EVALUATION OF WOUND HEALING POTENTIAL OF BIOFABRICATED GOLD NANOPARTICLE [GNP] IN FULL THICKNESS EXCISIONAL WOUND IN RAT MODELS

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By

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DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY
Bihar Veterinary College, Patna-800014
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CERTIFICATE- I

This is to certify that the thesis entitled, **"EVALUATION OF WOUND HEALING POTENTIAL OF BIOFABRICATED GOLD NANOPARTICLE [GNP] IN FULL THICKNESS EXCISION WOUND IN RAT MODELS"** submitted in partial fulfilment of the requirement for the award of the degree of Master of Veterinary Science in the discipline of **VETERINARY SURGERY AND RADIOLOGY** of the faculty of Post-Graduate Studies, Bihar Animal Sciences University, Patna, is a bonafide research work carried out by **Dr. SWETA KUMARI, Registration No-VM0023/2019-20** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation have been fully acknowledged.

Place: Patna **(GYANDEV SINGH)**

Date: (Major Advisor)

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CERTIFICATE- II

This is to certify that the thesis entitled, **"EVALUATION OF WOUND HEALING POTENTIAL OF BIOFABRICATED GOLD NANOPARTICLE [GNP] IN FULL THICKNESS EXCISION WOUND IN RAT MODELS"** submitted by **Dr. SWETA KUMARI, Registration No-VM0023/2019-20** to the Bihar Animal Sciences University, Patna, in partial fulfilment of the requirements for the degree of Master of Veterinary Science in the discipline of **VETERINARY SURGERY AND RADIOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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Place _____

Date _____ ***(Sweta Kumari)***

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

<	-	Less than
>	-	More than
%	-	Percentage
GNP	-	gold nanoparticle
MGNP	-	biofabricated gold nanoparticle
M.O.	-	*Moringa oleifera*
NS	-	Normal saline
B	-	Betadine
T/t	-	treatment
S. aureus	-	Staphylococcus aureus
Cq	-	cycle threshold
Rt-PCR	-	reverse transcriptase polymerase chain reaction
c-DNA	-	Complementary Deoxyribonucleic acid
ANOVA	-	Analysis of Variance
AOAC	-	Association of Official Analytical Chemist
DMRT	-	Duncans multiple range test
BW	-	Body weigh
µg	-	micro gram
mg	-	mili gram

DNA	-	Deoxyribo nucleic acid
RNA	-	Ribonucleic acid
HP	-	Hydroxyproline assay
ROS	-	Reactive oxygen species
g	-	Gram
IU	-	International Unit
Kcal	-	Kilocalorie
Kg	-	Kilogram
ml	-	Millilitre
U/L	-	Unit per Litre
WHO	-	World Health Organization

DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY

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Title of thesis**: EVALUATION OF WOUND HEALING POTENTIAL OF BIOFABRICATED GOLD NANOPARICLE [GNP] IN FULL THICKNESS EXCISION WOUND IN RAT MODELS**

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ABSTRACT

Wound has been set down as one of the commonest affliction in the mammals' body since the ages. Wound in the modern medicine has been defined as a discontinuity in the epithelial lining of the skin or mucosa resulting from physical or thermal damage. In the simplified manner any breach in the continuity of skin is considered as wound. Antibiotic resistance is a big issue in wound healing which is a result of indiscriminate would be developed due to indiscriminate use of antibiotics. Use of Gold nanoparticle are a meaningful research around the world in different area including electronics, nanotechnology and biomedicine. GNP has potent antioxidative properties and plays an important role in wound healing. Gold nanoparticle is modern biochemical applications both in vitro and in vivo.

The research work was conducted in 42 healthy male wistar rats equally divided into three groups comprising 14 animals in each group at day 0 of study. The animals of group I served as healthy control and treated with normal saline. The group II animals was treated with betadine and group III animals were treated with biofabricated gold nanoparticle. Treatment was done at first 5 days from wound creation. Tissue is collected from 7^{th} days and 14^{th} days from wound creation, and showed the effect of topical application of gold nanoparticle in full thickness excisional wound in rats. My study was done to see the wound healing efficacy and molecular perception of wound healing process following topical application of biofabricated GNP in full thickness excisional wound in rat models.

Re-epithelialization and improved formation of hair follicles and consider it as healthy sign of healing process. Collagen is directly proportional to the value of Hydroxyproline. Collagen has role in later stages of wound homeostasis and re-epithelisation process in reparative wounds as evident by the significant increase in Hydroxyproline value among the rats which is treated with biofabricated gold nanoparticle. Higher concentration of Hydroxyproline indicates speedy healing that also reflected by the increased cellular DNA content during the re-epithelisation process.

The gene was down regulated by 6.54 fold in MGNP treated group whereas it was 1.75 fold down regulation in Betadine treated group on 7^{th} day. After 7^{th} day of treatment the gene expression was going to be higher but still it was down regulated. The amplitude of the fold change was -0.93 and -0.37 on day 14^{th} in MGNP and Betadine treated groups in comparison to the control group.

In excision wound, biofabricated GNP showed faster healing compared with betadine and normal saline respectively. In present study more or less there was complete development of all the normal cells including epidermis, dermis & hypodermis components in rats treated with biofabricated gold nanoparticle group. Epithelisation was observed from the first day up to last of the experiment i.e. 14th day.

1. INTRODUCTION

Animal health issue has been considered on top priority throughout the twenty first century and many intensive work has been done to solve the issue of animal health. The skin plays very important role by giving protection of human and animal body, from various environmental hazard. Disruption of skin epithelial layer integrity, causes structural and functional disturbance (Enoch and Leaper. 2008). Factors affecting wound healing includes trauma, hygiene condition and ischemia (Branom and Rappl. 2001). Cutaneous wound healing is an essential physiological process with the collaboration of many cell strains and their products. The wound-healing process takes place in four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro. 2004). These phases of wound healing and their bio physiological functions should occur in the sequence, at a specific time, and continue for a specific duration up to optimal intensity (Mathieu *et al*., 2006).

Normal Wound-healing Process-

Phase	**Cellular and Bio-physiologic Events**
Hemostasis	1. vascular constriction 2. platelet aggregation, degranulation, and fibrin formation (thrombus)
Inflammation	1. neutrophil infiltration 2. monocyte infiltration and differentiation to macrophage 3. lymphocyte infiltration
Proliferation	1. re-epithelialization 2. angiogenesis 3. collagen synthesis 4. ECM formation (ECM, extracellular matrix.)
Remodeling	1. collagen remodeling 2. vascular maturation and regression

Antibiotic resistance is a big issue in wound healing which is a result of indiscriminate would be developed due to indiscriminate use of antibiotics (Singh *et al*., 2014). Use of gold nanoparticle are meaningful research around the world in different area including electronics, nanotechnology and biomedicine (Mokammel *et al*., 2019). Nanoparticle can be broadly classified into five important categories viz, semiconductor quantum dots, magnetic nanoparticle, polymeric particle, carbon-based nanostructure and metallic nanoparticle (Hung *et al*., 2007). Each of these nanostructures have characteristic properties and supplication. Among all the metals, gold is the most widely used and considered metal for biological applications.

Gold nanoparticle (GNP) are different from gold particle which is larger in size and inert solid whereas, GNP having antioxidant property (Nafisi *et al*., 2017). GNP has potent antioxidative properties and plays an important role in wound healing (Muthuvel *et al*., 2014). Gold nanoparticle is modern biochemical applications both in vitro and in vivo (Kim *et al*., 2015). Property of gold nanoparticle are depended upon its different shape and size (Nafisi and Mailbach, 2017). The synthesized nanoparticle showed synergistic effect with antibiotics against the micro-organism including Bacillus subtilis, Staphylococcus aureus, Escherichia coli etc. Important characteristics of gold nanoparticle includes-(a) gold core is basically inert and non-toxic (b) multifunctional gold nanoparticle can be created through thiol linkage (Manju *et al*., 2010).

Nanotechnology is an antidote manufacturing technology that allows the long-established trend towards smaller, faster, cheaper materials and devices. Gold nanoparticles (GNPs) are the most amicable nanomaterial for preparation of engineered nano-platforms in smart sensing devices. Biomedical applications using GNPs have become very fruitful research area in the recent years. A large variety of possible biomedical applications has been examined, i.e., drug and gene delivery etc. Previously it was reported that gold nanoparticles could be synthesized by using yeast, fungi, bacteria, plant extracts which provides an inspiration for studies on green chemistry routes. Present investigation is to synthesize stabilized GNPs by reduction of aqueous chloroauric acid solution using *Moringa oleifera* pod extract at normal room temperature.

GNPs can directly target the bacterial call wall, or they can bind to bacterial DNA, blocking the double-helix from uncoiling during replication or transcription, thus exerting bactericidal and bacteriostatic properties. As a result, they can inhibit multidrug-resistant

pathogens, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Moreover, GNPs prevent the reactive oxygen species, thus acting as antioxidant, aiding the healing process (Vijay Kumar *et al*., 2019)

Bio fabrication is a secret weapon to advance manufacturing healthcare and economics (Pavolich., 2016). Moringa oleifera commonly called drumstick or horse radish tree is used in Indian medicine in many more fiIed (Ranjan *et al*., 2009), and the alcoholic extract of the leaves of *Moringa oleifera* have analgesic activity (Sutar *et al*., 2008).

Objectives

1. **To study the wound healing potency of biofabricated gold nanoparticle in rat.**
2. **To observe gross and histomorphological changes in full thickness excisional wound in rat.**

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2. REVIEW OF LITERATURE

General consideration about integumentary system

As we know skin is considered as the largest organ of the body, which acts as barrier to protect the animal and humans from microbial infection and from environmental deterrents. It helps in regulation of homeostasis of the body and has the receptors to perceive the senses of heat, cold, touch and nociception. Skin has different morphologically distinct layers i.e. epidermis, dermis and hypodermis. Epidermis has capability of self-renewals and the loss from the desquamation from stratum corneum is corrected from the cellular growth generated through lower epidermis. Since epidermis is considered as the outermost layer of skin and thus it provides toned skin structure and prevent from water insult. Protective action of the skin is primarily provided by stratum corneum. Underlying this layer, viable epidermis (50–100 μm thick) is present which is responsible for generation of stratum corneum (Bouwstra and Ponec, 2006). From the inside to the outside these layers are the stratum basale, the stratum spinosum and the stratum granulosum. Melanocytes, cells responsible for skin pigmentation, which is present in the epidermis layer of the skin. The dermis layer composed of connective tissue, sweat glands and hair follicles, whereas the hypodermis or deeper subcutaneous tissue is layered from fat and connective tissue.

The skin of any living organism is not morphologically uniform structure, it covers whole body surface and constitute 15% of rat total body weight of rat. The structure of all mammalians' skin is similar and which is a layered structure. The epidermis, which is the external layer of the skin, is an ectodermal origin. Other layers: dermis, subcutaneous adipose tissue, nerves, musculature and vessels are mesodermal origin (English *et al*., 1994 and Elwell *et al*., 1990). Rats can be used as an animal model of a disease and its complications or as an effectiveness of the therapy. Rat skin can be used to assess the impact of nutritional deficiencies or aging (Thomas *et al*., 2005). The epidermis of rat is keratinized, multilayer flat epithelium which cover the dermal layer of skin. Dermis is contains connective tissue containing collagen, giving the skin considerable resistance, as well as elasticity and lissomness (Karimi *et al*., 2015). It contains numerous blood vessels and nerve endings which is helpful to feel touch or pressure, as well as cold and heat receptors.

Unlike a human skin, rat skin consist of an additional layer called panniculus. It is a layer of muscle differentiated in terms of the amount and thickness of muscle fibers. In

females, panniculus sometimes appears as a layer of dividing cells, especially in the area where the tissue transforms into mammary glands. The muscle is interrupted by the occurrence of fat tissue foci containing bundles of connective tissue, in which vessels and nerves are present (English *et al.,* 1994, Elwell *et al.,* 1990). In mammals, the skin maintain, the integrity of the organism with the external environment. An important role of the skin is protection against the adverse effect of the external environmental factors. A dense barrier is the stratum corneum of the epidermis and its good regenerative properties ensure its continuity. The outer layers of the epidermis are colonized by bacterial micro flora which gives protection to the body and prevents entry of adverse substance, as well as against the loss of water. Which ensures the homeostasis of the system. The skin is resistant to mechanical injuries due to its elasticity and stretch. The presence of fat in the subcutaneous tissue is absorbed by excessive external pressure. In case of rats, a brown fat is the main fat tissue and it is involved in the thermoregulation process (Sundberg *et al*., 2018)

Wound

Wound has been set down as one of the commonest affliction in the mammals' body since the ages. Wound in the modern medicine has been defined as a discontinuity in the epithelial lining of the skin or mucosa resulting from physical or thermal damage (Dhivya *et al*., 2015). In the ancient traditional medicine and Ayurveda literature, wound has been synonymously known as Varna and defined as discontinuation of membrane lining and leaves a scar after healing (Kar and katiyar, 1989). According to Boateng *et al.*, (2008), wound is a sharp injury that damages the skin's dermis. In the simplified manner any breach in the continuity of skin is considered as wound.

Vranaropaka term is used to define the drugs of natural origin to be used as wound healing agents in Ayurveda (Biswas and Mukherjee, 2003). Interestingly, Vranashotha; i.e. swelling or inflammatory reactions were considered to be the early phases of wound healing as described in modern day medicine. Therefore, the remedial measures in herbal entities may suggest the future agents of wound healing.

