Evaluating the synergistic effect of Chrysin & Enrofloxacin loaded Hydrogel for Acute cutaneous full thickness wound healing.

Thesis submitted in partial fulfilment of the requirement

for the degree of Master of Pharmacy

Under the guidance of

PROF. SANMOY KARMAKAR

Submitted by

Ankita Das (B. Pharm)

Class Roll no: 002211402042

Exam Roll no: M4PHP24004

Registration Number: 163683 of 2022 - 2023

Department Of Pharmaceutical Technology

Jadavpur university
Kolkata, India-700032

2024

CERTIFICATE

This is to certify that Ankita Das, Final year Masters of Pharmaceutical Technology (M. Pharm) examination student of Department of Pharmaceutical Technology, Jadavpur University, Class Roll No. 002211402042, Registration No. 163683 of 2022 - 2023, Examination Roll No. M4PHP24004 has completed the Project work titled, "**Evaluating the synergistic effect of Chrysin & Enrofloxacin loaded Hydrogel for Acute cutaneous full thickness wound healing.** " under the guidance of **Prof. Sanmoy Karmakar** during his Master's Curriculum. This work has not been reported earlier anywhere and can be approved for submission in partial fulfilment of the course work.

Supervised by

Sanmoy Karmakar

Prof Sanmoy Karmakar

Professor

Dept of Pharmaceutical Technology

Jadavpur University

Kolkata 700032

Professor
Dept. of Pharmaceutical Technology
Jadavpur University
Kolkata - 700 032, W.B. India

Forwarded by

28/8/24.

Prof (Dr.) Amalesh Samanta

HOD

Dept of Pharmaceutical Technology

Jadavpur University

Kolkata 700032

Head
Dept. of Pharmaceutical Technology
Jadavpur University
Kolkata - 700 032, W.B. India

Dipak Laha 28.8.24

Signature of the Dean

DEAN
Faculty of Engineering & Technology
JADAVPUR UNIVERSITY
KOLKATA-700 032

CERTIFICATE

This is to certify that Ankita Das, Final year Masters of Pharmaceutical Technology (M. Pharm) examination student of Department of Pharmaceutical Technology, Jadavpur University, Class Roll No. 002211402042, Registration No. 163683 of 2022 - 2023, Examination Roll No. M4PHP24004 has completed the Project work titled, " **Evaluating the synergistic effect of Chrysin & Enrofloxacin loaded Hydrogel for Acute cutaneous full thickness wound healing.** " under the guidance of **Prof. Sanmoy Karmakar** during his Master's Curriculum. This work has not been reported earlier anywhere and can be approved for submission in partial fulfilment of the course work.

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Signature of External

Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research as part of my work on "**Evaluating the synergistic effect of Chrysin & Enrofloxacin loaded Hydrogel for Acute cutaneous full thickness wound healing.**". All information in this document have been obtained and presented in accordance with academic rules and ethical conduct. I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Name: Ankita Das

Exam Roll Number: M4PHC24005

Registration Number: 163683 of 2022 - 2023

Thesis Title: **Evaluating the synergistic effect of Chrysin & Enrofloxacin loaded Hydrogel for Acute cutaneous full thickness wound healing.**

Ankita Das.
28/08/2024
Signature With Date:

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ANKITA DAS

Class Roll No: 002211402042

Exam Roll No: M4PHP24004

Dedicated to

My Loving Parents

and

My Lab-family

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CHAPTER 1: INTRODUCTION

1.1: wound healing

The skin is the largest organ in the human body, primarily serving as a protective barrier against environmental threats. Its integrity is essential for maintaining overall health and preventing the entry of pathogens, as well as minimizing water loss. When large portions of the skin are damaged due to injury or illness, it can lead to significant disability or even death. In the United States, more than 1.25 million people suffer from burns each year, and 6.5 million individuals are affected by chronic skin ulcers, often resulting from pressure, venous stasis, or diabetes mellitus (Singer and Clark, 1999). Injury to the skin initiates a cascade of events including inflammation, new tissue formation, and tissue remodelling, which finally lead to at least partial reconstruction of the wounded area. The repair process is initiated immediately after injury by the release of various growth factors, cytokines, and low-molecular weight compounds from the serum of injured blood vessels and from degranulating platelets. Disruption of blood vessels also leads to the formation of the blood clot, which is composed of cross-linked fibrin, and of extracellular matrix proteins such as fibronectin, vitronectin, and thrombospondin. Apart from providing a barrier against invading microorganisms, the blood clot also serves as a matrix for invading cells and as a reservoir of growth factors required during the later stages of the healing process. Within a few hours after injury, inflammatory cells invade the wound tissue. Neutrophils arrive first within a few minutes, followed by monocytes and lymphocytes. They produce a wide variety of proteinases and reactive oxygen species as a defence against contaminating microorganisms, and they are involved in the phagocytosis of cell debris. In addition to these Défense functions, inflammatory cells are also an important source of growth factors and cytokines, which initiate the proliferative phase of wound repair. The latter starts with the migration and proliferation of keratinocytes at the wound edge and is followed by proliferation of dermal fibroblasts in the neighbourhood of the wound. These cells subsequently migrate into the provisional matrix and deposit large amounts of extracellular matrix. Furthermore, wound fibroblasts acquire a contractile phenotype and transform into myofibroblasts, a cell type which plays a major role in wound contraction. Massive angiogenesis leads to the formation of new blood vessels, and nerve sprouting occurs at the wound edge. The resulting wound connective tissue is known as granulation tissue because of the granular appearance of the numerous capillaries. Finally, a transition from granulation tissue to mature scar occurs, characterized by continued collagen synthesis and collagen catabolism. The scar tissue is mechanically insufficient and lacks appendages, including hair follicles, sebaceous glands, and sweat glands. Scarring can also be excessive,

leading to hypertrophic scars and keloids. In contrast, wound healing in mammalian embryos until the beginning of the third trimester results in essentially perfect repair, suggesting fundamental differences in the healing process between embryonic and adult mammals. In addition to the importance of cell-cell and cell matrix interactions, all stages of the repair process are controlled by a wide variety of different growth factors and cytokines. Multiple studies have demonstrated a beneficial effect of many of these growth factors, e.g., platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), and granulocyte-macrophage colony stimulating factor (GM-CSF) on the healing process, both in animal models and in patients suffering from different types of wound healing disorders. However, the roles of endogenous growth factors in the healing response have been only partially elucidated, and in most cases, the suggested function of these molecules is based on descriptive expression studies and/or functional cell culture data. However, in vivo functions of many growth factors remain largely unconfirmed. The main goals in wound treatment are to achieve rapid wound closure and to ensure the formation of a functional scar. These objectives are crucial for reducing the risk of complications such as infections and prolonged healing times, thereby improving patient outcomes and quality of life.

1.2: Stages of wound healing

Wound healing is a dynamic, interactive process involving soluble mediators, blood cells, extracellular Matrix, and parenchymal cells. Wound healing has four phases — haemostasis, inflammation, tissue formation, and tissue remodelling — that overlap in time (Singer and Clark, 1999).

The first stage of physiological or acute wound healing is dedicated to haemostasis and the formation of a provisional wound matrix, which occurs immediately after injury and is completed after some hours. Furthermore, this phase initiates the inflammatory process. Sometimes this phase is also described as the ‘lag-phase’, in which the organism must manage the recruitment of the many cells and factors for the healing process in the absence of the mechanical strength of the wound. When a skin injury extends beyond the epidermal layer, blood and lymphatic vessels are damaged, leading to a flushing of the wound to remove microorganisms and antigens. Clotting cascades are initiated by clotting factors from the injured skin (extrinsic system), and thrombocytes become activated for aggregation by exposed

collagen (intrinsic system). Simultaneously, injured vessels undergo vasoconstriction for 5 to 10 minutes, triggered by platelets, to reduce blood loss and form a blood clot rich in cytokines, growth factors, fibrin molecules, fibronectin, vitronectin, and thrombospondins. This clot acts as a provisional matrix, providing a scaffold structure for the migration of leukocytes, keratinocytes, fibroblasts, and endothelial cells, as well as serving as a reservoir of growth factors. Vasoconstriction leads to local perfusion failure, resulting in decreased oxygen supply, increased glycolysis, and pH changes. Subsequently, vasodilation occurs, allowing thrombocytes to invade the provisional wound matrix. In addition, platelets influence the infiltration of leukocytes by the release of chemotactic factors. Both platelets and leukocytes release cytokines and growth factors to activate the inflammatory process (IL-1, IL 1B, IL-6 and TNF-alpha), stimulate the collagen synthesis (FGF-2, IGF-1, TGF-B), activate the transformation of fibroblasts to myofibroblasts (TGF-B), start the angiogenesis (FGF-2, VEGF-A, HIF-1alpha , TGF-beta) and already support the reepithelialisation process (EGF, FGF-2, IGF-1, TGF-alpha) . The vasodilation can also be recognized by a local redness (hyperaemia) and by an oedema of the wound (Strodtbeck, 2001).

The inflammatory phase of wound healing is initiated during the haemostasis and coagulation phase. It involves two main stages: an early phase characterized by neutrophil recruitment and a late phase marked by the appearance and transformation of monocytes into macrophages. Neutrophils are recruited to the wound site due to the response of the activated complement pathway, degranulated platelets, and bacterial degradation by-products. They play a crucial role within the first few days after injury by phagocytosing bacteria, secreting proteases to degrade necrotic tissue, and releasing inflammatory mediators such as TNF-α, IL-1, and IL-6, which amplify the inflammatory response and stimulate the production of growth factors like VEGF and IL-8 (Daley et al., 2005). Neutrophils also initiate debridement by releasing antimicrobial substances and proteinases. Around three days post-injury, macrophages enter the wound area, performing phagocytosis of pathogens and cell debris and secreting growth factors, chemokines, and cytokines. These molecules support the ongoing healing process and activate the proliferative phase of wound healing. Macrophages play diverse roles, including host defense, modulation of inflammation, removal of apoptotic cells, and support of cell proliferation and tissue restoration. They synthesize potent growth factors such as TGF-β, basic FGF, PDGF, and VEGF, which promote cell proliferation and the synthesis of extracellular matrix molecules by resident skin cells. The inflammatory response is crucial for supplying growth factor and cytokine signals necessary for subsequent repair mechanisms (Koh and

DiPietro, 2011). Cellular activity predominates in this phase, with major events including reepithelialisation, angiogenesis, and fibroplasia. Reepithelialisation involves the restoration of an intact epidermis after injury. This process encompasses several steps: migration of adjacent epidermal keratinocytes into the wound, proliferation of keratinocytes to supplement the advancing epithelial tongue, differentiation of neoepithelium into a stratified epidermis, and restoration of an intact basement membrane zone (BMZ) connecting the epidermis and underlying dermis. Keratinocyte migration is an early event in reepithelialisation, occurring within 24 hours of injury. Keratinocytes migrate from wound edges and remaining skin appendages, with stem cells from hair follicles contributing to this process. Various cellular changes prepare keratinocytes for migration, including flattening, elongation, and development of pseudopod-like projections. Despite migrating, keratinocytes proliferative potential is inhibited. Keratinocyte migration is facilitated by elements such as the extracellular matrix (ECM), integrin receptors, matrix metalloproteinase (MMPs), and growth factors. A provisional matrix composed of fibrin, fibronectin, and type V collagen aids migration by enabling keratinocytes to dissect under eschar and debris covering the wound. Keratinocytes interact with the provisional matrix via surface integrin receptors, and binding to newly formed collagen molecules in the wound bed guides migration. MMPs, particularly MMP-9 and MMP-1, play crucial roles by degrading basement membrane components and facilitating keratinocyte migration into the wound (Li et al., 2007).

