrix for subsequent cell migration and differentiation [33]. Anthraquinones, emodion, aloeemodin and rhein have been shown to stimulate the growth, increase cell number and DNA synthesis [34].

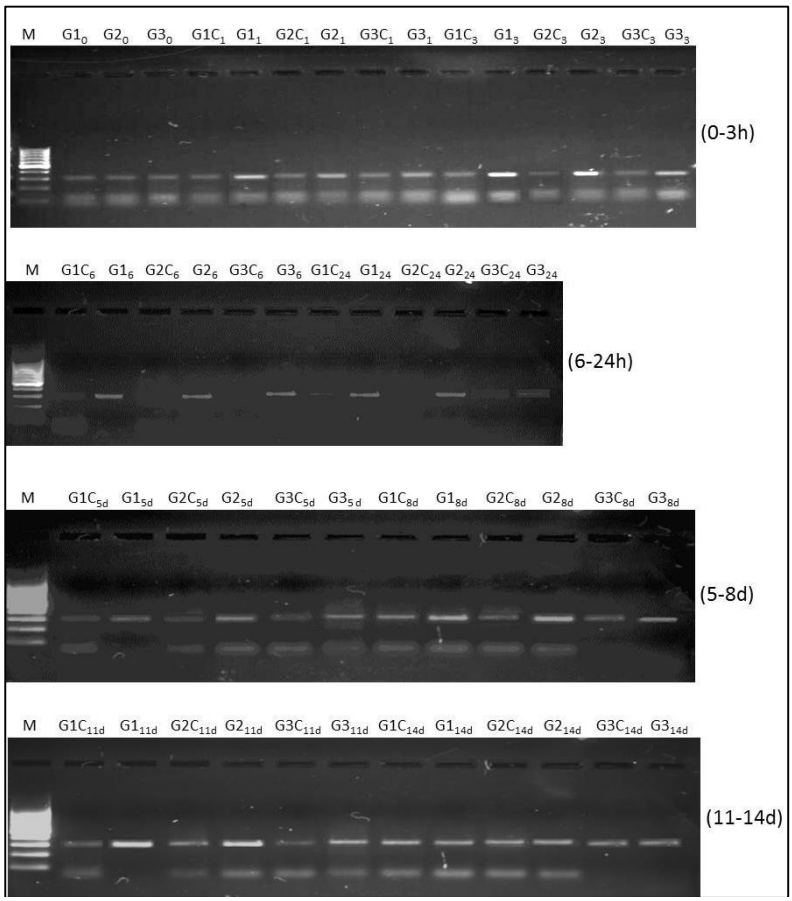

Fig 4: Expression of K^ mRNA (326 bp) in awound tissue of treated and control animals.

The transcription factor *Egr-1*also termed NGF 1-A and *Krox24*belongs to the *Egr* family (*Egr-1to -4*) of Zinc finger proteins [35, 36]. It activates cell division 20 gene (cdc20) which are involved in cell proliferation, migration and cell differentiation [37]. In addition this gene has also been associated with wound healing [38]. The group A and group B *Rheum emodi* treated wounds as shown in fig. 5. Showed immediate enhanced expression of *Egr-1* than group C treated wounds after creation of wound.

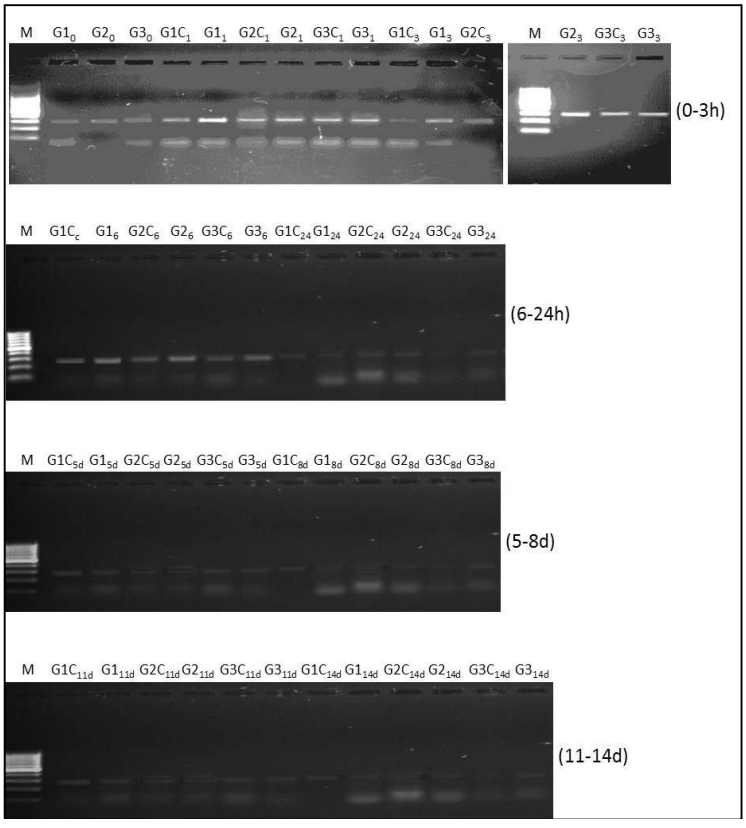

Fig 5: expression of EGR1 mENA (294 bp) in wound tissue of treated and control animals.

Rheum emodi contains many physiologically active constituents that exhibit, antimicrobial, anti-inflammatory, anti-diabetic, anticancer, immunomodulatory, and wound-healing effects. The ingredients whether acting alone or in synergistic manner, include anthraquinones, glycosides, flavonoids, tannins, terpenes, saponins and different derivatives of oxanthrone. In light of the many pharmacologic activities of the components a further understanding of these individual components and of their synergistic effects is essential if it is to be successfully developed for therapeutic purposes.

The group A and group B treated wounds exhibited early and enhanced expression of *Ccr1, EGF, K6* and *Egr-1* as against group C treated wounds. The reason for this may be the advancing age in group C animals [39], increased age results in delayed appearance of epidermal growth factor (EGF), basic epidermal growth factor (bEGF), transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF) A and B [40,41]. These growth factors have been reported to stimulate various aspects of wound healing.

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