Wound can be classified on the basis of different criteria. According to the nature and duration of healing process, wounds are classified as acute and chronic wounds (Szycher and Lee, 1992; Robson *et al*., 2001). An acute wound is claimed to be generated suddenly after an accidental insult or during surgical procedures *i.e*. in the form of surgical site infection. The healing of the acute wound depends on depth, size and extent of damage to the dermis and

epidermal layers and generally heals within 8-12 weeks' time (Schreml *et al*., 2010; Rajendran and Anand, 2011). Acute wounds may also be summarized into incision wound in which incomplete thickness damage and wound including special tissue lack. Chronic wounds are acute wounds have not progressed through the stages of healing normally and generally heals slowly and partially. Chronic wounds commonly present with chronic diseased conditions and this further impairs either the blood circulation to that site or the abnormal cellular functioning at that site (Bryant *et al*., 2015). Although the treatment of chronic wound needs to be marked out, but the diagnosis and therapeutic interventions of these chronic co-morbid conditions also needs to be treated. It is important to not only to treat the wound but also diagnose and address the underlying cause of wound to minimize risk of further chronic wounds. The delayed wound healing due to impaired immunity, co-morbid issues, leg ulcer, burns and metabolic diseases progresses into chronic wound condition and do not follow the normal course of healing process (Lazarus *et al*., 1994; Bischoff *et al*., 1999). As per to the reports of Gupta *et al*., (2004); lower extremities of the body showed most percentage of acute (47.94%) and chronic (74.21%) wounds as compared to the other sites of the body. Wound may also be classified as open and closed wound depending on the discontinuity in the skin. Open wound is defined as kind of damage where the skin is torn, ruptured or cut, whereas, closed wound develops due to blunt force trauma to the body tissue and culminated into contusion. There may be several etiological agents or factors that are associated with the creation of wound including trauma, scalds, animal bite, venous compromisation, burn, immunodeficiency, metabolic diseases, nutritional insufficiency etc. In the context of these factors, the underlying pathophysiology of wound healing may have multifactorial dimensions to cure the healing and breach process (Shankar *et al*., 2014).

The cutaneous wound healing is a stereotypical pathophysiological process enclose collaborative efforts of various cellular strains and their products (Shaw and Martin, 2009). There is involvement of different overlapping phases, although in order to set out involved physiological reactions these phases of wound healing are often put down in a sequential manner i.e. hemostasis, inflammation, cellular migration and proliferation, protein synthesis and wound contraction and finally the remodeling phase (David and Heather, 2000). According to Enoch and Leaper (2008) healing of an acute wound involves a series of arranged events and a healed wound is described as one in which the connective tissue has been repaired and re-epithelisation has occurred by regenerative process and additionally, the structure has accomplish normal anatomical and physiological shape.

The Wound Healing Process

The wound healing process is classically defined as a series of continuous, sometimes overlapping, events. These are homeostasis, inflammation, proliferation, epithelisation, maturation, and remodeling of the scar tissue (Stadelmann *et al*., 1998). Homeostatic events occur straight away after injury. The subjection of sub endothelial collagen and the formation of thrombin lead to the activation of the platelets, located in the intravascular space. Activated platelets take part in a trigger role in a number of events: (i) activation of the coagulation cascade. This eventually leads to the formation of a fibrin clot that acts as cross bridge for other cells that later enter the wound; (ii) activation of the complement system; (iii) platelet degranulation: cells release an assembling of cytokines, growth factors, and vasoactive substances from the platelet α-granules, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), endothelial growth factor (EGF), platelet-derived angiogenesis factor, serotonin, bradykinin, platelet-activating factor, thromboxane A2, platelet factor IV, prostaglandins, and histamine. These platelet releasates starts the early events of wound healing (Stadelmann *et al.,* 1998). The inflammatory phase begins immediately after injury and may carry on with for up to 6 days (Simpson *et al*., 1997). Growth factors released from the platelets diffuse into tissues surrounding the wound and chemotactic ally draw inflammatory cells into the injured area. Neutrophils are the first inflammatory cells to enter the wound, followed by monocytes. Once chemo taxis is completed, local mediators activate the inflammatory cells. Activated neutrophils release a number of lysosomal enzymes (such as elastase, neutral proteases, and collagenase) which proteolytically remove damaged components of extracellular matrix (ECM) (Simpson *et al*., 1997). Activated monocytes acquire the macrophage phenotype and aid in host defence ((Simpson et al., 1997). The proliferation phase is characterized by the formation of the ECM and the beginning of angiogenesis. The primary cells involved in this phase are fibroblasts and endothelial cells. They proliferate in response to growth factors and cytokines that are released from macrophages, platelets and mesenchymal cells, or have been stored in the fibrin clot. In addition to chemo tactically drawing fibroblasts into the wound, PDGF, FGF, and EGF induce fibroblast activation and proliferation (Witte and Barbul, 1997). During the first 2-3 days after-injury, fibroblasts activity predominantly involves migration and proliferation. After this time, fibroblasts release collagen and glycosaminoglycan (mainly hyaluronic acid, chondroitin4-sulphate, dermatan sulphate, and heparin sulphate) in response to macrophage-released growth factors, hypoxia and by-

products of anaerobic metabolism. The combination of collagen and fibronectin forms the new ECM, which is essential for the development of granulation tissue that eventually fills the wound (Stadelmann *et al*., 1998). Angiogenesis accompanies fibroblast proliferation and allows nutrients and healing factors to enter the wound space. It is also essential for the growth of granulation tissue. The principle growth factors that regulate angiogenesis are FGF, released by damaged endothelial cells and macrophages, and vascular endothelial growth factor (VEGF) which is released by keratinocytes and macrophages (Stadelmann *et al*., 1998). The maturation phase usually begins 3 weeks after injury and can take up to 2 years to complete (Cooper, 1990). Unlike uninjured skin, the arrangement of newly formed collagen fibres in the wound is random and disorganized. The remodeling of collagen fibres into a more organized lattice structure gradually increases the tensile strength of the scar tissue, though this never exceeds 80 percent of the strength of intact skin. Remodeling of the ECM involves a balance between collagen synthesis and degradation, which is operated by several enzymes, like matrix metalloproteinase (MMPs), neutrophil released elastase and gelatinize, collagenases and stromelysins (Madden and Peacock, 1968). Wounds that do not heal within three months are considered chronic. In acute wounds, there is a precise balance between production and degradation of molecules such as collagen; in chronic wounds this balance is lost and degradation plays too large a role. Chronic wound bed has been demonstrated to differ from acute wounds for a higher concentration of proteases such as MMPs (Bennett and Schultzand, 1993) lower levels of growth factors and cytokines (Chen *et al*., 1999). A high and prolonged proteolytic activity may lead to the degradation of growth factors, detaining the wound in the inflammatory stage for too long (Yager *et al*, 1997)

Historical perspective

Historically, interest in nanotechnology is highlighted by the presentation entitled “There’s a Plenty of Room at the Bottom” by Richard P. Feynman during American physical society meeting at California in 1959 (Feynman, 1960). Dr. Feynman described a process by which individual atoms and molecules could be employed, serving as possible contender for future innovation and development. At present, nanoparticles can be broadly classified into five different categories viz. semiconductor quantum dots, magnetic nanoparticles, polymeric particles, carbon-based nanostructures and metallic nanoparticles (Huang *et al*,. 2007). These are known as engineered nanoparticles as they do not occur naturally. Each of these nanostructures have its own properties and applications. For example, semiconductor quantum dots display fluorescent properties that are useful for biological labeling and

imaging (Bruchez *et al*,.2016), and the magnetic properties of magnetic nanoparticles provide powerful gadgets for cell sorting, magnetic resonance induction, drug delivery and magnetic hyperthermia therapy (Steehall *et al*., 1985). In contrast to the other groups, potential supplications of metallic nanoparticles have proven to be the most flexible because of the ease in synthesis and control over size, shape, composition, structure and assembly. This results in fine tenability of their optical properties which forms the basis for various applications (Sayad, Link and Aguilar 2014). Among all the metals that are commonly used for making nanoparticles, gold is the most widely used and studied metal for biological applications.

Nanotechnology studies the synthesis, structure and dynamic of atomic and molecular Nano metric particles (maximum diameter of 100 nm), termed nanoparticles, upon which Nano products are fabricated (Niska *et al*., 2018).

Gold nanoparticle (GNP) constitute a solid choice when it is used for wound therapy due to their chemical stability and capacity of absorbing near infrared light, while at the same time, being relatively easy to synthesize (Niska *et al*., 2018). Gold nanoparticle can either directly target the bacterial cell wall, or they can bind to bacterial DNA, blocking the double helix from uncoiling during replication or transcription, thus employ bactericidal and bacteriostatic properties as a result, they can inhibit multidrug –resistant pathogens, such as S. aureus, P. aeruginosa. Moreover gold nanoparticle prevent the formation of reactive oxygen species, thus acts as antioxidants, aiding the healing process (vijay *et al*., 2019). Low level of ROS are capable to protect against invading pathogens and act as arbitrator for intracellular signaling (Schafer *et al.,* 2008). However, excessive production of ROS ultimately impairs wound healing by inhibiting growth, causes cellular damage, which results in poor neovascularization (Panieri *et al*., 2013) Wound healing can be promoted by the presence of antioxidant by reducing the level of ROS. Gold nanoparticles have excellent antioxidant properties, biocompatible, high surface reactivity, and nontoxic. Thus, it has been considered as better option for therapeutic purpose.

Gold nanoparticle (GNP) have antibacterial and healing properties in both in vitro and in vivo from histopathological examination (Arafa *et al*., 2018). GNP prevent the formation of reactive oxygen species thus acting as antioxidant aiding the healing process (Kumar *et al*., 2019). Low concentration of GNP amplify the keratinocyte growth and differentiation but higher level is associated with cytotoxicity (Lu. *et al*., 2010). GNP Stimulate angiogenesis

and fibroblast proliferation rapid wound closure without causing cell toxicity (Marza *et al*., 2019). The size of GNP control the properties of the nanoparticle and the application for which they are used. (Rowles *et al*., 2013). Topical application of GNP causes significantly increase in the healing process through anti-inflammation and turns new blood vessels into mature non leakage vessel (Chen *et al.*, 2015). GNP coated on a hydrocolloid membrane to treat cutaneous wound in rat modals (Kim *et al*., 2015).

Gold nanoparticle can be well planned in the therapy of burns also and able promote to healing and inhibit microbial colonization (Arafa *et al*., 2018).

Biofabrication of nanoparticle

Biofabrication of nanoparticles by using plant sources is considered as most vital practice for nanoparticle syntheses, as the use of plant materials not only makes the process eco-friendly but also its super abundance makes it less expensive.

Gold, as well as other metal nanomaterial, are synthesized conventionally by physical and chemical methods. However, these methods have several limitations such as the use of harsh chemicals, extreme synthesis conditions, arduous as well as less productive. In addition to these drawbacks, these processes create an environmental risk as they involve toxic chemicals that have detrimental health implications on human beings. Hence, the need for a less toxic, inexpensive and applicable method of nanoparticle synthesis is in excess demand in medical, pharmaceutical and agriculture industries. The heterogeneity of the biological system allure the attention of many researchers for the synthesis the nanoparticles (Sanna and Shedbalkar *et al*., 2014)

Moringa oleifera Lam. is the most widely learned species of the monogenetic family Moringaceae (order Brassicales), which includes 13 species of trees and shrubs distributed in sub-Himalayan ranges of India, Sri Lanka, North-eastern and South-western Africa, Madagascar and Arabia (Fahey *et al*., 2005). *M. oleifera* is one of the most useful tropical trees. The relative ease with which it propagates through both sexual and asexual means and its low demand for soil nutrients and water after being planted makes its production and management easy. Introduction of this plant into a farm which has a bio diverse environment can be beneficial for both the owner of the farm and the surrounding eco-system (Foidl *et al*., 2001). Biosynthesis of nanoparticles can be attained by using plant extract, bacteria and fungi. The use of microorganisms in the biosynthesis of nanoparticles has several advantages

over plants as there is no need of substantial purification of nanoparticles after synthesis (Shedbalkare *et al*., 2014)

WHO has endorsed to *Moringa* as a substitute to imported food supplies for the treatment of malnutrition. Besides being edible, all parts of the *M. oleifera* have long been used for the treatment of unlimited diseases, and for that reason, in many case, it is been called as "Miracle Tree" (Mbikay, 2012). Now a days, some parts of this plant have gaunt much recognition and have been studied for its various biological venture, including ant atherosclerotic (Chumark *et al.*, 2008), immune boosting (Miyoshi *et al*., 2004), cardiovascular diseases, antiviral (Murakami *et al*., 2015), antioxidant, antimicrobial, anti-inflammatory properties (Kumar Gupta *et al*., 2013 , Dawood and Fathy, 2021).

Tumor repressive effects in skin papilloma genesis, hepatocellular carcinoma, colon cancer, and myeloma (Brunelli *et al*., 2010). Leaves of *M. oleifera* have been used as antiulcer, diuretic, anti-inflammatory, wound healing (Farooq *et al*., 2012), antifungal activity (Chuang *et al*., 2007), potent CNS calmative action (Pal *et al*., 1995), and antifertility activity (Tahiliani and Kar, 2000). Major Phytoconstituents present in *M. oleifera* leaf are niazinin, niazimicin, β-sitosterol, glucomoringin, *n*-benzyl thiocarbamates, kaempferol, other natural antioxidant molecules including vitamins, minerals, and carotenoids (Anwar *et al*., 2007; Jaiswal *et al*., 2009).

Animal models

Rodents

Rodents have been imputed with commonly used animal model in wound healing studies (Murray *et al*., 2017). The human skin although shows certain differences in healing process, but the current advances have broaden the horizon of rodent wound healing model. Application of advanced silicone splint in rodents enhance healing in refined manner via granulation and re-epithelisation (Isakson *et al*., 2015). Although earlier only contraction has been prescribed as main mechanism coddled in rodent healing; but studies of Chen *et al*. (2016) has reevaluated the ground of contraction along with re-epithelisation in wound healing of rodent models.