Remodelling is a crucial phase of wound healing characterized by the deposition of matrix and subsequent changes over time. This process occurs throughout the entire repair process, starting with the replacement of the fibrin clot formed in the early inflammatory phase by granulation tissue rich in type III collagen and blood vessels during the proliferative phase. Subsequently, this granulation tissue is replaced by a collagenous scar predominantly composed of type I collagen, with fewer mature blood vessels. Collagen fibres, constituting approximately 80% of the dry weight of normal human dermis, provide structure, strength, and stiffness to dermal tissue. In healthy adults, type I collagen accounts for about 80% of collagen, while type III collagen constitutes around 10%. Tensile strength, a functional assessment of collagen, increases during wound healing, reaching up to 40% of preinjury strength at 1 month and continuing to increase over 1 year, potentially reaching 70% of preinjury strength. The turnover of collagen involves the degradation of type III collagen and increased synthesis of type I collagen, primarily regulated by matrix metalloproteinase (MMPs). MMPs are induced during wound repair in response to cytokines, growth factors, and cell contact with the extracellular

matrix. Their catalytic activity is controlled by tissue inhibitors of metalloproteinase (TIMPs), which bind to MMPs and inhibit their activity. The balance between MMPs and TIMPs is critical for proper wound repair and remodelling (Visse and Nagase, 2003).

Table: 1

1.3: Models of acute wound healing:

Wound model	**overview**	**advantages**	**disadvantages**
Incision	Involves incision beyond epidermis and dermis and into the subcutaneous tissue	• Low risk of infection • Ease of execution and reproducibility	Not ideal for histological assessment and evaluation of wound biochemistry and reepithelialisation
Superficial excision	Involves removal of stratum corneum as well as the stratum granulosum, exposing the basal cell layer	Cost-effective, ease of use, and non-invasive method that is sufficient to stimulate an inflammatory response	Reproducibility is difficult due to various factors such as tape strength, site of application, application pressure, and the force and velocity of removal
Partial-thickness excision	Involves removal of epidermis and dermis	Used to represent partial-thickness burn wounds	Most partial-thickness injuries are caused by burns, which involve a different healing process than that of excision

			wounds
Full-thickness excision	Involves removal of epidermis, dermis, subcutaneous fat, and panniculus carnosus	Large cavity permits collection of significant amounts of wound tissue for examination of all dermal components Allows for histological assessment	Risk of infection

1.4: Full-thickness cutaneous wound model:

Full-thickness excision wounds are amongst the most popularly used models to study acute wound healing processes. Making these wounds involves the complete removal of the mouse skin, including the epidermis, dermis, subcutaneous fat, and the underlying panniculus carnosus using a biopsy punch. These wounds are made almost exclusively on the dorsum of the animal. A contentious issue in the field of acute wound healing is related to the fact that rodent skin contracts easily and results in healing primarily via contraction. This contrasts with human wound healing, which occurs through granulation tissue formation and re-epithelialization. Thus, to make a more clinically relevant excision wound model in mice, a novel model was developed whereby a silicone splint device was glued (or sutured) on the wound margins, effectively minimising skin contraction and allowing wound closure to occur primarily through granulation tissue formation and re-epithelialization. However, this model has its disadvantages given that sutures and/or glue are added to the wound, therefore, introducing additional constraints to the healing process. Furthermore, it should be noted that added mechanical tension plays a key role in scar formation through a variety of molecular signals. Thus, any added extrinsic force from splints may also result in altered gene expression and signalling pathways that could affect normal wound healing making the healing different than human healing. Nevertheless, this model has been used extensively in recent years, particularly in studies utilising transgenic mouse strains. However, a more recent study has

challenged the claim that the healing of un-splinted excision mouse wounds occurs primarily by contraction. By makings marks on the skin of mouse strains, at the time of wounding, the investigators calculated the percentage of wound closure associated with contraction and re-epithelialization at various times post-wounding and concluded that re-epithelization-associated wound closure was always more prevalent than that of contraction. Thus, although contraction of mice skin should be accounted for, it does not exclude the study of un-splinted mouse wounds, especially in studies aimed at understanding wound healing processes at the early stages of healing. One critical issue is to start with wounds larger than 6 mm in diameter. This size wound results in healing without contraction during the first 6–7 days post-wounding allowing for examination of the very early stages of healing without the interference of contraction. Wounds much smaller than 6 mm in diameter heal by contraction. In mice, full-thickness excision wounds are often used for histological assessment of the wound bed, including granulation tissue formation, collagen deposition, neovascularization, and re-epithelialization. The larger wound bed generated in excision wounds allows for more tissue to be collected to perform histological, molecular, and biochemical assessments, whilst also making these wounds particularly susceptible to infections. Wounds covered with occlusive dressings, which retain wound exudate, may be used to study exudate composition such as cytokines, proteins, and nutrients. Investigators have consistently used mice primarily, because of the ability to use genetically modified strains. Thus, many excisions wounds have been performed on genetically modified mouse strains to elucidate the function of specific genes and their related signalling pathways during wound healing.

1.6: TLRs and TLR signalling:

Toll-like receptors are a group of highly conserved molecules that allow the immune system to sense molecules that are present in most classes of pathogens such as bacteria and viruses, but not the host, and to coordinate defence mechanisms against these pathogens. The recognition of pathogen-associated molecular patterns (PAMPs) by TLRs is a cornerstone of innate immunity and provides a quick and highly efficient response to pathogens in both vertebrate and invertebrate species(Janeway and Medzhitov, 2002). The human TLR family consists of currently ten members, which are structurally characterized by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a Toll/interleukin (IL)-1 receptor (TIR) domain in their intracellular domain (Akira and Takeda, 2004). The existence of many TLRs enables the innate immune system to discriminate between PAMPs that are characteristic of

different microbial classes and launch specific defense mechanisms. TLR4 senses Gram-negative bacteria by binding lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria. TLR2 heterodimers recognize cell membrane components of Gram-positive bacteria. TLR3 and TLR7 sense viral infections by recognizing double-stranded and single-stranded RNA, respectively. TLR9 recognizes nonmethylated CpG-containing DNA from bacteria and viruses. TLRs that mainly serve to detect bacterial LPS and lipoproteins are located on the cell surface. TLRs such asTLR3, TLR7, TLR8, and TLR9 that mainly recognize viral RNA and bacterial DNA are in late endosome lysosomes in which these materials are processed, and host DNA is not present, thus avoiding aberrant self-recognition. Some TLRs such as TLR2 and TLR4 have been suggested to also detect endogenous ligands termed DAMPs that are released from injured and inflamed tissue. A wide range of DAMPs including HMGB1, hyaluronan, S100 proteins, heat shock protein 60, and the alternatively spliced extra domain A of fibronectin have been suggested to activate TLRs (Miyake, 2007),(Kluwe et al., 2009).

1.7: Toll like receptors and skin:

Intact skin acts as a physical barrier against pathogenic microorganisms, preventing infection by the majority of bacteria, viruses, and fungi (Pasparakis et al., 2014).Additionally, various innate immune cells present in the skin, such as dermal mast cells, phagocytes, dendritic cells like Langerhans cells, along with those recruited from the bloodstream, express Toll-like receptors (TLRs) for pattern recognition. Upon detection of invading pathogens through recognition of pathogen-associated molecular patterns (PAMPs), TLR activation triggers a pro-inflammatory defense response, promoting processes like phagocytosis, immune cell recruitment, and antigen presentation (Terhorst et al., 2010) TLRs are not only expressed by immune cells but also by various non-immune cells within the epidermis and dermis, which play crucial roles in wound healing. Keratinocytes, the primary cells of the epidermis, express TLRs 1–6 and TLR9 and 10(Miller and Modlin, 2007a). Unlike specialized immune cells, keratinocytes and other epithelial cells form the boundary with the external environment and are constantly exposed to microbes and PAMPs. They maintain a delicate balance between tolerance of commensal organisms and detection of infection and injury, triggering subsequent inflammatory responses (Huebener and Schwabe, 2013)The expression of TLRs by keratinocytes varies depending on the cell's position within the epidermis. For example, TLR5 is predominantly expressed in the basal layers, while TLR9 is expressed more in differentiated cells of the upper epidermal layers (Miller et al., 2005).. However, all TLRs are functional and

produce distinct immune responses. Activation of keratinocyte TLRs by their ligands results in the release of various cytokines and chemokines, such as TNF-α, IL-8, CCL2, and CCL20, involved in inflammation. TLR3 and TLR9 activation specifically leads to the production of CXCL9 and CXCL10, which are associated with T-memory cell activation and type 1 interferon production, respectively (Miller and Modlin, 2007b).Fibroblasts are the major cellular components in the dermis. In the proliferation phase, skin resident fibroblasts or fibroblasts that have differentiated from blood-borne fibrocytes produce ECM molecules to provide structural support to the re pairing tissue. Emerging evidence shows that fibroblasts can also participate in regulation of inflammation. They have been found to express the full range of human TLRs from 1 to 10 (Jang et al., 2012). Fibroblasts can be induced to produce a variety of cytokines and growth factors such as VEGF, PDGF, FGF2, EGF, TGF-b, MMPs, and tissue inhibitors of MMPs. Studies have demonstrated in vitro activation of TLRs 2, 3, 4, 5 and 9 resulted in production of interferon-γ, CXCL9, CXCL10 and CXCL11, important in the recruitment of T-cells and NKcells (Miller and Modlin, 2007c)TLR4 activation in dermal fibroblasts has been demonstrated to result in IL-6, IL-8 and monocyte chemotactic protein (MCP) (Wang et al., 2011a). Microvascular cells such as dermal endothelial cells have been shown to highly express TLR4 and to a lesser extent TLR2. In vitro treatment with the exogenous TLR4 ligand LPS resulted in NFkB activation. Likewise, exposure to the endogenous derived ligand hyaluronan induced IL-8, a potent chemokine, stimulating the recognition of tissue injury and promoting initiation of the early stages of the wound healing process (Taylor et al., 2004). TLR activation in these cells may play an important role in wound healing, especially in the remodelling phase.