Rats and mice are commonly associated with the preclinical studies and also with the chronic wound healing oddities (Isakson *et al*., 2015) described that rodent model were placed in 78% of studies associated with the excisional, incisional and burn wound injury to make out the efficacy of different agents in wound healing and reformative process. The sex

difference also lies within the same species (Wong *et al.*, 2011) also detailed that skin of male mice was 40 % stronger as compared to its counter part due to thickened dermis layer. Stage of hair follicular growth also affects the healing process as reported by Ansell *et al*., (2012) as healing is stimulated in late anagen as compared to catagen or telogen stage of hair growth. Thus wounding must be carried out experimentally at resting stage i.e. exogen or late telogen stage.

Excisional wound healing model

Excisional model of wound healing necessitate the debridement or removal of desired histological building and could be superficial, partial thickness, or full thickness (Seaton *et al.*, 2015). It has been associated with several diseased model including mice, rats, rabbits and pigs to portray the molecular cascading and ascertain wound healing efficacy in impaired or delayed/ chronic wounds (Wong *et al*., 2011).

Expression gene

The reactive oxygen species are short-lived entities and electron acceptors that are continuously produced by all cells at low levels during the course of normal aerobic metabolism (Bickers *et al*, 2006 and Schafer *et al*., 2008). In wounded and inflamed tissues, the synthesis of ROS by inflammatory cells contributes to the defense against invading pathogens and mediates intracellular pathways (Schafer *et al*., 2008). However, excessive amounts of ROS have deleterious effects on lipids, proteins and nucleic acids of cells involved in skin repair leading to tissue damage (Schafer *et al*., 2008). The COX is present in three isoforms: COX-1, COX-2 and COX-3. The COX-1 is normally expressed in the body and has many physiological functions as thromboxane A2 synthesis in platelets (Lee *et al* .,2006). The COX-3 was recently identified in canine and human cortex and it has been involved in a central mechanism of pain and fever (Chandrasekharan *et al.*,2002). The COX-2 is not normally expressed in the most cells, but is rapidly induced in response to inflammatory stimuli producing prostaglandins, such as prostaglandin E2 (PGE2) (Lee *et al* .,2006). In chronic venous leg ulcers, the excessive expression of inducible nitric oxide synthase (iNOS), COX-2 and high PGE2 levels on wound bed contributes to chronic inflammation observed in these lesions (Abd-El-Aleem *et al*., 2001). Thus, the persistent infiltration of inflammatory cells associated to the increase in the ROS production and prostaglandins may contribute to non-healing of chronic lesions. Therefore, the

administration of anti-inflammatory and antioxidant compounds may be a good therapeutic strategy to promote the wound healing. The effect of decreased COX-2 activation on cutaneous wound healing of acute lesions is still controversial. Some studies propose that decreased COX-2 activation decreases inflammatory response in sponge implants and promotes the closure of excisional lesions, while others propose that celecoxib administration reduces the wound closure and scar formation in incisional and excisional lesions of rodents (Wilgus *et al*., 2003). In addition, other studies also suggest that celecoxib administration does not alter cutaneous wound healing of rat acute incisional lesions in mice sponge implants (Blomme *et al*., 2003)

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3. MATERIALS AND METHODS

This research study was designed to work out the molecular operation of wound healing success of biofabricated gold nanoparticle in rat model. The research work was performed in Department of Veterinary Surgery and Radiology and Veterinary Biochemistry, Bihar Veterinary College, (Bihar Animal Sciences University), Patna, Bihar, INDIA.

3.1. Procurement and Maintenance of Experimental Animals

The research work was conducted in 42 healthy male wistar rats weighing 200-250 grams. The rats were procured from S. N. CHAKRBAORTY-31/1G RD KOLKATA Laboratory Animal Resources (LAR) and kept for two weeks in Laboratory Animal House in the Department of Veterinary Surgery and Radiology, BASU, PATNA for acclimatization. During the experimental period, rats were maintained under proper managemental conditions with 12 hour light-dark cycle and provided with balanced feed and clean drinking water throughout the experimental period. After two weeks of acclimatization all the rats were randomly and equally divided into three groups comprising 14 animals in each group at day 0 of study. The animals of group I served as healthy control and treated with normal saline. The group II animals were treated with betadine and group III animals were treated with biofabricated gold nanoparticle. Treatment was done at first 5 days from wound creation. Tissue was collected on 7^{th} days and 14^{th} days from wound creation. The experiment was conducted after the approval of Institutional Animal Ethics Committee and approval no (IAEC/BVC/21/12)

3.2. Agent used for treatment

3.2.1. Material used for preparation of biofabricated GNP

Moringa leaf extract, 1% auric chloride (50% Au in $HAuCl_4.3H_2O$), filter paper, syringe filter (size 0.45µm), aqua regia, magnetic stirrer, conical flask**.**

3.2.2. Preparation of biofabricated gold nanoparticle

Ten gram Moringa leaf extract was mixed with 90 ml of distilled water in a beaker. Mixed solution was stirred overnight on magnetic stirrer. Mix volume was maintain 100ml with distilled water. Solution was centrifuge with 10,000 rpm for 20 min. after centrifugation supernatant was collected in fresh centrifuge tube and again centrifuge 10,000 rpm for 20

min. Triple layer filter paper was used for filtration of supernatant to get Moringa leaf broth; filtered broth was again filtered with syringe filter.

All the glass wares used in the experiment were thoroughly cleaned in aqua regia and the rinsed with distilled water, acetone and methanol, finally they were rinsed with distilled water. For bio fabrication of GNP the Moringa leaf extract was distilled in1:2, 1:5, 1:10, and 1:20 with distilled water. Among these dilution 1:5 was optically converts the aurocholoric acid to GNP. So for further MGNP preparation this dilution was used.

To prepare MGNP, 100 µl gold solution was mixed with in 22.90 ml high purity liquid chromatography and heated in the domestic microwave for 10 sec, the Moringa leaf extract was added slowly to the solution and again microwaved for 20 sec. The color of the solution was changed from yellow to reddish purple.

Characterization of prepared GNP was done by U.V. spectroscopy and electron microscopy analysis.

Fig. No.: 1

Fig. No.: 2

Fig. No.: 3

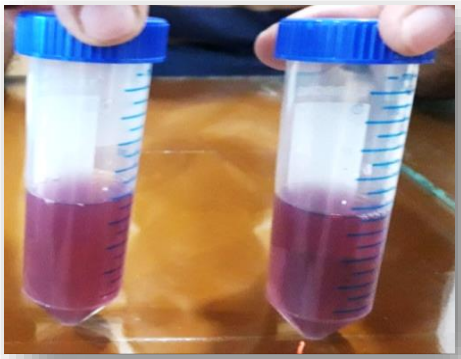

Fig. No.: 4

Fig. No. : 1-2 showed the filtration of Moringa broath, **Fig. No. : 3** showed the heating Moringa broath with HPLC and, 1% auric chloride (50% Au in $HAuCl_4.3H_2O$). **Fig. No. : 4** showing prepared **MGNP**

3.3. Chemicals and Reagents

- RNA isolation kit [promega].
- Power up SYBR green master mix were procured from Thermo fisher Scientific, USA.
- Hydroxyproline assay kit was procured from Sigma.
- 100 bp plus DNA ladder were obtained from Thermo Scientific, USA.
- Trizol reagent was procured from Invitrogen
- Ethanol, NaOH, HEPES were procured from Sigma.
- RNA was checked by Biophotometer (Eppendorff, Jenway)
- Vernier caliper
- Agarose, Hi Media Laboratories Pvt. Ltd., Mumbai
- Masson's trichome, Haemotoxylin and eosin stains were obtained from Hi Media Laboratories Pvt. Ltd., Mumbai.
- Plastic wares including micro centrifuge tubes (1.5 & 2 ml), micro tips (2-200 µl and 200-1000 µl), centrifuge tubes (15 ml and 50 ml) and gloves were purchased from Tarsons Products Limited, Kolkata, India.

3.4. Induction of wound

Initially the rats were restrained in ventral recumbency and preanaesthesized by butorphanol @ 2 mg/kg b.wt. After that induction of anesthesia by 3-5% isoflurane, and maintained by 1.5-3% isoflurane and the surgical area was shaved with shaving blade, after that surgical area was dried and prepared aseptically with spirit followed by betadine application. A sterilized B.P. handle with sharp blade was used to create full thickness excisional wound 2 x 2 cm measured by vernier calipers. The single surgical wound was created at dorsal lumber region. Open excision type wounds of $\approx 2\times2$ cm^2 were surgically created on the back of the animals to the depth including the *panniculus carnosus*, of all the three groups as per the standard protocol (Singh *et al.,* 2019). Animals was kept individually in properly disinfected polypropylene cages. Surgical techniques involved in the creation of wound were performed according to the procedural norms described by Institutional Animal Ethics Committee.

Fig. No.: 5

Fig. No.: 6

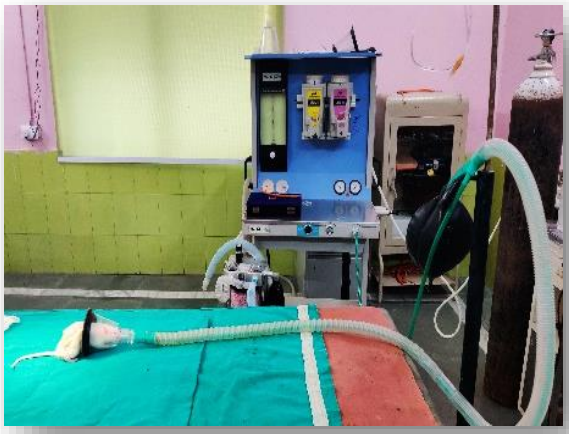

Fig. No.: 7

Fig. no. 5 to 7: sedation, induction and maintenance of anaesthesia

3.5. Gross studies and wound area measurement

Each group of animals were examined for the estimation of wound closure at weekly interval till 14th day. Wounds of animal was digitally photographed and used digital vernier caliper for measurement on postoperative days 0, 7, 14 after creating the wound to assess the quality of healing and healed area percentage. The area of wound contraction was measured at weekly interval till 14th day post lesion by Wilson's formula as follows:

$$\% \text{ Wound contraction} = \left[\frac{Heald\ area}{Total\ wound\ area}\right] x10$$

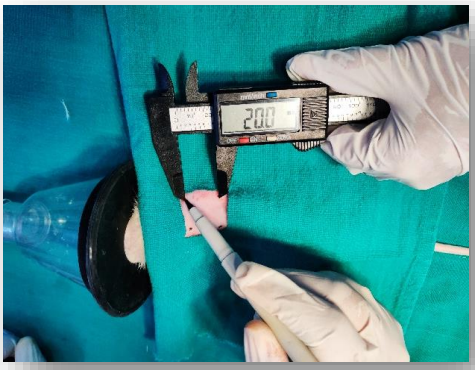

Fig. No.: 8

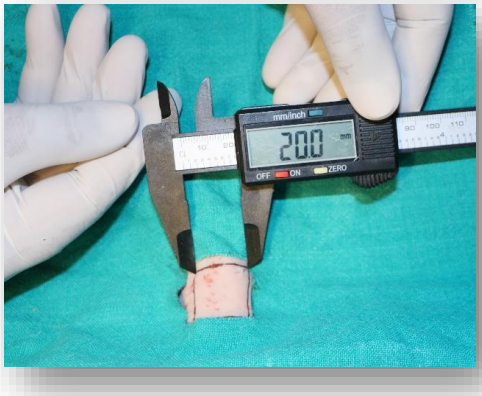

Fig. No.: 9

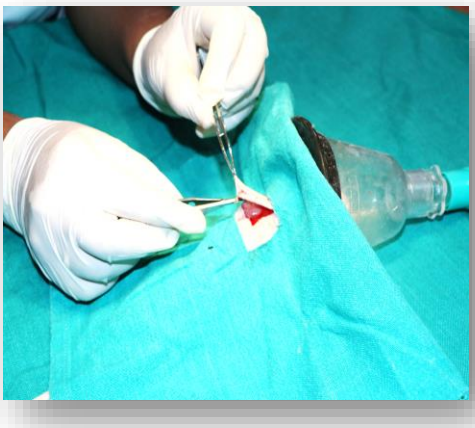

Fig. No.: 10

Fig. No.: 11

Fig 8 to 11 creation of wound and tissue collection proper measurement with vernier caliper

3.6. Collection of tissue samples

Animals from each sub-group were anaesthetized properly and tissue samples of healed skin were collected on day 7th and 14th day from each group. Half part of tissue was preserved in 10% neutral buffer formalin for Haemotoxylin and Eosin staining and Massons trichrome staining and another half portion of the tissue were preserved in -80°C after dipping the tissue in liquid Nitrogen for gene expression study and some portion of skin tissue was immediately used for hydroxyproline assay and DNA estimation as per the standard protocol of S V total Promega.

3.7. Biochemical profiling from tissue samples

3.7.1. Hydroxyproline assay in tissue samples

The skin samples from wound edges of animals of different groups were collected at 7th and 14th days of interval for estimation of Hydroxyproline content (unit) to evaluate the progress of wound healing. The tissue homogenates were prepared from collected skin tissue samples from rat skins of each group and absorbance was noticed at 560nm with the help of spectrophotometer. Concentration of hydroxyproline were measured by following the of SIGMA-AL protocol.

Fig. No.:12

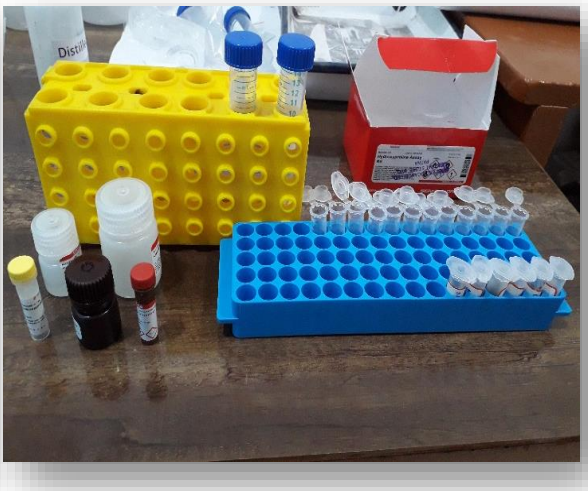

Fig. No.:13

Fig –12 and 13 sample preparation and hydroxyproline estimation with the SIGMA-AL KIT

3.7.2. DNA estimation in tissue samples

For the estimation of DNA content in the rat skin tissue samples, of from each group collected at 7th and 14th days of interval. The homogenates were prepared from collected skin tissue samples from rat skins of each group for calculation the concentration and purity of DNA. DNA was extracted from tissue homogenate by following the instruction of promega kit protocol. The isolated DNA samples were re-suspended in TE buffer and DNA content was measured spectrophometrically in µg/ml by determining A260/280 ratio.