CHAPTER 2: AIM & OBJETIVE & PLAN OF WORK

2.1: AIM & OBJECTIVE OF THE WORK

- ✓ Hydrogel formulation.
- ✓ Evaluation of synergistic effect of Chrysin & Enrofloxacin loaded in formulation.
- ✓ Evaluation of the wound healing activity of the formulation In-vivo assay.

2.3: PLAN OF WORK

- ✓ Hydrogel Formulation.
- ✓ API release parameter check from formulation
- ✓ Evaluation of Antimicrobial assay
- ✓ Acute skin irritation assay
- ✓ Evaluation of In-vivo wound healing potential.
- ✓ Wound size Measurement.
- ✓ Tissue collagen marker determination.
- ✓ Histopathology.

CHAPTER 3: LITERATURE REVIEW

- TLR activation by endogenous ligands in both immune and non-immune skin cells generates alarm signals, triggering sterile inflammation in response to tissue injury. However, the impact of TLR activation on wound healing extends beyond this initial recognition of cellular damage. Depending on factors such as location, timing, and degree of activation, TLR activation can either promote or inhibit the process of wound healing and tissue regeneration (Wang et al., 2011b). In both in vitro and in vivo studies, it has been observed that TLR4 expression increases within the first 12–24 hours following injury and gradually returns to baseline levels by day 10, primarily in epidermal keratinocytes. Studies using TLR4 deficient mice have shown significantly impaired wound healing during the initial 1–5 days post-injury, with no difference observed compared to wild-type mice by day 10 (Chen et al., 2013a). This impairment was associated with alterations in cytokine release and inflammatory cell infiltration, characterized by decreased IL-1β and IL-6 levels and an increase in neutrophil, macrophage, and T-cell infiltrates at specific time points (Chen et al., 2013b).
- studies involving TLR2 and TLR4 deficient mice have demonstrated impaired wound healing at days 3 and 7 post-injury. However, in these studies, there was also a decrease in neutrophil and macrophage infiltration, along with reduced expression of TGF-β and CCL5. These findings suggest that activation of TLR4 and TLR2 may have a beneficial effect on wound healing during the early stages following acute injury, particularly in the absence of other influences on TLR expression, signaling, and activation (Suga et al., 2014).
- In addition to decreased healing time, wounds in TLR2 deficient diabetic mice exhibited reduced NFκB activation, as well as decreased release of IL-6 and TNF-α. Furthermore, in the same study, comparing diabetic wild-type mice to non-diabetic controls revealed significantly increased expression of TLR2 mRNA and protein, along with activation markers such as elevated expression of MyD88, IRAK, and NFκB. Similarly, TLR4 mRNA and protein expression, as well as IL-6, TNF-α, and NFκB activation, were increased in diabetic wild-type mice compared to non-diabetic animals, with corresponding reductions in these parameters observed in TLR4 deficient diabetic mice (Dasu and Jialal, 2013).Studies have shown increased expression of TLR2, TLR4, and MyD88 in diabetic wounds compared to non-diabetic wounds, indicating that in diabetes, TLR2 and TLR4 mediated hyperinflammation impairs wound healing. This persistent activation of TLR2 and TLR4 is also associated

with other chronic non-healing wounds like chronic venous ulceration (Pukstad et al., 2010).

- In wound healing studies using TLR3 deficient mice, delayed wound healing was observed compared to wild-type controls. This delay was accompanied by decreased recruitment of neutrophils and macrophages, as well as reduced levels of CXCL2, CCL2, and CCL3 chemokines. Conversely, topical application of the TLR3 agonist poly(I:C) accelerated wound healing in both human and mouse wounds compared to controls. This acceleration was associated with increased recruitment of neutrophils and macrophages, along with upregulation of CXCL2 (Lin et al., 2011).Similarly, TLR9 deficient mice exhibited delayed wound healing compared to wild-type mice. However, topical administration of the TLR9 agonist CpG ODN to wounds resulted in significantly improved healing times. This improvement was associated with increased infiltration of macrophages and increased production of vascular endothelial growth factor (VEGF). (Lin et al., 2012),(Sato et al., 2010)
- Oral mucosal wound is compared to skin wound, which shows that there is reduced scar formation in oral mucosal wound .previous studies suggest that intrinsic differences in inflammation, growth factor production, levels of stem cells, and cellular proliferation capacity may underlie the exceptional healing that occurs in mucosa (Wong et al., 2009),(Mak et al., 2009). Study has been designed to compare the transcriptomes of oral mucosal and skin wounds in order to identify critical differences in the healing response in murine model. Study demonstrates that TLR4 is upregulated and activated in early skin wound healing; functional mutation of TLR4 results in altered inflammatory cell infiltration, differential cytokine production, and impaired wound closure; and IL-1b production by injured keratinocytes is induced through the TLR4-p38/JNK pathway. The results provide evidence that TLR4 is a critical regulator in wound inflammation, and that this receptor supports the creation of an optimal early inflammatory environment in healing skin.(Chen et al., 2010)
- according to Mark. et.al, in case of hyperglycaemia and ischaemia,study investigates the effect of high glucose and hypoxic conditions on TLR4 activation and signalling in vitro and in vivo. Innate immune Toll-like receptor 4 (TLR4) mediated inflammation has been implicated in the systemic pathogenesis of diabetes and may contribute to impairment of wound healing. In vitro experiments using fibroblasts cultured at physiological glucose concentrations (5.5 mM) showed that exposure to high glucose

levels (up to 25 mM) in hypoxic conditions led to increased TLR4 protein expression, apoptosis, and interleukin (IL)-6 release. Inhibition of TLR4 with a neutralizing antibody and a specific TLR4 antagonist mitigated the detrimental effects of high glucose and ischemia.In vivo experiments involved inducing diabetes in wild-type (WT) and TLR4 knockout (KO) C57BL/6 mice using streptozocin injection, followed by femoral artery ligation to induce hindlimb ischemia and infliction of a full-thickness 4 mm skin wound below the knee. Wound healing was assessed using digital planimetry on days 3, 7, and 14 post-surgeries.Results showed that diabetic ischemic wounds exhibited significant impairment in wound healing at day 14 compared to non-diabetic wounds. However, diabetic ischemic wounds in TLR4 KO mice demonstrated significantly improved healing rates at all-time points compared to WT mice. This suggests that TLR4 plays a crucial role in mediating the impaired wound healing observed in diabetic ischemic conditions.Hypoxia stimulates upregulation of TLR4 protein expression and this effect is exaggerated by hyperglycaemia. In TLR4 KO mice, there is a significant improvement in the healing of diabetic ischaemic wounds compared with WT. It is suggested that a synergistic effect between hypoxia and hyperglycaemia impairing wound healing exists, through TLR4 mediated inflammation (Portou et al., 2020).

- according to medi . et.al ., role of TLR4 in corneal epithelial wound healing is evaluated. In study examining TLR4 expression during in vivo corneal epithelial wound healing in mice, immunostaining revealed prominent TLR4 expression in the epithelium 8 hours after wounding. In primary or telomerase-immortalized human corneal epithelial cells (HCEC), scratch assays showed a significant 4-fold increase in TLR4 expression 6 hours after in vitro scratch wounding. Confocal microscopy confirmed membrane localization of the TLR4/MD2 complex.Treatment of HCEC with the TLR4 activator lipopolysaccharide (LPS) resulted in significant increases in migration, proliferation, and wound closure. Conversely, inhibition of TLR4 led to significant decreases in these processes. Addition of LPS to wounded HCEC also increased the expression of inflammatory cytokines IL-6, TNF-α, CXCL8/IL8, and CCL5/RANTES at both mRNA and protein levels. Additionally, LPS increased the activation of p42/44 and p38 signalling pathways in wounded HCEC. These findings suggest that TLR4 activation enhances corneal epithelial wound healing through the promotion of migration, proliferation, and cytokine expression. These results suggest that epithelial wounding induces the expression of functional TLR4. Toll-like receptor

4 signaling appears to contribute to early corneal epithelial wound repair by enhancing migration and proliferation (Eslani et al., 2014)

- According to peng et.al.,study investigates the clinical significance of miR-146a as a biomarker in M2 macrophage polarization in diabetic wound healing. Initially, it has been found that there is reduced miR-146a in macrophages of diabetic patients. Next, dual-luciferase assay verified that toll-like receptor 4 (TLR4) was a target gene of miR-146 and was negatively regulated by miR-146. Moreover, after ectopic expression and depletion experiments of miR-146 and/or TLR4, lipopolysaccharide-induced inflammatory response of macrophages was detected. The results revealed that over expression of miR-146a promoted the M2 macrophage polarization by suppressing the TLR4/nuclear factor-kappaB (NF-κB) axis, to enhance wound healing in diabetic ulcers. Further, mouse models with diabetic ulcers were established to investigate the effects of miR-146a on diabetic wound healing *in vivo*, which revealed that miR-146a promoted wound healing in diabetic ulcers by inhibiting the TLR4/NF-κB axis. In conclusion, we demonstrate that miR-146a can induce M2 macrophage polarization to enhance wound healing in diabetic ulcers by inhibiting the TLR4/NF-κB axis (Peng et al., 2022).
- Hypertrophic scars are proliferative diseases of dermal fibroblasts that produce abundant amounts of collagen and extracellular matrix in the skin after severe burns, inflammation, and trauma. Hypertrophic scars affect the daily life of patients and cause a series of problems. The biological mechanism of hypertrophic scar formation is still unclear and has received much attention in plastic surgery. According to ping et.al.,a hypothesis has been established that LPS can activate TLR4 signalling, leading to the overexpression of collagen I and TGF-β and the induction of hypertrophic scar formation. LPS is used to validate the role of the TLR4 signalling pathway in 3T3-L1 cells in vitro and hypertrophic scar mouse models to determine the role of the TLR4 signalling pathway in proliferative scar formation in vivo. The results suggested that LPS leads to the activation of the TLR4 pathway in fibroblasts, and inhibitor experiments confirmed that TLR4 is involved in the expression of collagen I by regulating the NF-κB pathway. The mouse skin wound model experiments demonstrated that TLR4 is involved in wound healing and scar formation. Our experiments demonstrated that the TLR4-IRAK4-NF-κB pathway is involved in the production of hypertrophic scars and wound healing.

- . Cell proliferation and death are key components of wound healing and tissue repair. Telocytes (TCs) represent a newly discovered cell type that can protect tissue from acute injury via cell–cell communication with adjacent cells.according to wang.et.al.,a study had done by using a mouse model of skin wound healing and lipopolysaccharide (LPS) induced cell injury to evaluate the effects of TCs on skin wound healing in vivo and in vitro. : Immunohistochemical staining was performed to evaluate the alteration of TCs in tissues from normal and chronic wound patients. Then, a male C57BL/6 mouse wound model of the back was established. The mice were divided randomly into three groups, and wound healing was estimated according to the wound healing rate and histology. An LPS-induced co-culture model of a mouse lung telocyte cell line (TCs) with human keratinocyte (HaCaT), human dermal microvascular endothelial cell (HDMEC) or murine fbroblast (L929) cell lines was established to analyse the efects of TCs on constitutive cell types of the skin. Cell proliferation, migration and apoptosis were examined, and reactive oxygen species (ROS) and infammatory factors in HaCaT cells, HDMECs, and L929 cells were detected to study the mechanisms involved in TC protection in skin wounds. TCs were signifcantly increased in tissues from chronic wound patients compared with healthy controls.Wound healing was signifcantly improved in wound mouse models treated with exogenous TCs compared with LPSinduced models. TCs reversed the LPS-induced inhibition of HaCaT cells and HDMECs and reduced the LPS-induced apoptosis of HaCaT cells and the death ratios of HDMECs and L929 cells. TCs reversed LPS-induced ROS in HDMECs and L929 cells and decreased infammatory factor mRNA levels in HaCaT cells, HDMECs and L929 cells. TCs reduce wound healing delay, and infammatory responses caused by LPS might be mediated by infammatory inhibition, thus restricting apoptosis and promoting migration of the main component cell types in the skin (Wang et al., 2020)

CHAPTER 4: METHODOLOGY

4.1. HYDROGEL DRESSING:

Unlike traditional dressings, such as bandages and gauzes, hydrogel dressings are widely acknowledged for their excellent properties, including mechanical properties that are compatible with biological tissues and exceptional water retention capacity which can keep the wound moist and continuously absorb exudate. In addition, their opportune biodegradation avoids secondary damage during dressing replacement, making them ideal wound dressing materials (Hu and Xu, 2020). (Liang et al., 2021). Furthermore, compared to other emerging dressings, such as foam and films, hydrogels possess a three-dimensional porous network structure like that of a natural ECM, providing a framework for cells to proliferate and migrate. More importantly, hydrogel dressings can be structurally and biochemically designed and functionally integrated to acquire various advantageous properties (Brumberg et al., 2021) (Wang et al., 2021), (Firlar et al., 2022) (Luo et al., 2022). of which anti-inflammatory hydro gel dressings are foremost representatives. Natural or synthetic polymers are combined by physical or chemical cross-linking methods to present different functions and properties. Physical cross-linking mainly includes hydrophobic association, hydrogen bonding and ionic interactions. The polymers are connected by covalent bonds in chemical cross linking, including disulphide, a Schiff base and borate ester bond. By integrating drugs, small bioactive molecules and novel biomaterials into a hydrogel matrix, anti-inflammatory hydrogel dressings can scavenge excessive free radicals, sequester chemokines, and promote M1-to-M2 polarization of macrophages, thereby resolving excessive inflammation in the wound and thus promoting wound healing. Over the past 5 years, intensive research has been con ducted on anti-inflammatory hydrogels.

4.2. DRUG OF CHOICE:

4.2.1. CHRYSIN: Chrysin is a flavonoid (dihydroxyflavone) that is mainly found in honey, propolis (Jung, 2016), mint, and blue passionflower (Cho, 2004). Chrysin has a broad spectrum of biological functions, including anti-inflammatory, anti-oxidant, and pro-apoptotic (Deldar et al., 2018). It also acts as a potent stimulator for keratinocyte differentiation and accelerates the natural healing process. Despite so many biological applications, the significant challenges that have been seen in Chrysin are its poor solubility and low stability in-vivo. However, Chrysin is soluble in ethyl alcohol and DMSO, so that the approaches can be made in the field of wound healing with Chrysin. When the Chrysin has been introduced with any polymers, the

cell's terminal differentiation is enhanced even to a greater extent (Jaraczewski et al., 2013).. So Chrysin was a new and innovative drug to work with, affecting the genes contributing to wound healing and clarifying the potential application in wound healing.

4.2.2. ENROFLOXACIN:

Enrofloxacin (EF) is a fluoroquinolone antibiotic used in veterinary against both Gram negative and positive bacteria with lower minimum inhibitory concentration (Lai et al., 2013). The drug is released rapidly during inflammation at early stages of the wound healing process.

4.3.*IN-VITRO* STUDIES:

4.3.1. SOLUBILITY STUDY OF DRUGS:

The solubility study of the compounds that are either insoluble or show poor solubility in water plays a significant role in the chemical, pharmaceutical, biotechnological industries, etc. Also, to decide appropriate solvents for chrysin, it is vital to know the solubility of drugs or compounds in different solvents. Chrysin and Enrofloxacin have poor aqueous solubility.

4.3.1.1. Solubility Determination:

4.3.1.1.1. METHOD:

an excess amount (10 mg) of chrysin and enrofloxacin was added into 2 mL of solvent to solubilize the chrysin using a vortex mixer at 37C for 15 min. The equilibrated samples after vortexing were centrifuged at 3,000 rpm for 10 min. The supernatant from these sample(s) was filtered through a 0.22 μm syringe fi ter. The solubility analysis was performed in triplicates to avoid errors, and the mean-values have reported. The absorbance of chrysin and Enrofloxacin in the supernatant was determined with the help of a UV spectrophotometer (Bansal et al., 2022)From the obtained absorbance (n = 3). the solubility of drugs in different solvents (phosphate buffer), surfactants (Tween 80 and Tween 20), co-surfactants (PEG 200 and PEG 400) individually has analysed. The concentration of solubilized drugs was analysed quantitatively using the developed UV visible spectrophotometer method.

TABLE 2

4.3.1.1.2. OBSERVATION:

Solvent	Solubility of Chrysin in the solvent (µg/mL)	Solubility of Enrofloxacin in the solvent (µg/mL)
Tween 20	76.23	67.83
Tween 80	123.12	119.09
PEG 400	187.67	173.89
PEG 200	187.74	179.08
PBS	195.27	191.35

If solubility pattern is observed then both of the drugs are soluble in PBS.As Chrysin is a Flavonoid with -OH group as well as Enrofloxacin I a weak base pKa 6.0 and pH of PBS is 7.4 which is slightly alkaline which helps both the drugs to get ionize which increases solubility.

4.3.2. HYDROGEL FORMULATION:

4.3.2.1. Materials and Method:

Chrysin 5,7-dihydroxy, triethanolamine, Enrofloxacin were obtained from BLD Pharma, Sigma Aldrich Taufkirchen, Germany and Nanjing Kangmanlin biomedical technology China respectively. Carbopol 940 was obtained from Lubrizol (Wickliffe, OH, USA). Other ingredients and chemicals utilized in this investigation were of pharmaceutical and analytical grade.

4.3.2.2. Preparation of Carbopol Hydrogel and Gel strength Determination:

Carbopol 940 was used as gelling agent for preparing a topical hydrogel system. Carbopol 940 was prepared at different concentration 0.5%w/w,1%w/w, and 5%w/w by dissolving 0.5 g, 1g ,5g of it in 90 g of phosphate buffer solution and then making the final weight up to 100 g. These mixtures were kept over night to achieve complete swelling and homogenecity of Carbopol 940 into aqueous system (Algahtani et al., 2020b). Triethanolamine was added drop-

by-drop into an aqueous dispersion system of carbopol 940 to convert it into a hydrogel system. The prepared carbopol 940 hydrogel was evaluated for gel strength determination (Algahtani et al., 2020a). Carbopol 940 hydrogel weighing 60 g was placed in a 100 mL graduated measuring cylinder (1.5 inches in diameter). Subsequently, a disc (having a diameter of 3 cm and thickness of 3 mm) was placed on the gel surface with 30 g of weight mounted on it. Gel strength was determined by the time the disc took (in seconds) to sink 5 cm into the gel from the surface. An accurate amount of Chrysin and Enrofloxacin was dissolved in PBS with constant stirring and homogenization. Subsequently, triethanolamine (2–3 drops) was poured into the mixture to neutralize the aqueous dispersion system of carbopol 940 to a hydrogel system.

4.3.2.3. OBSERVATION:

The characteristic properties of hydrogels of substantial significance in treating wounds are gel strength, spreadability, and thixotropic characteristics. Ideally, the hydrogel strength should lie in a range of 25–50 s for topical administration because if it is less than 25 s, then the gel would not be able to adhere appropriately to the wound, and if it is more than 50 s, then it will be difficult to spread the gel uniformly over the wound bed. In the present study, carbopol hydrogel of different strengths was prepared 0.5% w/w, 1% w/w, 5% w/w. The gel strength was found to be 38.94±1.27s, 42.05±1.03s, 48.07± 1.67 s respectively. Carbopol 940 Hydrogels of gel strength of 1 % w/w were selected because their optimal characteristic is desirable for topical hydrogel preparation, mainly exploited in wound healing applications

TABLE 3

The percentage composition of the optimized formulation system for wound healing application shown I the table.

S.No.	Ingredients	Role	% Composition w/w
i	Carbopol 940	Gelling Agent	1
ii	Chrysin	API	0.5
iii	Enrofloxacin	API	0.5
iv	triethanolamine	pH adjuster	q.s.
v	Phosphate Buffer	Vehicle	q.s. to 100

4.3.3. Drug release parameter:

In vitro release of hydrogel was performed using vertical Franz diffusion cell at 37±0.5ºC. The area for diffusion was 2.14 cm2 and the receptor chamber volume was 5.0 mL Acetate cellulose membrane (0.45µm pore size) was fit between donor and receptor compartment. The receptor medium consisting of phosphate buffer (pH 7.4).1g of hydrogel containing 1000 µg of combination drug was evenly spread on the membrane surface. Half of a millilitre of the receptor medium was taken at pre-determined time intervals of 5 min ,30 min,1hr to till 12 hours replaced by an equal volume of fresh medium. Release amount is determined by % of drug release in time interval described in fig:1, which indicates that 60% of loaded drug had been released over 8 hours (Negi et al., 2019).