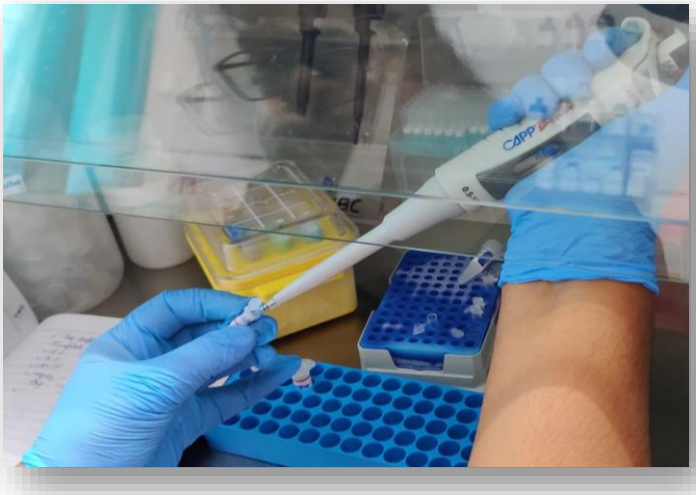

Fig. No.:14 extracted DNA from rat skin tissue sample

3.7.3. Expression profiling of genes

3.7.3.1. RNA isolation

At weekly interval the tissue samples from the wounded site were collected from rats of different groups and dip the tissue in liquid nitrogen for fixing the genes, after dipping tissues, were stored in RNA later at -80 ºC. Total RNA was isolated with S V total isolation system (promega) as per the manufacturer's instructions.

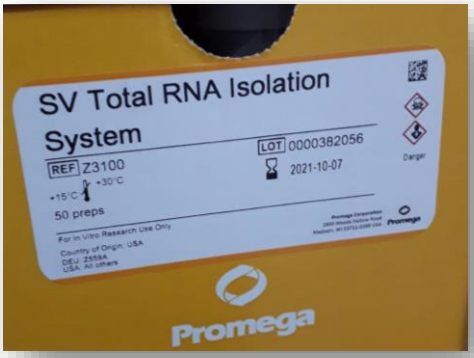

Fig. No.:15 (RNA isolaton kit)

Fig. No.: 16

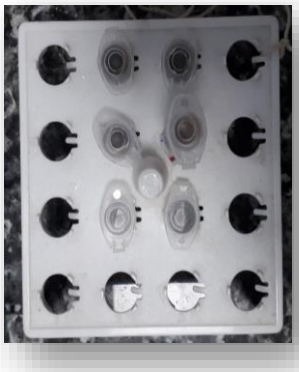

Fig. No.:17

Fig. No.:18

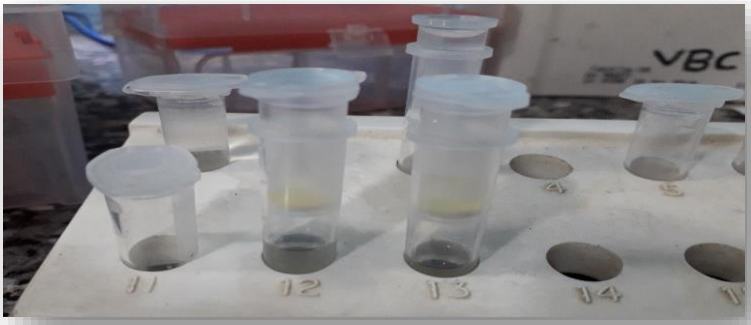

Fig. No.:19

Fig no-16 to 19 showed the methods of RNA extraction from rat skin tissue sample

The purity of the RNA was checked by Bio photometer (Eppendorff, Jenway) and quantitated as follows:

$$1OD_{260} = 40\ \mu g/ml$$

The RNA samples showing A_{260}/A_{280} ratio 1.8-2.0 and A_{260}/A_{230} ratio>1.6 were used for cDNA synthesis. The RNA concentration was estimated by reading the absorbance at 260nm and was checked for purity at 280nm in a spectrophotometer. For each sample, RNA were adjusted and used to synthesize cDNA with 1µl volume.

3.7.3.2. cDNA synthesis

Synthesis of cDNA was carried out from the mRNA present in the total RNA using Revert aid®. First strand cDNA synthesis kit (Promega) using moloney murine leukemia virus reverse transcriptase enzyme by following the manufacturer's instructions.

Table-1. Composition for cDNA synthesis

Composition	Volume
Product of RT system	5 µl
5x Reaction buffer	4 µl
$MgCl_2$	1.2 µl
Recombinant RNasin	0.1 µl
RT	1 µl
Nuclease free water	7.7 µl
PCR nucleotide mix	1 µl
Final volume=	**20 µl**

Table-2. Cycling condition for cDNA synthesis.

S.NO.	TEMPRATURE AND STEPS
1	25°C for 5 min
2	42°C FOR 1:00 Hr
3	70°C for 15 min
4	End and storage

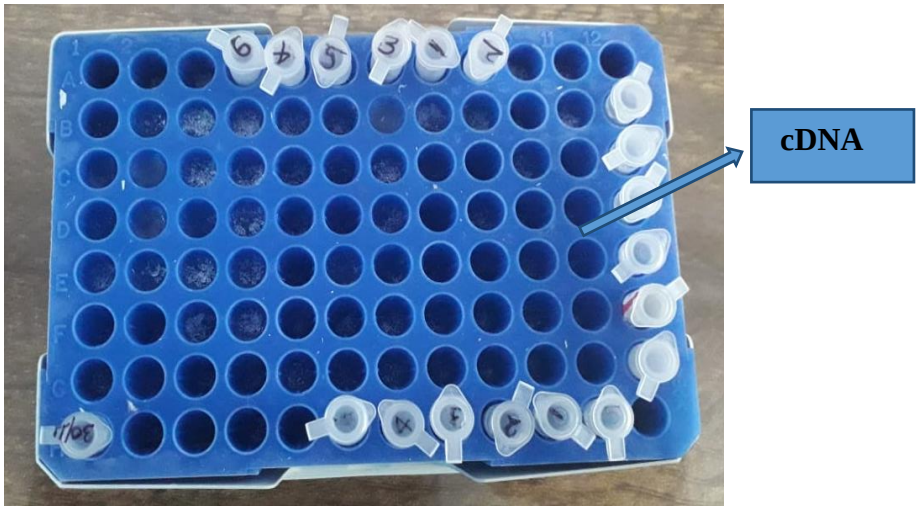

Fig. No.: 20(prepared cDNA)

3.7.3.3. Primer validation

Primer sequences were obtained from the previous publications and respective gene sequences were checked from NCBI nucleotide database. The cDNA was subjected to amplification with the respective primers at various annealing temperatures to optimize the PCR conditions. Reverse transcription real time (RT-PCR) analysis was carried out after checking each primer for its specificity.

Fig. No.: 21

Fig. No.:22

Fig 21and 22 showed the oligonucleotide activation before use

Table-3. Description of primers-

GENE	PRIMER SEQUENCE	AMPLICON SIZE (bp)	ANNEALING TEM (°C)	REFERENCE
GADPH	F5-`CCTGCACCACCAACTGCTTAG-3` R5`GTCTTCTGGGTGGCAGTGATG-3	109 bp	62°C	Barberio *et al.*, (2016
COX-2	F5`-AAAGCCTCGTCCAGATGCTA-3` R5´ATGGTGGCTGTCTTGGTAGG-3`	249 bp	59°C	Szekeres *et al.*, (2015)

3.7.3.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Performing PCR with standard GAPDH primers for quality check of prepared cDNA. The polymerase chain reaction was standardized for both housekeeping gene and expression gene using cDNA from rat skin tissue sample. The reaction was carried out at different annealing temperatures and the optimum annealing temperatures for different genes were determined (Table 4). The used protocol was as follows (Table 5) (Sambrook and Russel, 2001).

Table- 4: Composition of PCR mixture

Composition	Volume
PCR master mix (10X)	1.25 µl
2mM dNTP	1.0 µl
25Mm MgCl2	0.5 µl
Forward primer (10 pmol/µl)	0.5 µl
Reverse primer (10 pmol/µl)	0.5µl
Template (100ng/µl)	1.0 µl
Taq(5u/ µl)	0.2 µl
Nuclease free water	up to 12.5 µl

Table-5: Thermal cycling condition for PCR

S.NO.	STEPS	TEMPRATURE AND DURATION
1.	Initial denaturation	95°C for 3:00 min
2.	Denaturation	95°C for 0:30 min
3.	Annealing	59°C/62°C for 0:30 min
4.	Extension	72°C for 0:30 min
5.	Steps 2-4 repetition	35 cycles
6.	Final extension	72°C for 5:00 min
7.	Held at	4°C

3.7.3.5. Agarose gel electrophoresis

DNA amplified by PCR was standardized with agarose gel electrophoresis as described by Sambrook and Russel (2001). Ten mg agarose (1.5%) was dissolved in 90 ml 1X TAE buffer by heating and allowed to cool and 1.0 µl ethidium bromide (final concentration 0.5 µg/ml) was added and mixed thoroughly. The mixture was poured onto medium gel-casting tray fitted with acrylic comb and allowed to solidify. When 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. Four µl of 6X DNA loading dye was mixed with 8 µl of DNA samples and loaded into the wells.

Electrophoresis was carried out at 85-89 volts until the tracking dye reached the end of the gel. The DNA samples were visualized as bands under UV-illumination and documented. The size of the PCR products amplified was calculated from the standard 100 bp DNA ladder (thermo fisher).

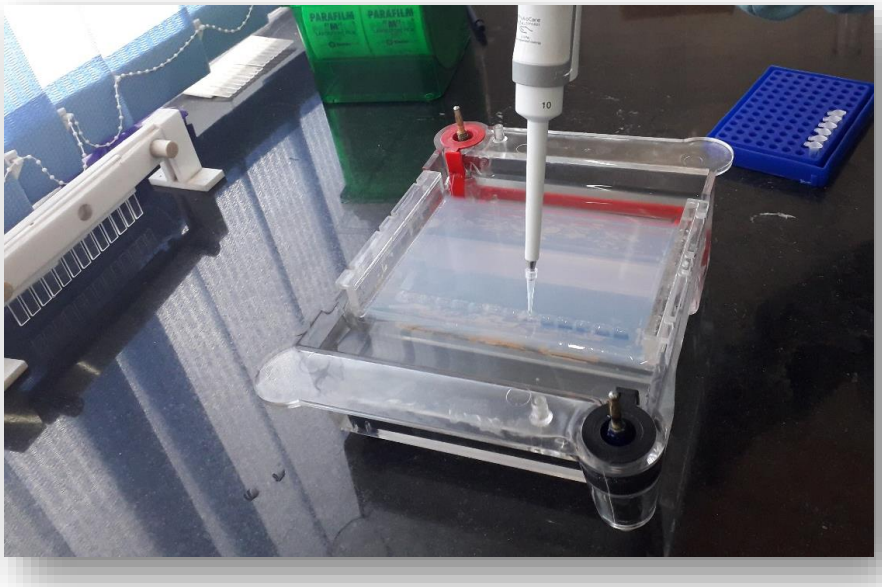

Fig. No.: 23 (loading of DNA sample and ladder)

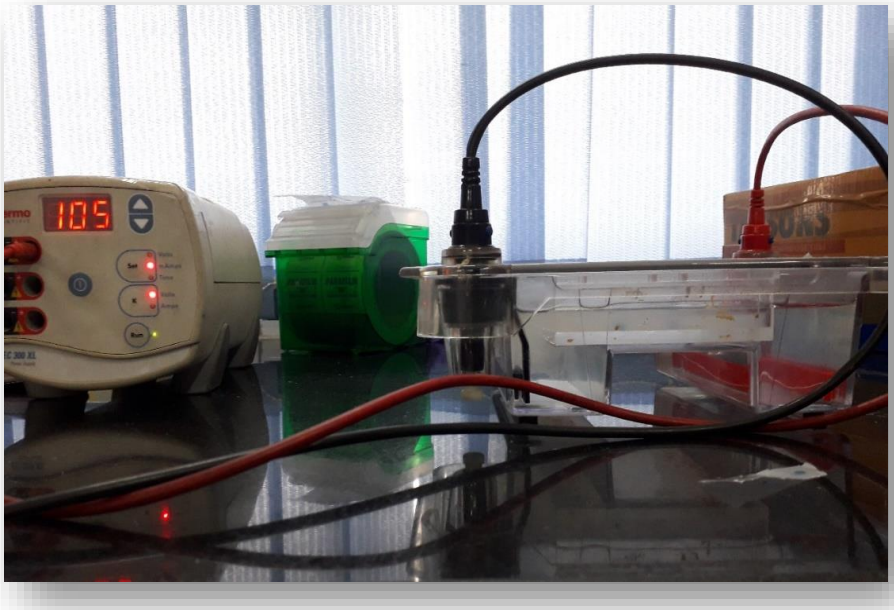

Fig. No.:24 (Gel Electrophoresis of sample DNA)

3.7.3.5. Reverse transcription real-time quantitative PCR (RT-PCR)

RT-PCR reactions were run on Real-Time PCR system (AriaMX Real-Time PCR system G8830A) and analyzed by using Design AriaMX and Analysis Software's v1.2.x.

3.7.3.6. RT-PCR reaction conditions

Table: 6 Composition of RT-PCR mixture

Composition	Volume
Syber green master mix(thermo fisher)	5 µl
Primer(F)	0.5 µl
Primer (R)	0.5 µl
Template DNA	1 µl
Nuclease free water	3 µl

Table :7 cycling condition of housekeeping gene

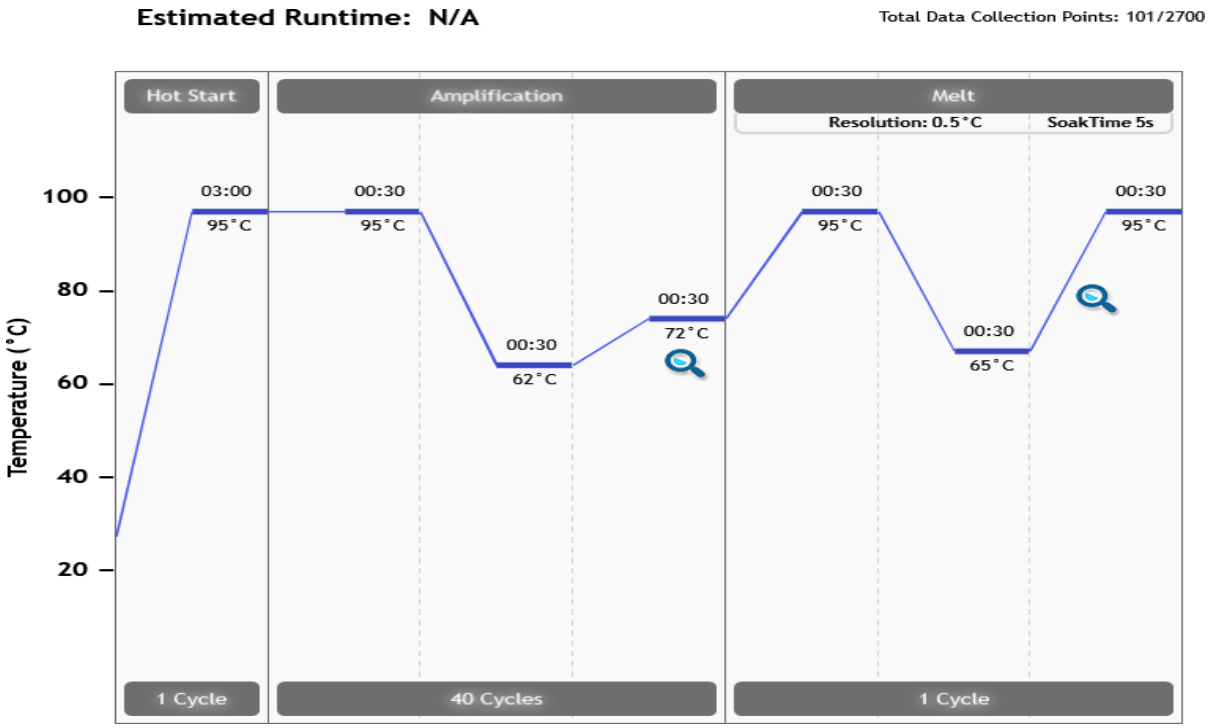

Fig. No.: 25 (Cycling Condition of housekeeping gene,GADPH)

I. Initial denaturation at 95°C for 3min

II. Denaturation at 95°C for 30 sec

III. Annealing at 62°C for 30 sec

IV. Extension at 72°C for 30 sec

V. Steps 1-4 repetition at 40 cycle

VI. Extension at 72°C for 30 sec

Table: 8 cycling condition of expression gene

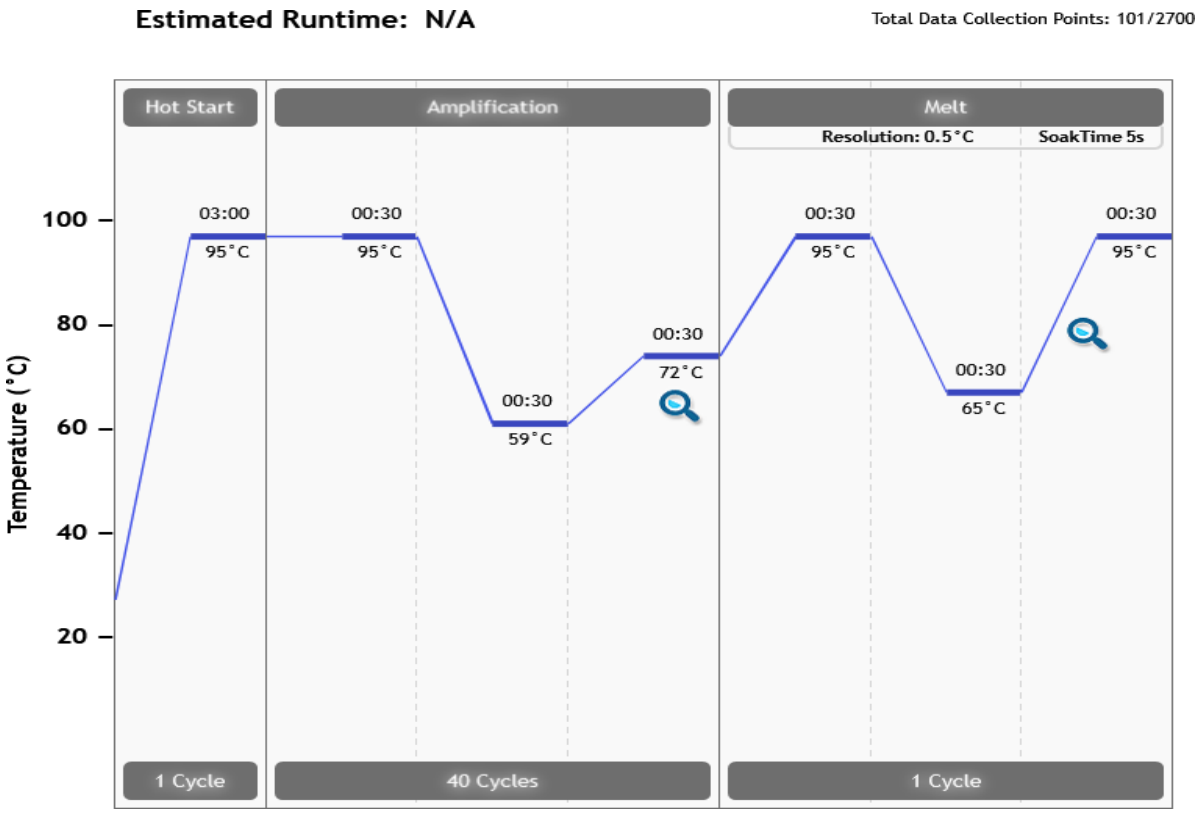

Fig. No.: 26 (Cycling Condition of Expression gene COX-2)

I. Initial denaturation at 95°C for 3min

II. Denaturation at 95°C for 30 sec

III. Annealing at 59°C for 30 sec

IV. Extension at 72°C for 30 sec

V. Steps 1-4 repetition at 40 cycle

VI. Extension at 72°C for 30 sec

Each sample was run in duplicate manner in RT-PCR. A dissociation curve was created at temperature of 60 °C through 95 °C to determine the specificity of the amplified product. The results were presented as cycle threshold values (C_T). When the fluorescence of the reporter dye is significantly higher than the background fluorescence this is the cycle number. C_T values were generated using the instruments automatically adjusted threshold. By detecting the C_T value of various dilution of cDNA samples the relative fold gene expression was calculated for estimating the fold gene expression level of expression gene (Kenneath and Thomas, 2001).

Formula for calculating fold gene expression level calculate by= $2^{-\Delta\Delta Ct}$

$\Delta\Delta Ct = \Delta Ct(\text{treated sample}) - \Delta Ct(\text{control sample})$

$\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{housekeeping gene})$

(Treated Sample) = Biofabricated Gold nanoparticle, Betadine

(Control Sample) = Normal Saline

(Gene of Interest) = COX-2

(Housekeeping Gene) = GADPH

3.7.4. HISTOPATHOLOGICAL EXAMINATION

3.7.4.1. Hematoxylin and Eosin staining

Tissue sample of rat skin from each group of animal collected at interval of 7th and 14th were fixed in 10% neutral buffer formalin. After fixation tissue sample of skin were dehydrated using graded alcohol series (50%, 70%, 80%, 90%, 100%) and embedded within paraffin wax at 60-62°C. Embedded tissue blocks were sectioned 4-5 µm thick sections using microtome and mounted on glycerol and albumin (1:1 ratio) coated slides. Deparraffinized the slides were using xylene and then slides were rehydrated with using isopropyl alcohol (95%, 90%, 80%, 70%) for 2-3 min each and finally in distilled water for 2 min. After absorption of the excess water, slides were stained using Haemotoxylin for 1-2 min. Again slides were washed with distilled water and then slides were rinsed with 95% alcohol to ensure complete dehydration and counterstained with eosin solution for 1min. Slides were dehydrated again by using 95% alcohol . Finally all slides were cleaned by xylene washing and mounted with DPX mounting medium. The obtained slides were examined and imaged under light microscope using different magnification.

Fig. No.:27

Fig. No.:28

Fig. No.: 29

Figure no-27 to 29 The embedded tissue within wax blocks, slide preparation and H and E stain

3.7.4.2. Masson's trichrome staining

Each group of slides at interval of 7th and 14th were processed using Masson trichrome staining method for checking collagen deposition. All slides were dipped in Bouin's solution for 1h at 56°C and washed with running tap water to remove yellow color, then stained with weigert's Haemotoxylin for 10 min and biebrich scarlet - acid fuschin for 10 min then washed with distilled water. Later, the slides were differentiated with phosphomolybdic- phosphotungstic acid solution for 10-15 min to enhance collagen staining. Then the slides were transferred to aniline blue solution for 5- 10 min. Following washing, slides were treated with 1% acetic acid solution for 2 min for stain differentiation, washed and then dehydrated using gradient alcohol concentrations. Finally, cleared using xylene. Then the slides were mounted with cover slip using DPX and observed under light microscope.

Fig. No.: 30 stain for masson trichome staining

Fig. No.: 31

Figure 31 Prepared slide to see the collagen thickness in collected skin

Statistical Analysis

- ANOVA (Analysis of variance) and Duncan's multiple range test (DMRT) were used to compare the means at different time intervals between different groups.

  (Snedecor and chochran, 1994)

- The subjective data generated from the scoring was analyzed by Turkey HSD testing among groups and within each group.

****

4. RESULTS AND DISCUSSION

Present study was aimed to evaluate the wound healing efficacy and molecular perception of wound healing process following topical application of biofabricated GNP in full thickness excisional wound in rat models. For study the efficacy and molecular dynamics, 42 rats were divided randomly and equally into five groups viz group I (healthy control), group II (betadine treated) and group III(biofabricated GNP) with 14 rats in each group. Experiment was conducted for 14 days and wound healing were assessed in animals of each group (n=6) at weekly interval of 7th and 14th day.

4.1. U.V. Spectrophotometical examination of MGNP

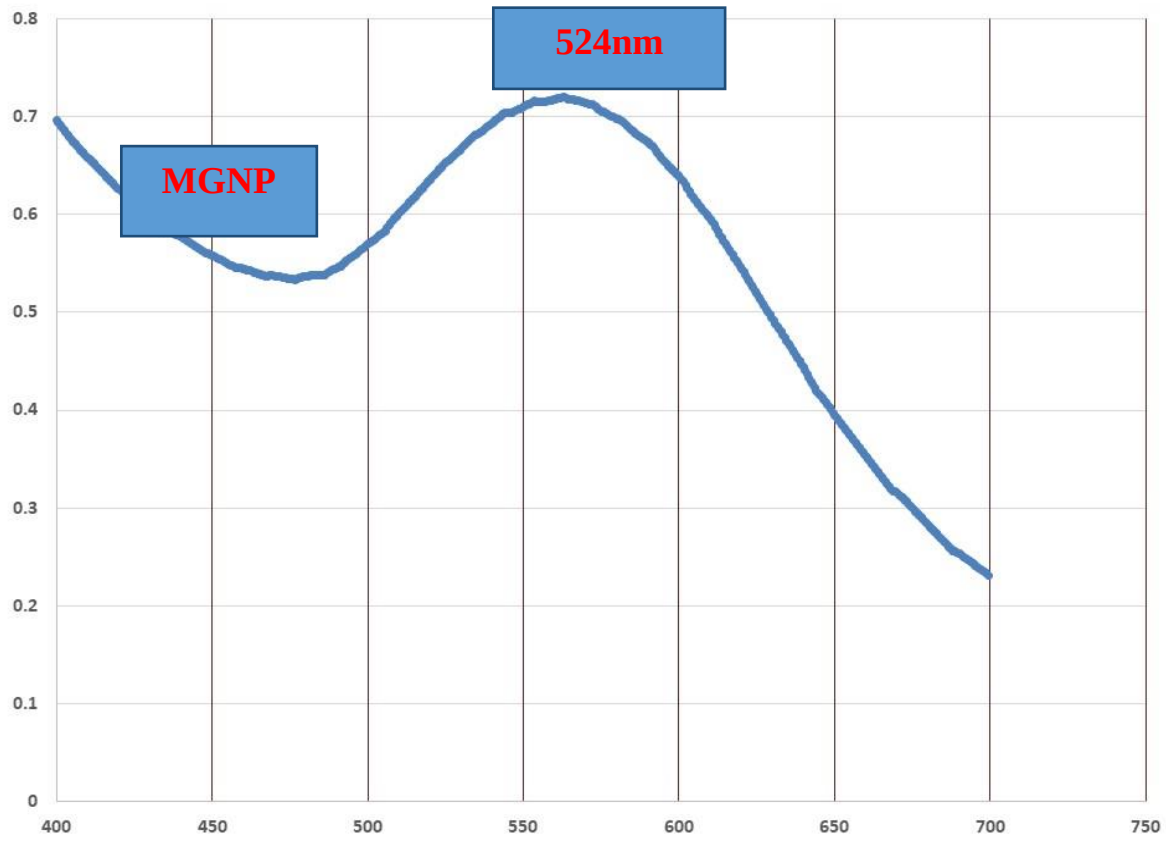

Fig. No.: 32 Spectrum analysis of MGNP (cold extract) Spectrum analysis of shown absorption peak of GNP 524nm (biofabricated with *Moringa oleifera)*