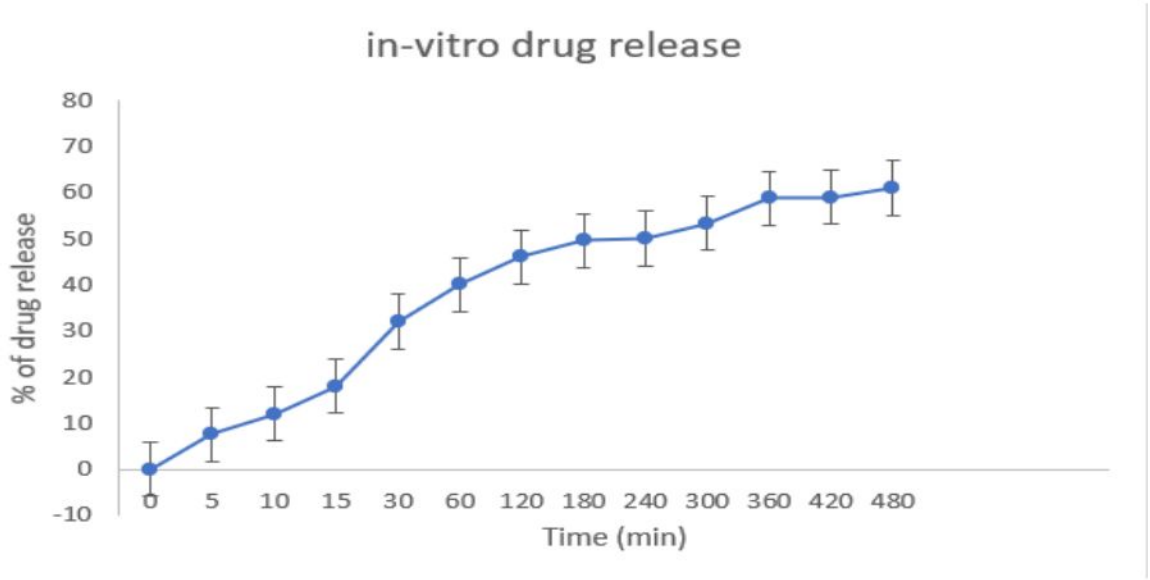


Fig1: Drug release profile

4.3.4. Rheology:

The developed chrysin & Enrofloxacin loaded hydrogel system was characterized for its rheology profile. The unique attribute of hydrogels is their sol–gel inter-convertible nature or thixotropic property. Due to this, they convert into a softer and spreadable sol form on the application of shear rate/stress, and once that force is withdrawn, they adhere to the surface over which they were spread in the same way. Rheological behaviour of the developed formulation is helpful in imparting the spreadability and extrudability characteristics. A

rotational viscometer Anton paar MCR 102 with a parallel plate was employed to estimate a rheological profile of the prepared hydrogel system at room temperature. Easy spreadability of the hydrogel formulation is highly desirable. To exhibit good spreadability, pseudoplastic rheological behaviour is desirable for the topical formulation, particularly for wound healing applications. The pseudo plastic behaviour of the developed formulation is helpful to attain the desired spreadability.

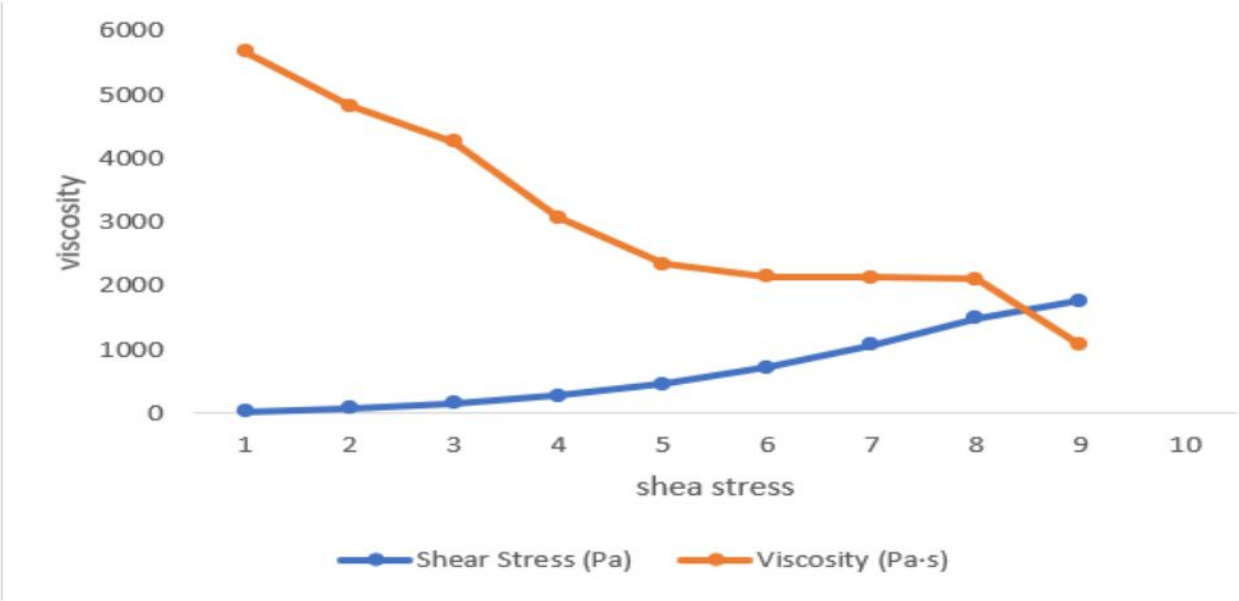


FIG: Viscosity of Hydrogel

It was observed that the hydrogel system has a thixotropic characteristic, non-Newtonian and pseudoplastic behaviour that is greatly desirable for ideal topical formulation. From fig it showed that increase of shear stress and decrease of viscosity as they are inversely proportional for non-Newtonian fluid, which indicates shear thinning. This would help in easy removal of gel during dressing (Li et al., 2021).

4.3.5. Antimicrobial assay:

Escherichia coli (ATCC 8739) and *Staphylococcus aureus* (ATCC 25923) both were used as bacteria models to evaluate the activity. The bacteria pre-culture was incubated under aerobiosis and moderate shaking for 24 h. The *E. coli* was kept at 37 °C and the *S. aureus* at 32 °C, considering the ideal temperature for each colony growth The bacterial kinetic was determined by measuring the absorbance at 620 nm wavelength hourly, following procedure, using a Shimadzu UVPC 2000 (Shimadzu Co. Kyoto, Japan) spectrophotometer.

4.3.3.1. OBSERVATIONS:

The time- kill kinetics antibacterial study of the combination formulation was carried out to assess the killing rate within a given contact time. This study was done according to standard guide for assessment of antimicrobial activity using time-kill kinetics procedure of Antimicrobial Susceptibility Testing Method. Microbial population at the initiation and completion was determined by spectrometric methods at interval of 30 min.

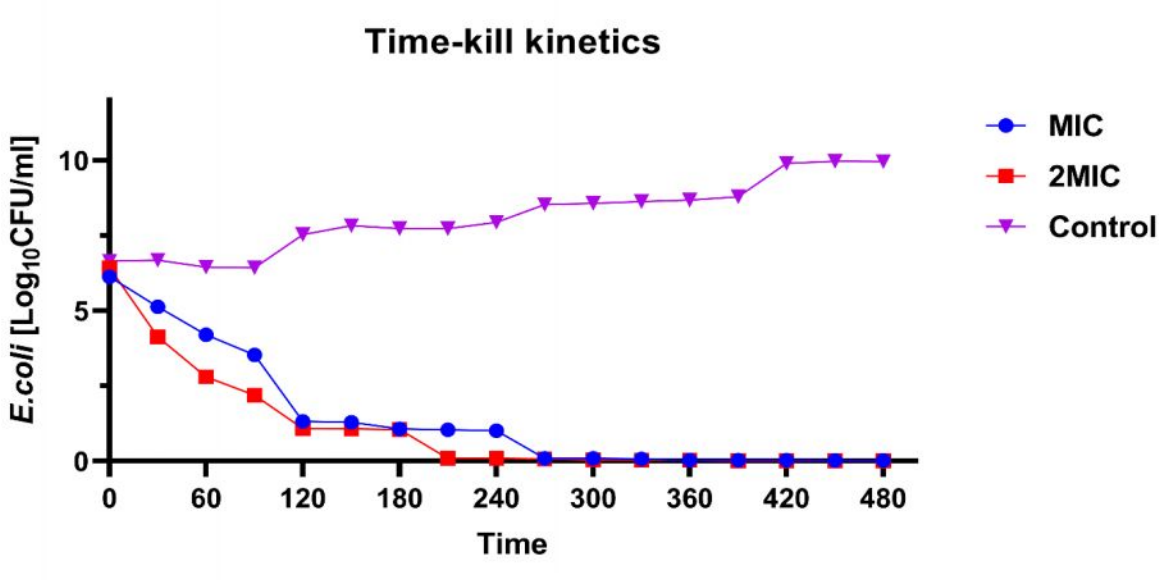


FIG: 4

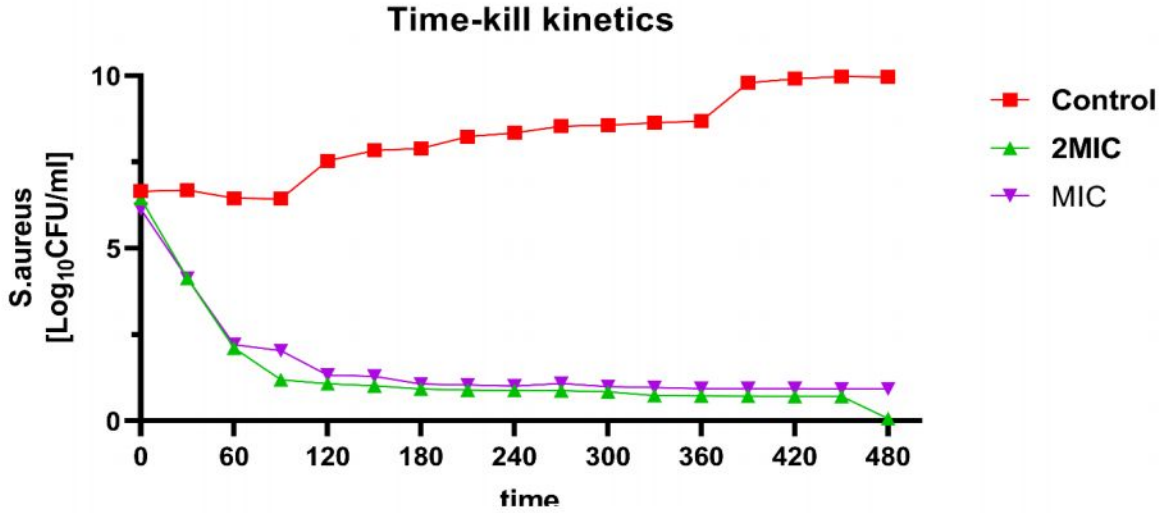


FIG:5

From the curve of time dependent kinetic study it demonstrates that the combination formulation has bacteriostatic activity for Gram (+) *S.aureus* bacteria and bactericidal activity against gram(-) *E.coli* bacteria. Where chrysin is a bacteriostatic only., & Enrofloxacin possess only bactericidal activity