4.2. Zeta sizing of prepared biofabricated

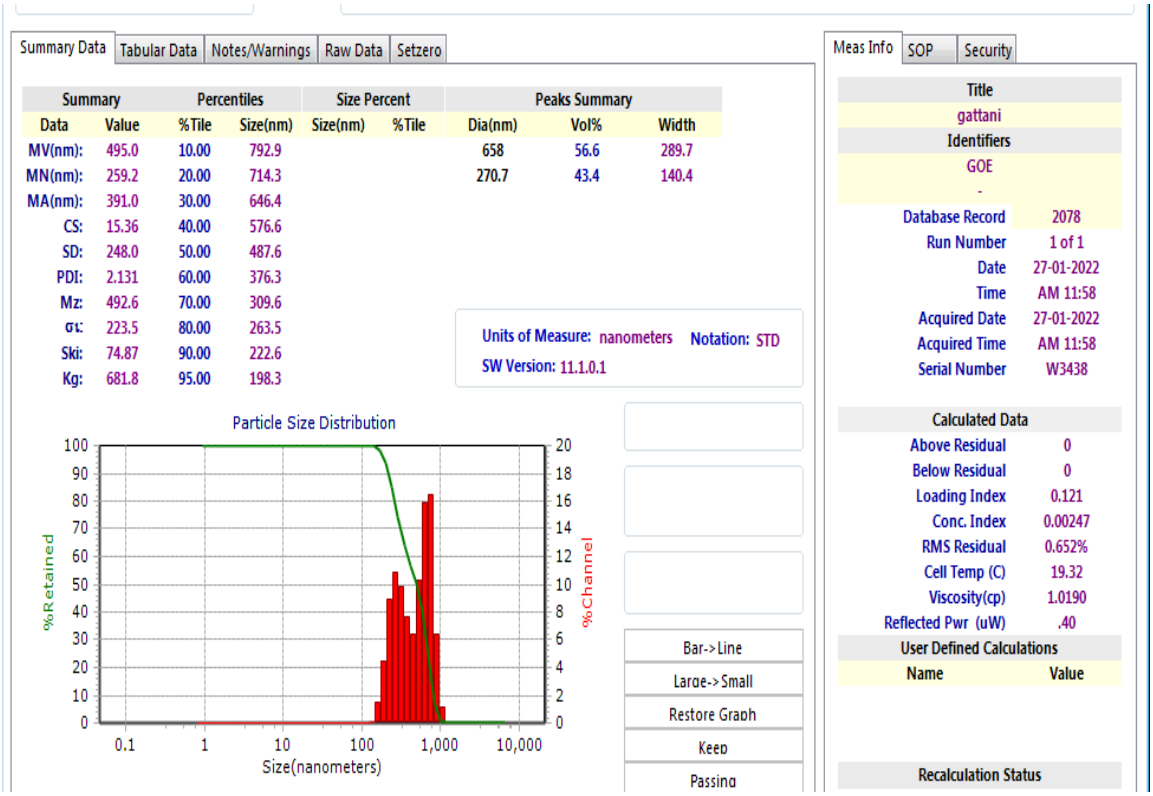

Fig. No.: 33 Zeta sizing of prepared MGNP measured in IVRI (Bareilly)

4.3. Measurement of wound contraction

Wound contraction (%) was calculated following the Wilson's formula and represented in table 9. Wound contraction is a measure of healing in lessoned site

The contraction of wound was measured by using Wilson formula, the value of wound contraction (%) in animals of group I, II and III, on 7th day were 10.88 ± 2.09, 28.81 ± 1.97 and 61.66 ± 1.71 respectively. On 7th day % of wound contraction in group III was significantly ($P < 0.05$) higher in rats were treated with biofabricated GNP in comparisons to group II and group I which were treated with betadine and normal saline respectively. Animal of group III showed maximum % of healing.

On 14th days of experimental trial the contraction of wound was measured by using Wilson formula % in all three groups I, II, and III were 39.91 ± 2.68, 80.35 ± 0.96 and 93.11 ±0.74 respectively. Significant ($P < 0.05$) increase in wound contraction (%) in animal of group III was noticed which was treated with biofabricated GNP. Animals of group III showed almost proper healing and showed as normal in comparisons to group I and group II.

Table no: 9 Effect of various treatment on wound contraction (%) in rats

[n=6]

Groups/days of treatment	7th day	14th day
Normal saline	$10.88^{Aa} \pm 2.09$	$39.91^{Ab} \pm 2.68$
Betadine	$28.81^{Ba} \pm 1.97$	$80.35^{Bb} \pm 0.96$
GNP	$61.66^{Ca} \pm 1.71$	$93.11^{Cb} \pm 0.74$

*Mean ±S.E. value bearing different upper case differ significantly within the column on respective days and the mean with different lower case superscript in row differ significantly ($p<0.05$).

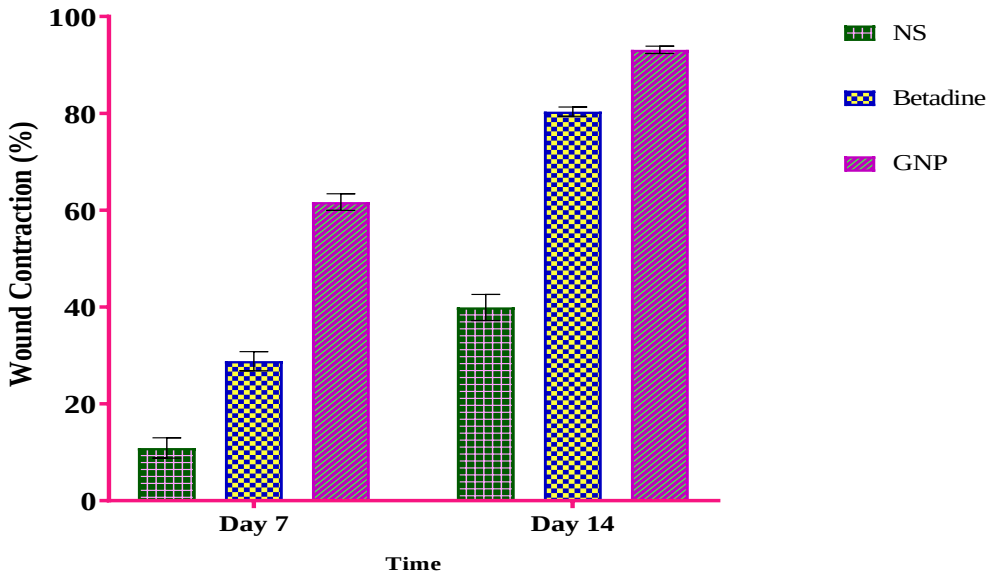

Fig. No.: 34 graphical representation of wound contraction %

Gross image for contraction of wound

Fig. No.: 35

Fig. No.: 36

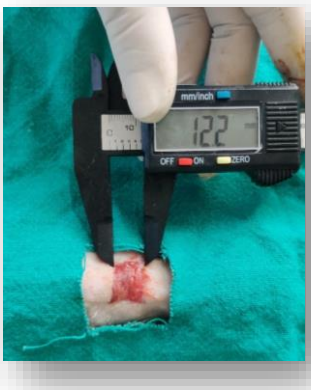

Fig. No.:37

Fig. No.: 38

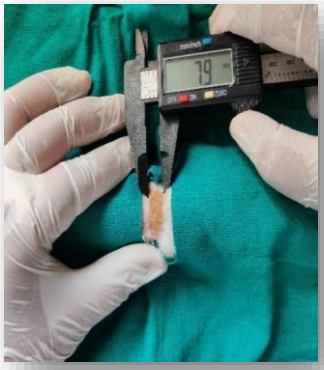

Fig. No.: 39

Fig. No.: 40

Fig 35 to 40 - Photographic representation of contraction rate showing percent wound contraction area of different post excision days of control, betadine and biofabricated gold nanoparticle treated groups of animal.

4.4 Biochemical profiling of skin tissue samples

Skin samples from the animals of each group were collected to assay the biochemical parameters viz. Hydroxyproline assay, RNA at weekly interval till 14th day of study.

4.4.1. Hydroxyproline assay in tissue sample

Tissue sample were collected from each group of animal at wounded site to estimate the healing status in various treatment in different group. The effect of different given treatment on hydroxyproline content (µg/mg of tissue) is given in the table10.

On 7th days of experimental trial, the value of hydroxyproline content in groups of animal's I, II and III were 3.93±0.14, 5.05±0.13 and 6.58±0.04 µg/mg of tissue. Significant ($P < 0.05$) increase in hydroxyproline content in collected tissue sample of rats which was treated with biofabricated GNP in comparisons to group II and group I which was treated with betadine and normal saline respectively. Animal of group III showed maximum hydroxyproline content.

On 14th days of experimental trial, the value of hydroxyproline content in group of animals' I, II and III 4.67 ± 0.10, 6.30 ± 0.11 and 8.16 ± 0.07 µg/mg of tissue collected from wounded site. Significant increase ($P < 0.05$) in hydroxyproline content in collected tissue sample in group III which showed faster healing of wound in comparison to group II and group I respectively.

Table10: effect of various treatment on hydroxyproline content (µg/mg of tissue)

Groups/days of treatment	7TH DAY	14TH DAY
NS	$3.93^{Aa} \pm 0.14$	$4.67^{Ab} \pm 0.10$
Betadine	$5.05^{Ba} \pm 0.13$	$6.30^{Bb} \pm 0.11$
GNP	$6.58^{Ca} \pm 0.04$	$8.16^{Cb} \pm 0.07$

*Mean± S.E. value of hydroxyproline concentration bearing different upper case differ significantly within the column on respective days and the mean with different lower case superscript in row differ significantly($p<0.05$).

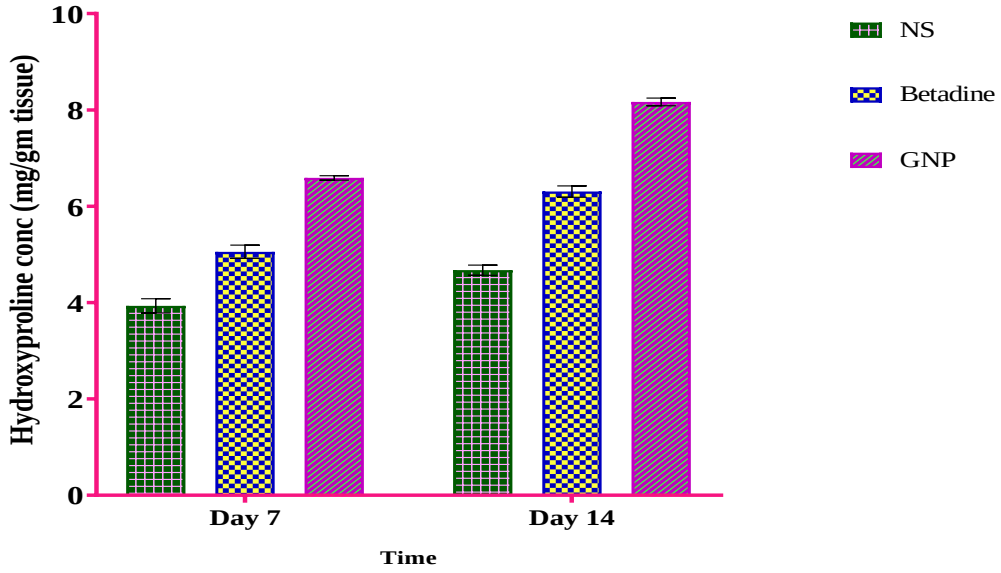

Fig. No.: 41 graphical representation of hydroxyproline content

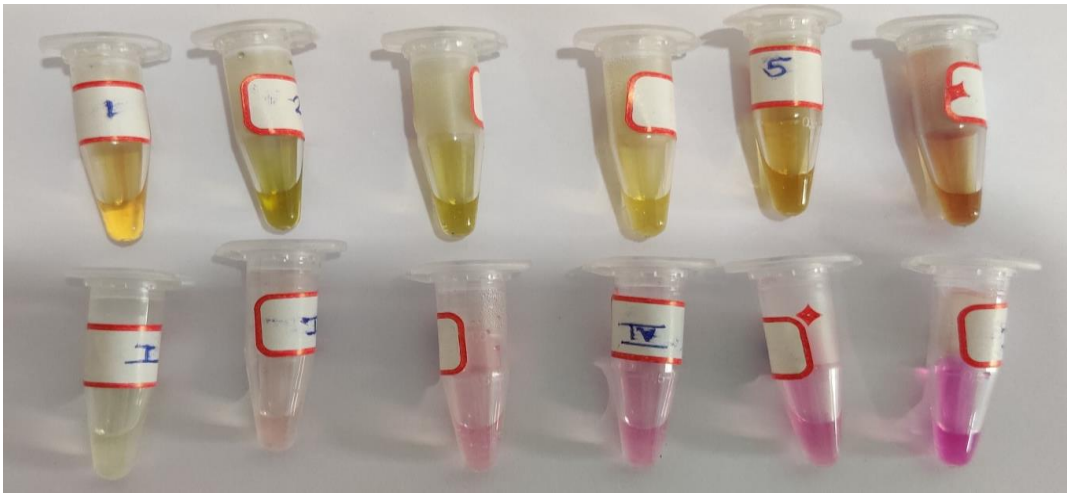

Fig. No.:42 colour change of sample after 3 hr of hot air oven

4.4.2. DNA estimation in tissue sample

Tissue sample were collected from each group of animal at wounded site to estimate the healing status in various treatment in different group. DNA value (µg/ml of tissue extract) in the representative skin sample obtained after 7^{th} and 14^{th} day of interval is given in the table No.11.