4.3.5. Enzyme linked immunosorbent assay:

In vitro enzyme-linked immunosorbent assay for the quantitative measurement of TNF-alpha in skin tissue culture supernatants. This assay employs an antibody specific for TNF-alpha coated on a 96-well plate. Standards and samples are pipetted into the wells and TNF-alpha present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti TNF-alpha antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a

TMB substrate solution is added to the wells and colour develops in proportion to the amount of TNF-alpha bound. The Stop Solution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm. This assay has been performed for every group and TNF-alpha was quantified for for every study group. Tissue sample was taken at 0 hr for on day0 followed by again skin tissue sample withdrawn at 3 hr. Then skin tissue sample was taken at day 3, day7, day14 for every study group.

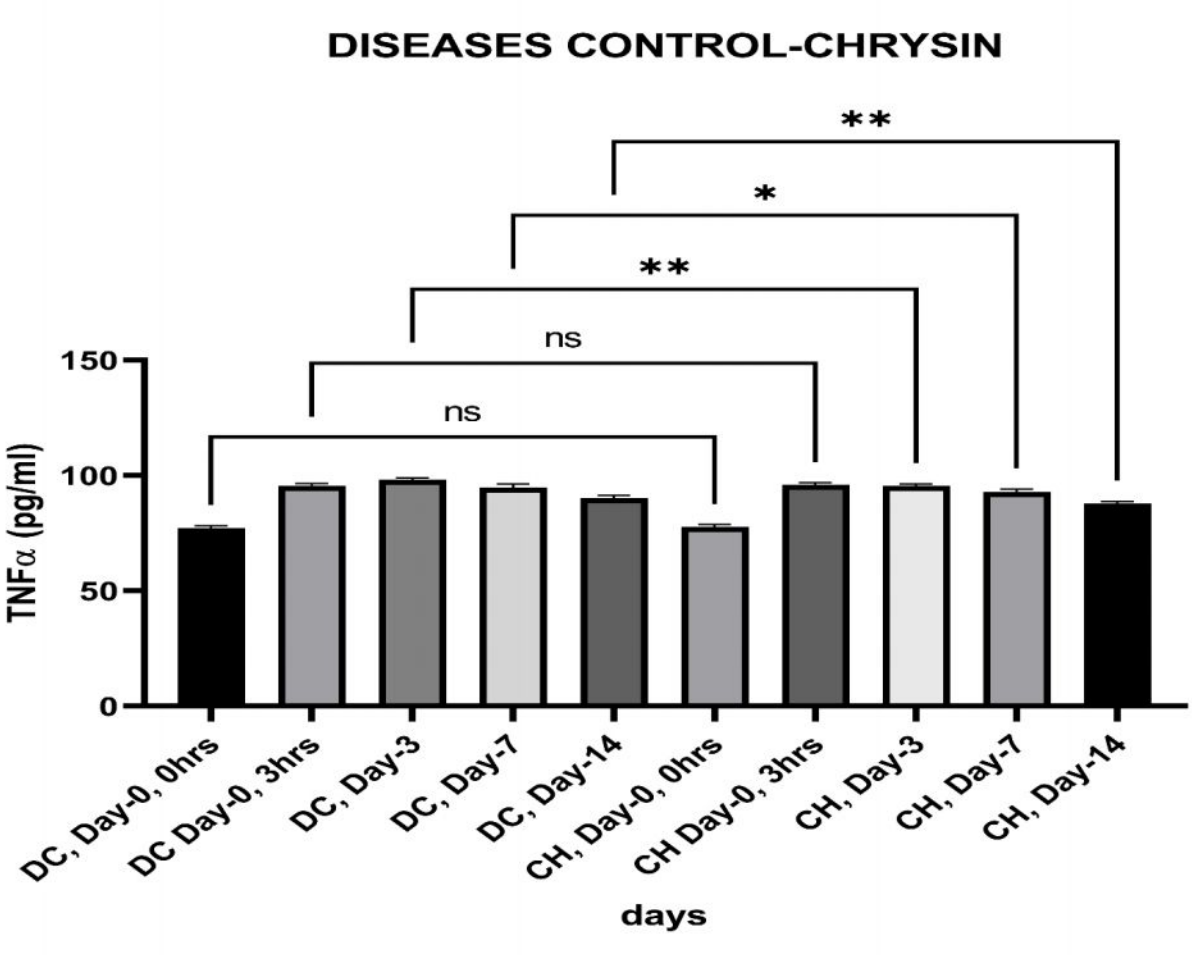


FIG: 6 **TNF-alpha value for diseases and**

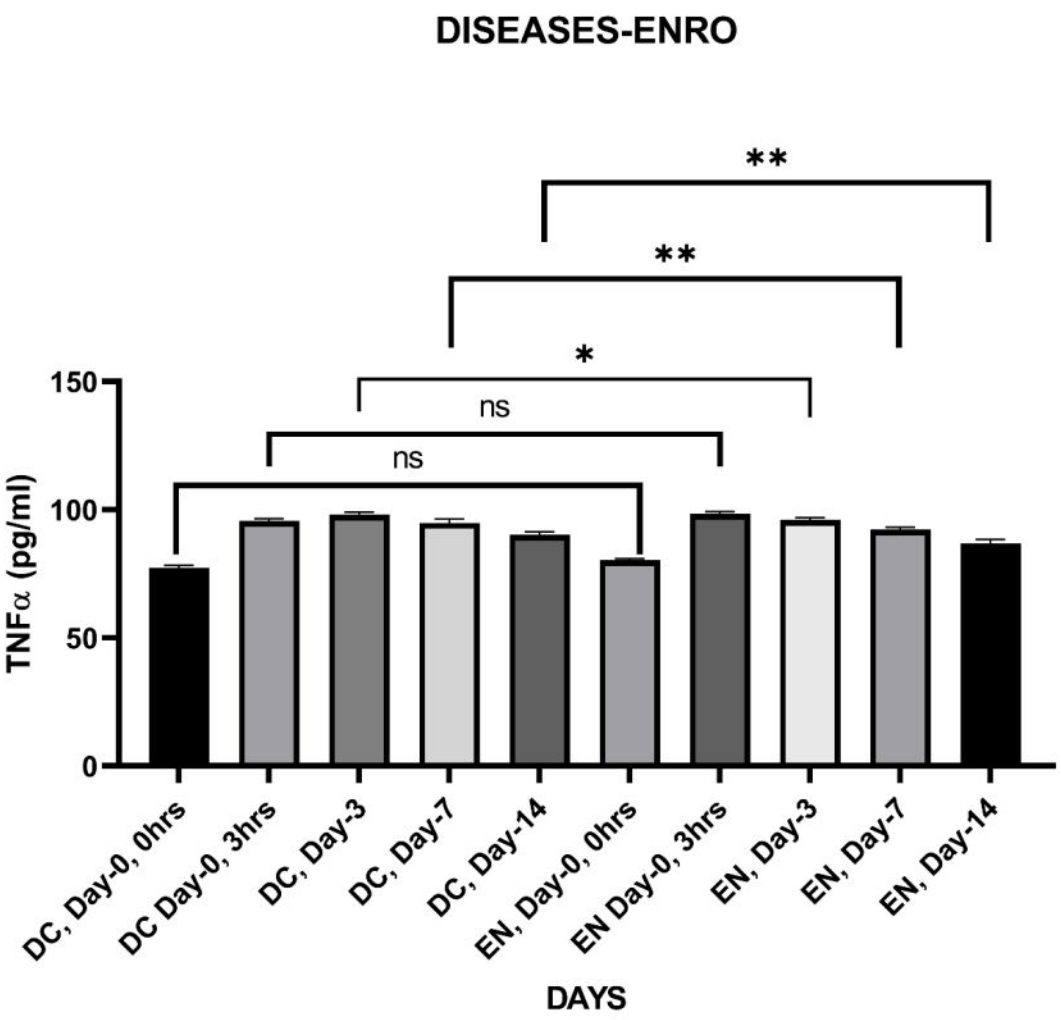


FIG7: TNF-alpha value for diseases and Enrofloxacin

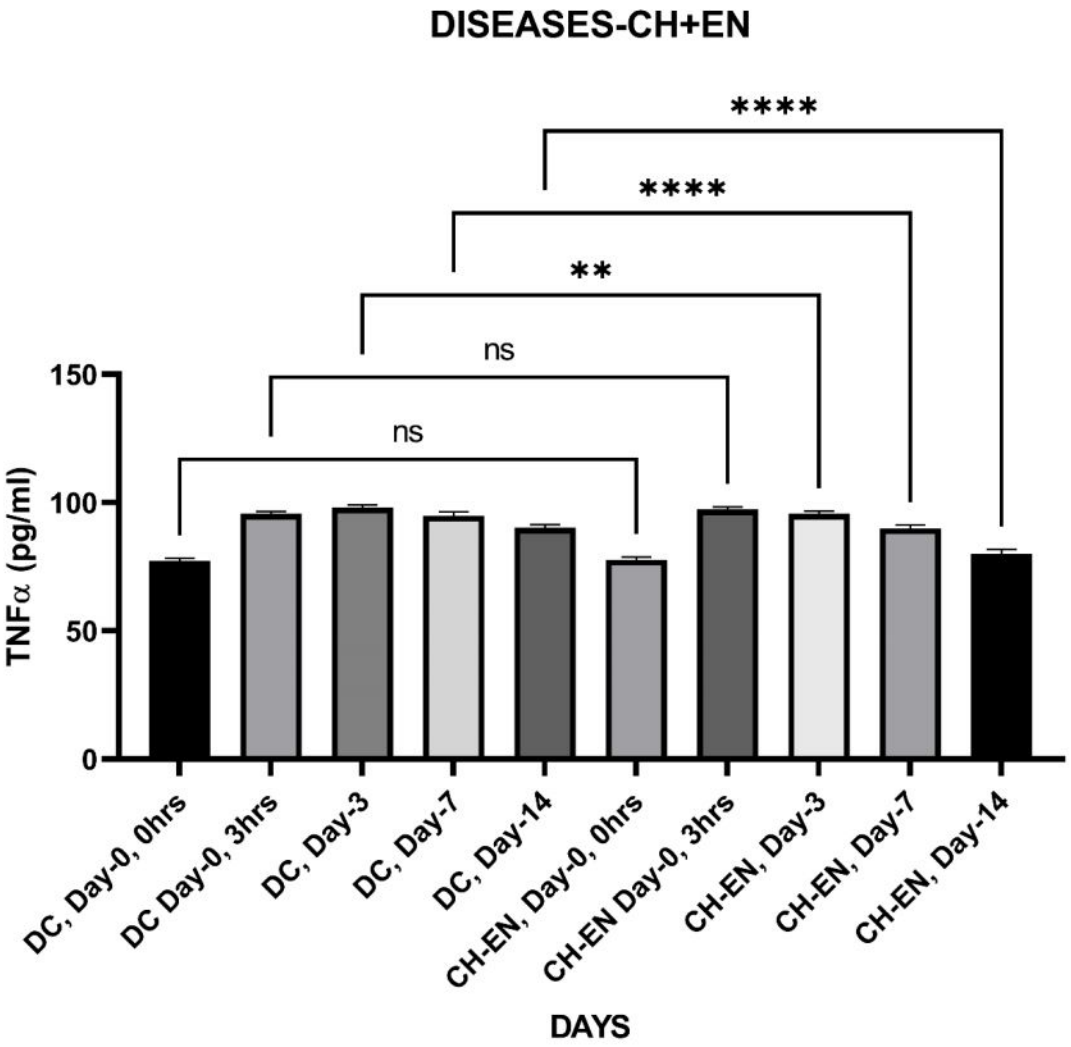


FIG:8 TNF-alpha value for diseases and combination gel

4.3.6.: Synergistic effect:

The prerequisite for synergism or antagonism determination is to know both the "potency" and the "shape" of the dose-effect curve for each drug. Therefore, it is necessary to calculate the does-effect parameters of each drug alone, as well as in combination and thereby determine the CI value. The above parameters can be easily and automatically determined from the median-effect equation using CompuSyn software (Available for free download from www.combosyn.com). The present study indicates that chrysin and enrofloxacin are moderately synergistic with CI values ranging from 0.836 to 0.715 for fa=0.5~0.97 as indicated by the and Fa-CI plot.

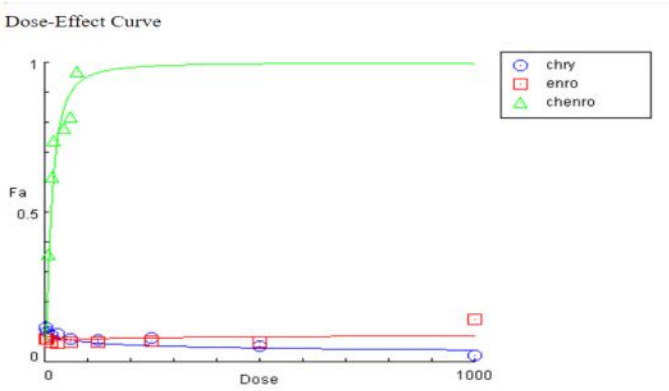


FIG: 9

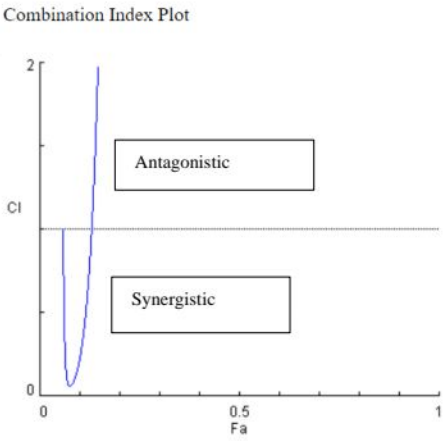


FIG:10

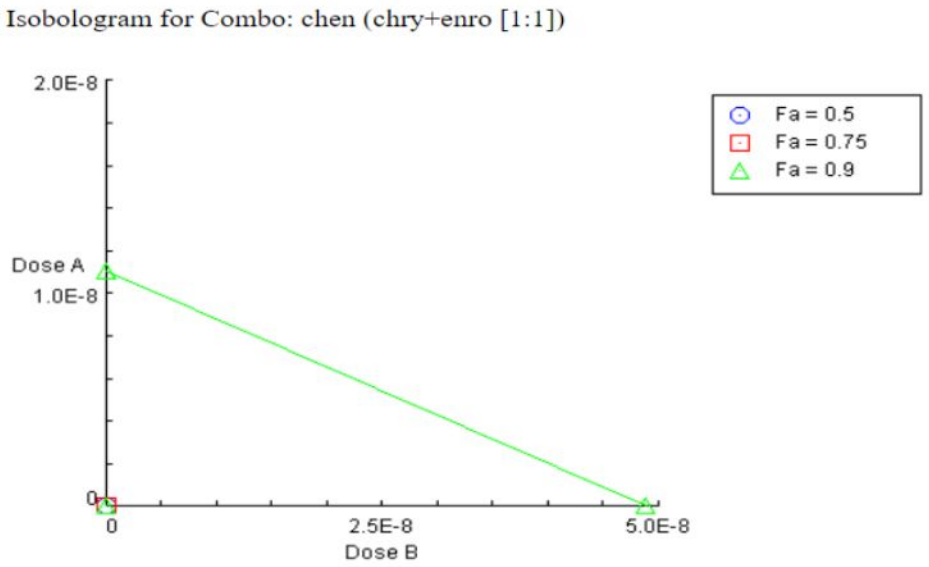


FIG:11

The isobolograms, as shown at fa=0.5, 0.75 and 0.9 in fig. the present studies in vitro showing synergy at broad dose ranges and broad effect ranges is consistent with the in vivo wound healing co-treatment at a given optimal specific dose and regimen of each compound.

CHAPTER 5: WOUND HEALING ACTIVITY OF HYDROGEL

5.1: Materials and methods

5.1.1: Animals

The State Centre for Laboratory Animal Breeding (SCLAB), Buddha Park, B 14, Block B, Kalyani, West Bengal 741235, provided male Wister rats (180-220 grams), aged between 6-7 weeks. Following that, animals were cared for properly in the departmental animal home both before and throughout the trial. Temperature was maintained between 26 and 20 degrees Celsius, relative humidity was 44-56%, and 12-hour cycles of light and dark was maintained with the right food and bedding. Prior to the primary animal experiment, the Institutional Animal Ethical Committee (IAEC) approved the experimental work.

5.3*: In – vivo* Wound Healing Studies

5.3.1: Excision Wound Model

First, the test rats were given ketamine to make them unconscious (Rhea and Dunnwald, 2020). After marking a 200 mm2 region on the dorsal of the experimental rats with a conventional ring, the hair was plucked from that location. Each rat had a properly executed excision of the desired area. After measuring the wound's area, this process was repeated every four days for the next 16 days. Measurements and calculations were made on a regular basis to account for changes in the damaged area and the pace of wound contraction. Following that, the duration of epithelization was also tracked, noted, and compared amongst the groups. Image J software was used to trace the wound.

% Wound contraction = Healed area∘ /Total wound area × 100,

(% healed area = original wound area – present wound area)

5.3.2: Treatment Protocol

All 30 Animals were divided into 5 groups as following **:** Gr I: Chrysin loaded hydrogel treated, Gr II: Enrofloxacin loaded hydrogel treated , Gr III: Chrysin + Enrofloxacin loaded hydrogel

treated, Gr IV: Diseases control, Gr V: Normal control (Turner et al., 2011)

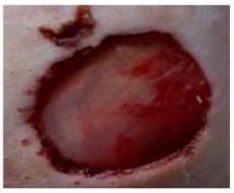
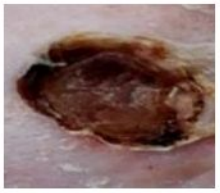
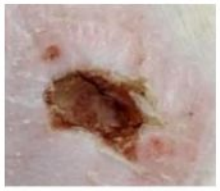
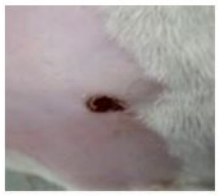
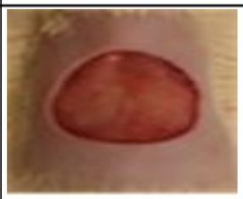
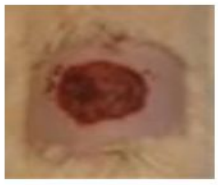
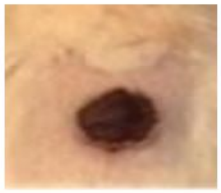
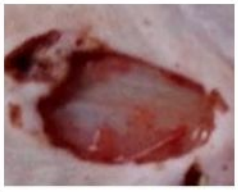
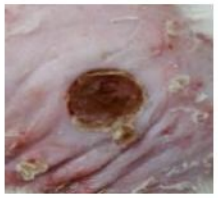
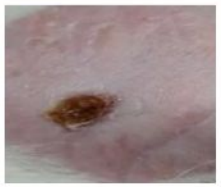
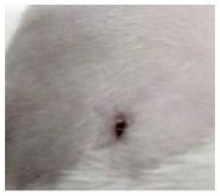
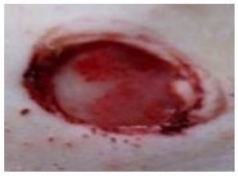
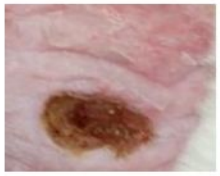
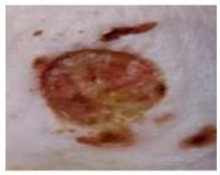
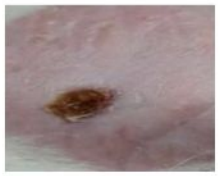
GROUPS	DAY0	DAY3	DAY7	DAY14
CHRYSIN TREATED [GROUP I]				
ENROFLOXACIN TREATED [GROUP II]				
COMBINATION DRUG [GROUP III]				
DISEASES GROUP [GROUP IV]				

Fig:12. Qualitative trend of the wound healing process of rat groups receiving no treatment (diseases control), chrysin gel, enrofloxacin gel, and combination drug loaded formulation. The photos were taken on Day0 wound creation day, Day 3, Day 7, and Day 14 of treatment.