On 7^{th} days of experimental trial, the value of DNA content in groups of animal's I, II and III were 53.18 ± 1.46, 301.33 ± 6.32 and 698.29 ± 9.56 µg/ml of tissue. Significant ($P < 0.05$) increase in DNA content in collected tissue sample in group III sample of rats which was treated with biofabricated GNP in comparisons to group II and group I which was treated with betadine and normal saline respectively. Animal of group III showed maximum DNA content were noticed on 7^{th} day of treatment.

On 14^{th} days of experimental trial, the DNA content in group of animal's I, II and III 199.13 ± 1.82, 415.73 ± 68.60 and 907.60 ± 73.93 µg/ml of tissue collected from wounded site. Significant increase ($P < 0.05$) in DNA content in collected tissue sample of group III animals showed faster healing in comparison to group II and group I, respectively.

Table11: effect of various treatment on DNA content (µg/ml of tissue)

Groups/days of treatment	7th day	14th day
Normal saline	53.18 ± 1.46	199.13 ± 1.82
Betadine	301.33 ± 6.32	415.73 ± 68.60
GNP	698.29 ± 9.56	907.60 ± 73.93

*Mean± S.E. DNA content differ significantly within the column on respective days and the mean with different treatment differ significantly with respective groups($p<0.05$).

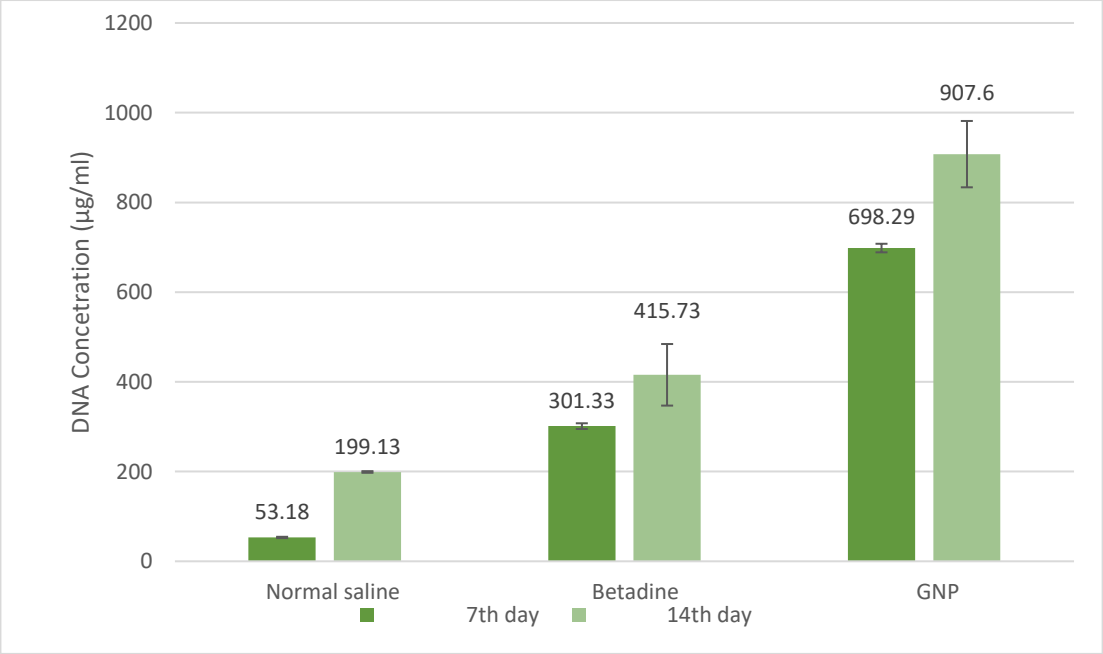

Fig. No: 43 graphical representation of DNA content

4.4.3. Expression profiling of genes

The RNA samples showing $\mathbf{A_{260}/A_{280}}$ ratio 1.8-2.0 and $\mathbf{A_{260}/A_{230}}$ **ratio>1.6** were used for cDNA synthesis. The RNA concentration was estimated by reading the absorbance at 260nm and was checked for purity at 280nm in a spectrophotometer. After purity checking of RNA prepared cDNA is used for Conventional PCR for GADPH and COX-2 gene standardization before rt-PCR by agarose gel electrophoresis by vilber gel documentation system.

4.4.3.1. Gel documentation analysis of GADPH gene and COX-2 gene

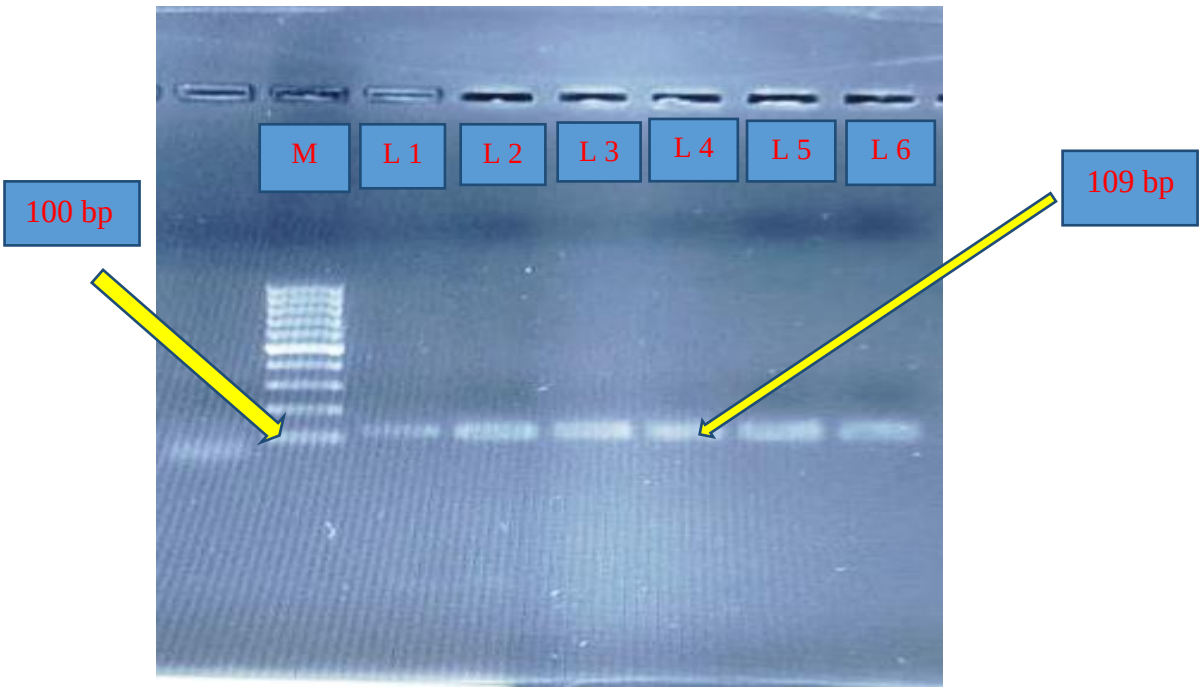

Fig. No.: 44 gel doc result of GADPH gene(lane M=100 bp DNA ladder and lane 1-6 PCR standardization product size)

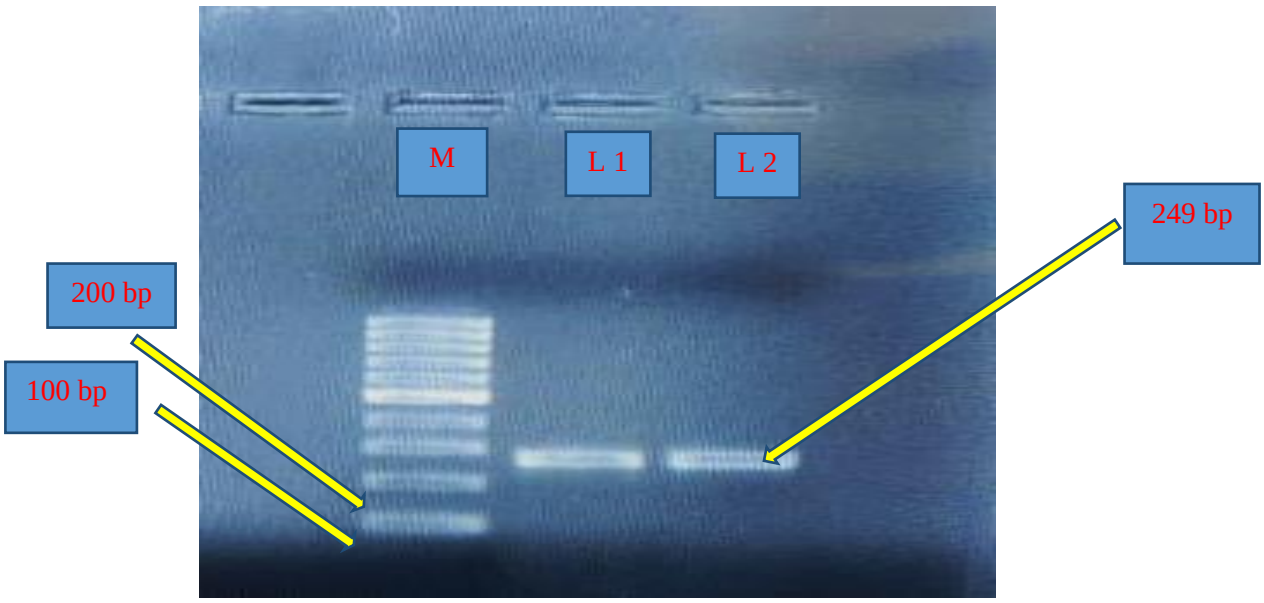

Fig. No.: 45 Gel doc result of COX-2 gene(lane M=100 bp DNA ladder and lane 1-2 PCR standardization product size)

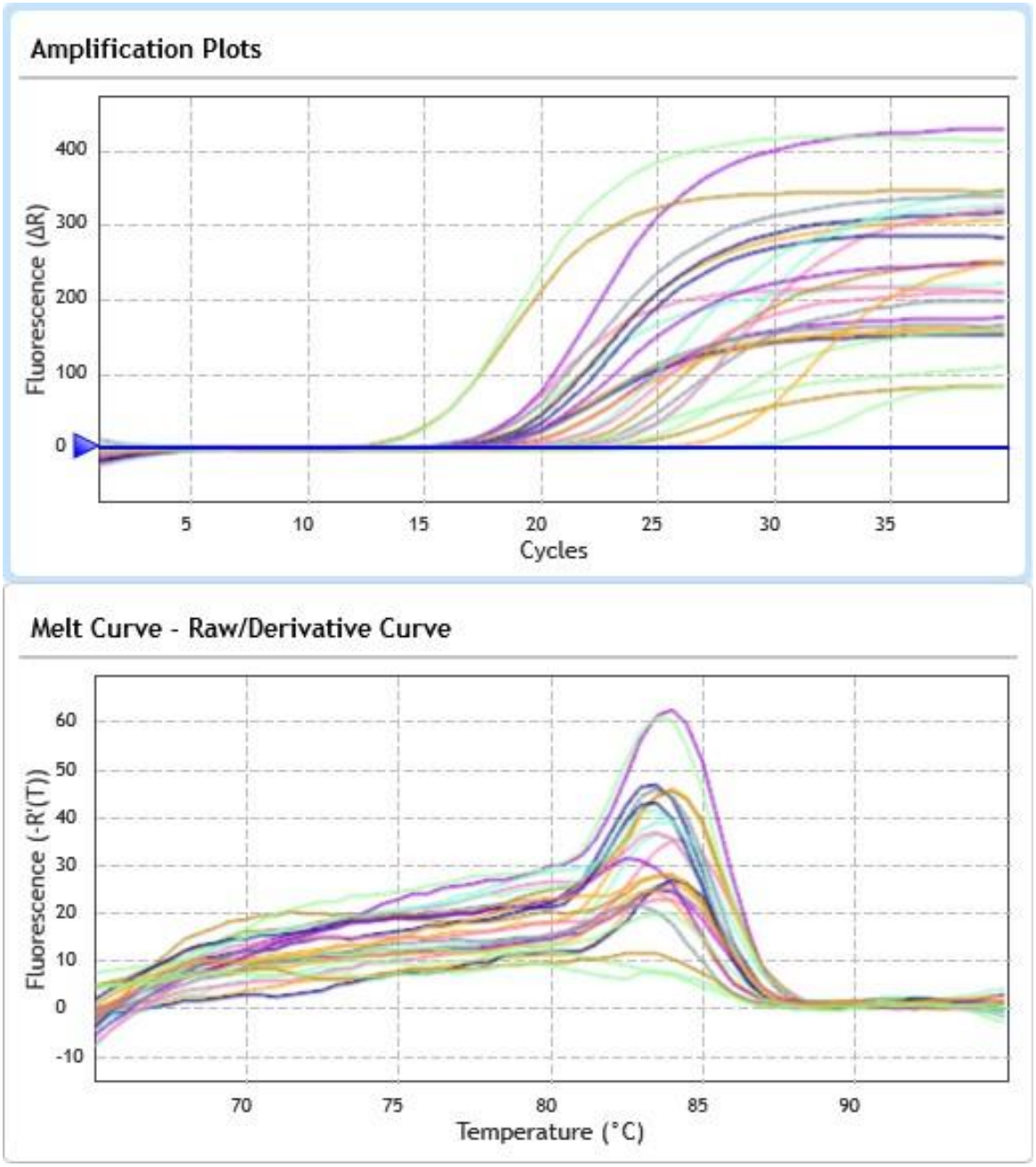

Fig. No.: 46 (a) Graphical representation of amplification plot and melting curve of GADPH (housekeeping gene). Amplification plot showed the amplified gene of the sample and melting curve showed the purity of gene of sample.