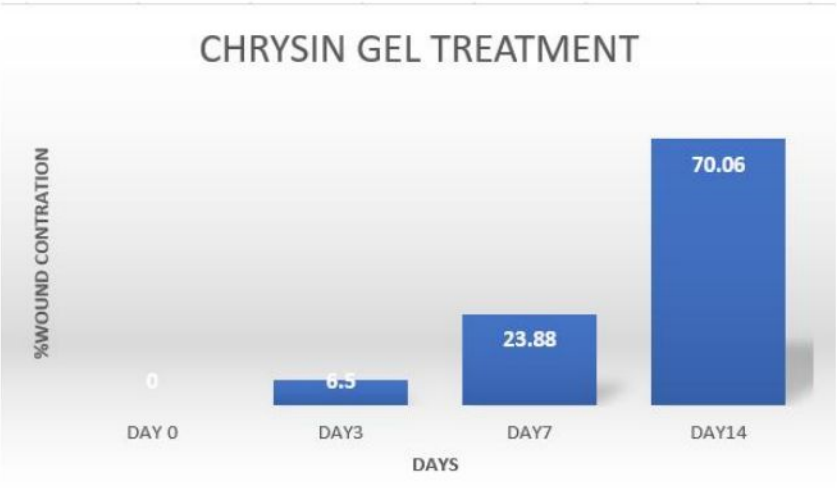


Fig: 13. Group I wound contraction

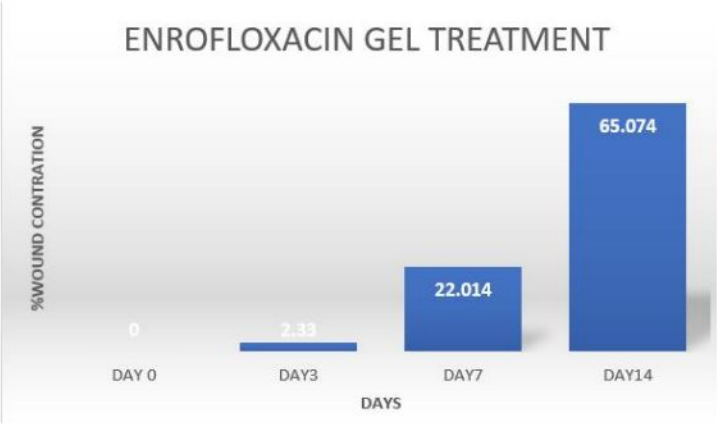


Fig: 14. Group II wound contraction

Fig: 15. Group III wound contraction

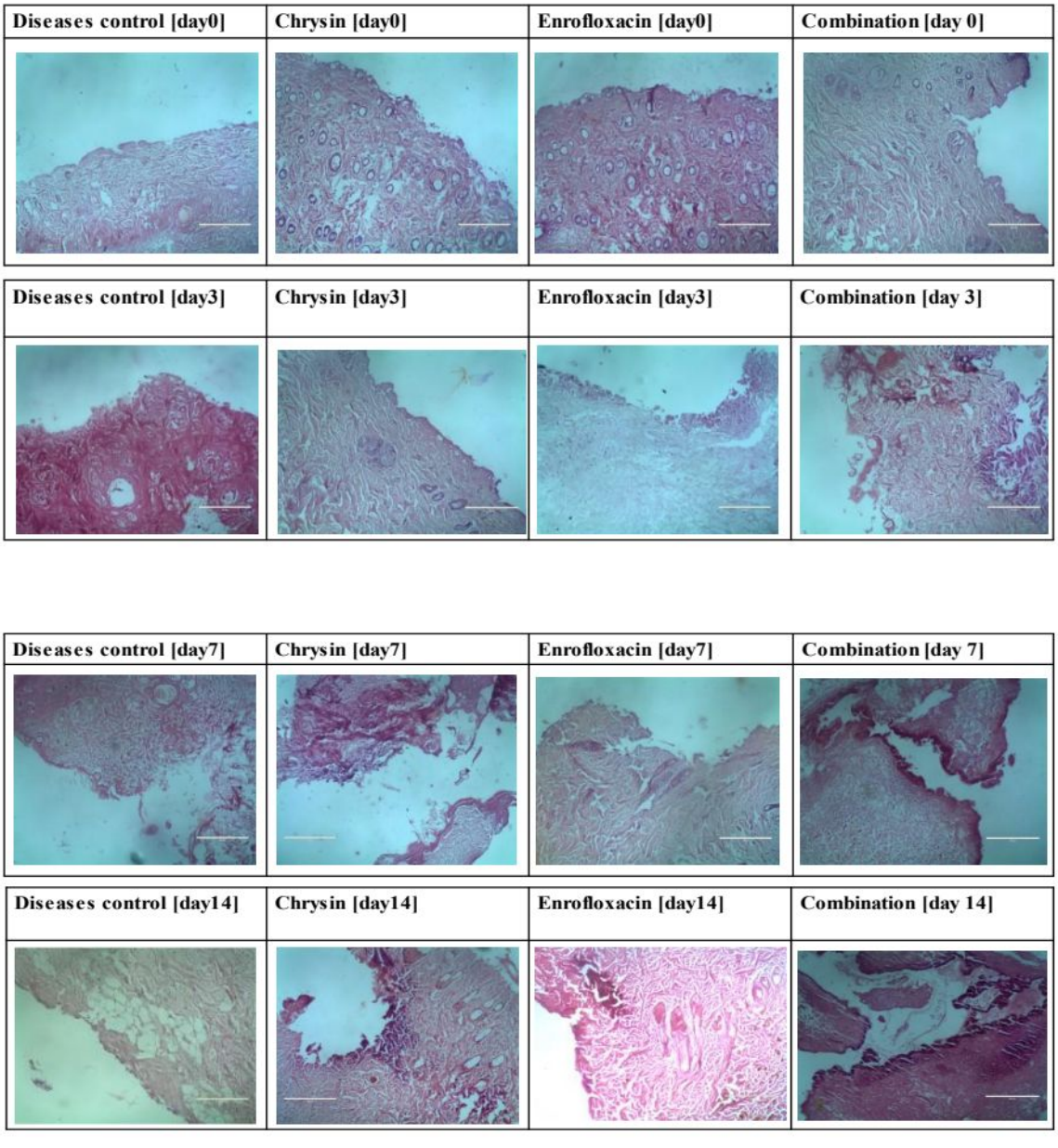
Fig: 16. Group IV wound contraction

5.3.3. OBSERVATION:

In Group I the percentage of wound contraction in day 14 is 70±5% in respect to day 0.in group II which receives only enrofloxacin treatment the percentage of wound closer is 65±5% with respect to day 0.Group III which receives the combination hydrogel chrysin and enrofloxacin loaded.shows the maximum rate of wound contraction i.e.106±5% in day 14.where as the group IV diseases controlshows lesser rate of wound contraction on 14^{th} day that is 39±5%.Hence Combination hydrogel is more effective than the single drug loaded hydrogels.

5.4: Histopathology

The samples of the group treated with the hydrogels and no treatment in diseases control group showed signs of collagen and re-epithelization, but these The results of the histopathological evaluation of skin samples treated with different formulations (including skin samples of the no treatment group) on day 14 post-wound are shown in Figure ..The samples of the group treated with the chrysin hydrogel, Enrofloxacin hydrogel and combined drug formulation showed signs of collagen and re-epithelization, but these findings were more pronounced in samples treated with the chrysin and enrofloxacin loaded hydrogel system. Figure confirms the hydrogel system's substantial wound healing potential (Masson-Meyers et al., 2020).



5.5. Discussion:

Being a flavonoid, chrysin has less cytotoxic effect against fibroblast which is key growth factor for wound healing with cell proliferative property Enrofloxacin is fluroquinolone antibiotic possess bactericidal activity against gram-positive and gram-negative bacteria whereas chrysin has bacteriostatic effect.

Time kill kinetics study showed that the combination of chrysin and enrofloxacin has bacteriostatic activity against gram positive bacteria where as it has bactericidal activity against gram negative bacteria. Moreover, according to solubility study both the drugs are soluble in phosphate buffer [pH6.4]. However, it is also noted that phosphate buffer will help to maintain the physiologic condition as well as the pH of formulation which is also compatible for skin and provide moisture to the wounded area which is necessary for fibroblast stimulation. For hydrogel drug release parameter shows 60% release from the matrix over the time period of 8 hr which is ideal. However, rheological study also shows that the hydrogel is non-newtonian has shear thinning property. These studies confirm that chrysin-enrofloxacin hydrogel is ideal as hydrogel formulation.

Synergistic study has been performed for the chrysin and enrofloxacin with fractional inhibitory concentration by using compusyn software where isobologram curve showed the potential synergistic at 1:1 ratio.

TNF-alpha, a significance inflammatory cytokine marker, is activated TLR4 mediated downstream signalling pathway during inflammatory condition. So, it is necessary to quantify this marker for every groups. From ELISA it has been seen that there was significant change in TNF-alpha concentration in study groups which are mentioned previously. Statistical data shows that concentration of TNF-alpha in combination group is significantly lesser than the individual chrysin and enrofloxacin group whereas TNF-alpha is significantly higher in diseases control group. However, sustained high concentration of TNF-alpha is responsible for prolong inflammation that ultimately slow down the wound contraction whereas lower concentration of TNF-alpha is one of the significant parameters of wound healing property. Due to antimicrobial effect of combination formulation inflammation is less. From histopathological study it has been seen that collagen formation is more in combination formulation treated group than the disease control group and individual chrysin gel and hydrogel gel.

5.6.CONCLUSION:

From this study, it is hypothesized that hydrogel formulation, combined with Chrysin & Enrofloxacin provide synergistically significant role in manifestation of wound healing as well as antimicrobial property with better release effect. moreover, this combination shows better bactericidal property in gram negative bacteria and significantly has more lowering effect in TNF-alpha than Chrysin & Enrofloxacin. So, it may effect on LPS-TLR4 signalling pathway where LPS is a gram-negative bacterial endotoxin and a significant role in TLR4 mediated induction of inflammatory cytokine like TNF-alpha, though further study is necessary however Chrysin & Enrofloxacin combination hydrogel may provide a stepping stone in therapeutic management with better wound healing effect.

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