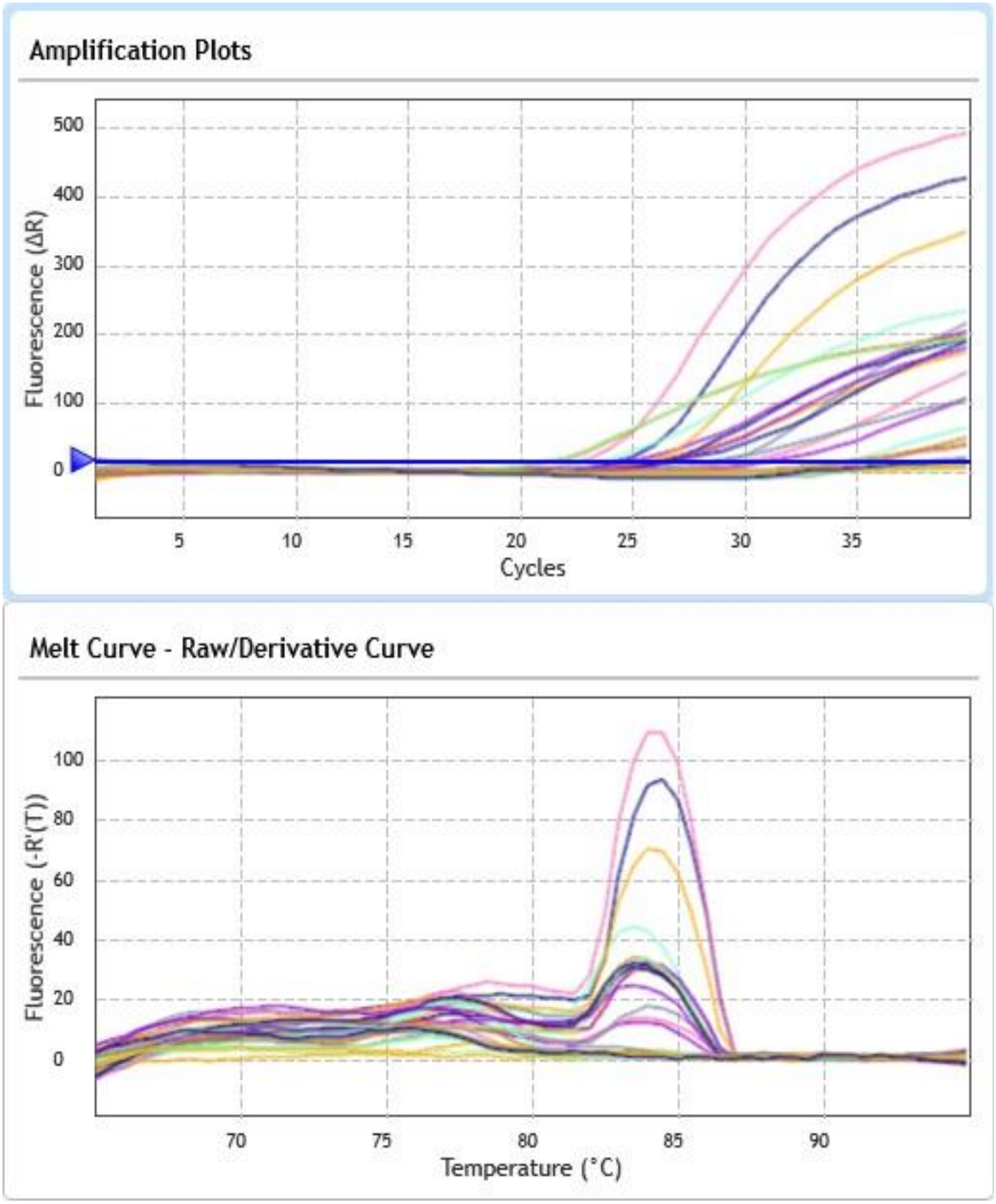

Fig. No.:46 (b) Graphical representation of amplification plot and melting curve of COX-2 (expression gene). Amplification plot showed the amplified gene of the sample and melting curve showed the purity of gene of sample.

Well	Well Type	Dye	Target	Replicate	Cq (ΔR)	Tm Product 1 (-R'(T))
A1	Unknown	SYBR	SYBR	---	18.04	84.00
A2	Unknown	SYBR	SYBR	---	18.26	84.00
A3	Unknown	SYBR	SYBR	---	16.56	83.50
A4	Unknown	SYBR	SYBR	---	16.67	84.50
A5	Unknown	SYBR	SYBR	---	19.37	83.50
A6	Unknown	SYBR	SYBR	---	22.09	83.50
A7	Unknown	SYBR	SYBR	---	21.43	84.00
A8	Unknown	SYBR	SYBR	---	21.44	80.00
B1	Unknown	SYBR	SYBR	---	17.37	84.00
B2	Unknown	SYBR	SYBR	---	17.65	83.50
B3	Unknown	SYBR	SYBR	---	23.10	83.50
B4	Unknown	SYBR	SYBR	---	22.91	83.50
B5	Unknown	SYBR	SYBR	---	16.81	84.00
B6	Unknown	SYBR	SYBR	---	16.59	83.50
B7	Unknown	SYBR	SYBR	---	13.21	84.00
B8	Unknown	SYBR	SYBR	---	13.72	83.50
C1	Unknown	SYBR	SYBR	---	19.71	83.50
C2	Unknown	SYBR	SYBR	---	18.19	83.50
C3	Unknown	SYBR	SYBR	---	20.72	83.50
C4	Unknown	SYBR	SYBR	---	20.51	83.50
C5	Unknown	SYBR	SYBR	---	18.27	82.50
C6	Unknown	SYBR	SYBR	---	18.55	83.00
C7	Unknown	SYBR	SYBR	---	24.44	83.00
C8	Unknown	SYBR	SYBR	---	23.80	83.50

Fig. No.: 47 Ct value of housekeeping gene

Well	Well Type	Dye	Target	Replicate	Cq (ΔR)	Tm Product 1 (-R'(T))
A1	Unknown	SYBR	SYBR	---	26.84	84.00
A2	Unknown	SYBR	SYBR	---	25.06	84.50
A3	Unknown	SYBR	SYBR	---	24.44	83.50
A4	Unknown	SYBR	SYBR	---	23.42	84.00
A5	Unknown	SYBR	SYBR	---	32.38	76.00
A6	Unknown	SYBR	SYBR	---	29.92	84.00
A7	Unknown	SYBR	SYBR	---	36.85	77.50
A8	Unknown	SYBR	SYBR	---	No Cq	70.00
B1	Unknown	SYBR	SYBR	---	No Cq	74.50
B2	Unknown	SYBR	SYBR	---	27.10	83.50
B3	Unknown	SYBR	SYBR	---	No Cq	76.50
B4	Unknown	SYBR	SYBR	---	31.27	77.00
B5	Unknown	SYBR	SYBR	---	25.86	84.00
B6	Unknown	SYBR	SYBR	---	29.90	84.00
B7	Unknown	SYBR	SYBR	---	22.38	83.50
B8	Unknown	SYBR	SYBR	---	22.06	84.00
C1	Unknown	SYBR	SYBR	---	26.43	84.00
C2	Unknown	SYBR	SYBR	---	27.91	83.50
C3	Unknown	SYBR	SYBR	---	35.42	77.00
C4	Unknown	SYBR	SYBR	---	36.89	76.50
C5	Unknown	SYBR	SYBR	---	27.68	83.50
C6	Unknown	SYBR	SYBR	---	No Cq	76.50
C7	Unknown	SYBR	SYBR	---	36.22	76.00
C8	Unknown	SYBR	SYBR	---	No Cq	76.50

Fig. No.: 48 Ct value of expression gene

Where Ct denotes the cycle number where the fluorescence generated by the rt-PCR produce is distinguishable from the background noise. In fig no 47 and 48 showed the Ct value of housekeeping gene and expression gene. On the basis of following ct value, put the value and calculate the fild gene expression of expression with respect to housekeeping gene expression.

By using the following formula the relative fold change in the gene expression was calculated on day 7th and 14th and compared the same with the day 0 of the study.

$$= 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \Delta Ct\ (treated\ sample) - \Delta Ct\ (untreated\ sample)$$

$$\Delta Ct = \Delta Ct\ (gene\ of\ interest) - \Delta Ct\ (housekeeping\ gene)$$

The gene was down regulated by 6.54 fold in MGNP treated group whereas it was 1.75 fold down regulation in Betadine treated group on 7th day. After 7th day of treatment the gene expression was going to be higher but still it was down regulated. The amplitude of the fold change was -0.93 and -0.37 on day 14th in MGNP and Betadine treated groups in comparison to the control group.

HISTOPATHOLOGICAL EXAMINATION

Haemotoxylin and Eosin staining

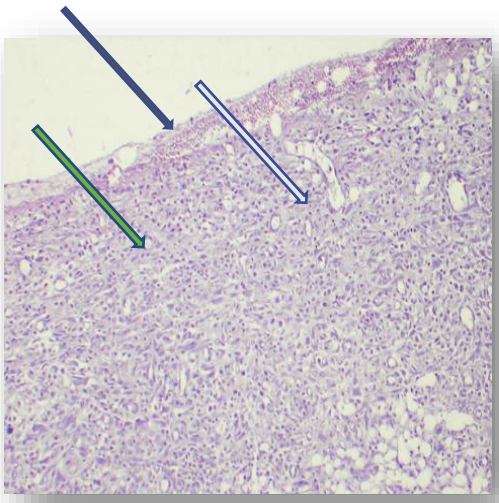

Fig. No.: 49

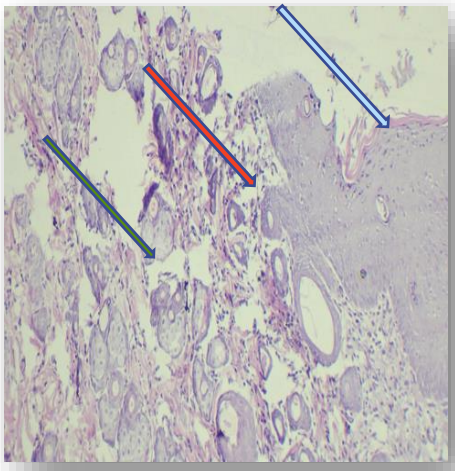

Fig. No.: 50

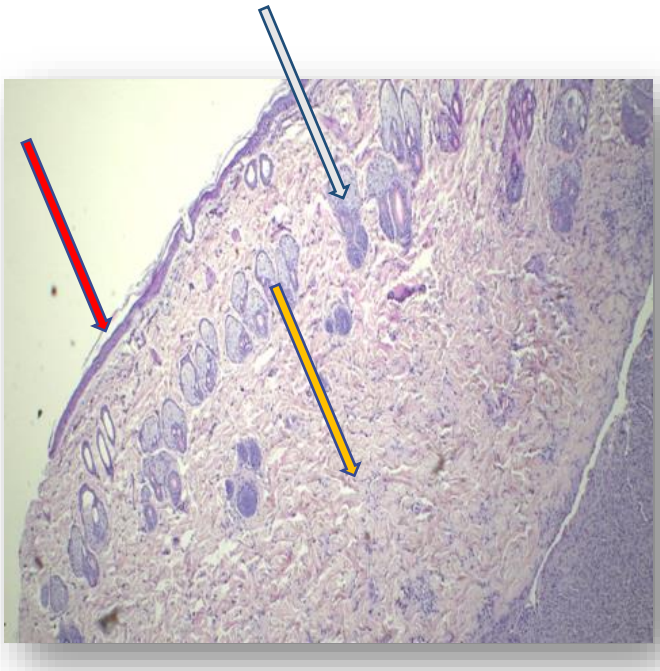

Fig. No.: 51

Photomicrograph showing healing pattern on day 7th with N.S., betadine and GNP. Healing tissue in GNP Treated wound was better with compact and wider adnexa, collagen fibre and epithelisation (H & E stain 10X)

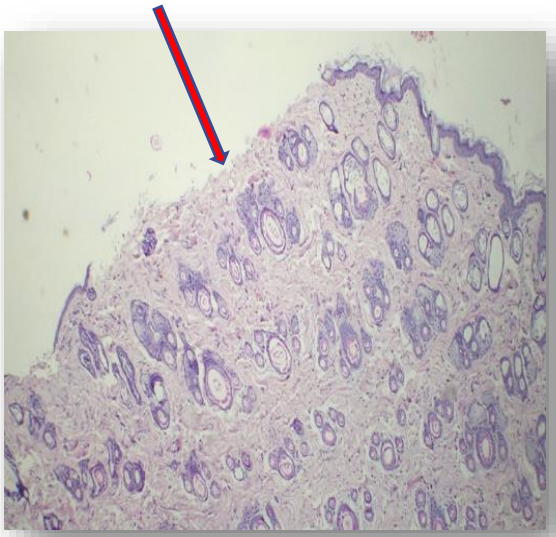

Fig. No.: 52

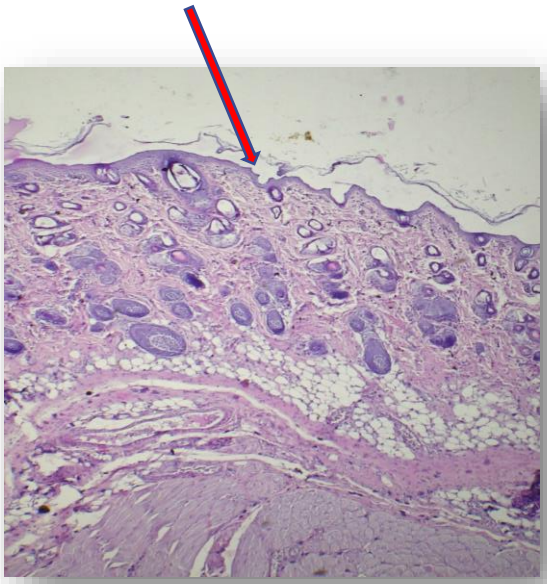

Fig. No.: 53

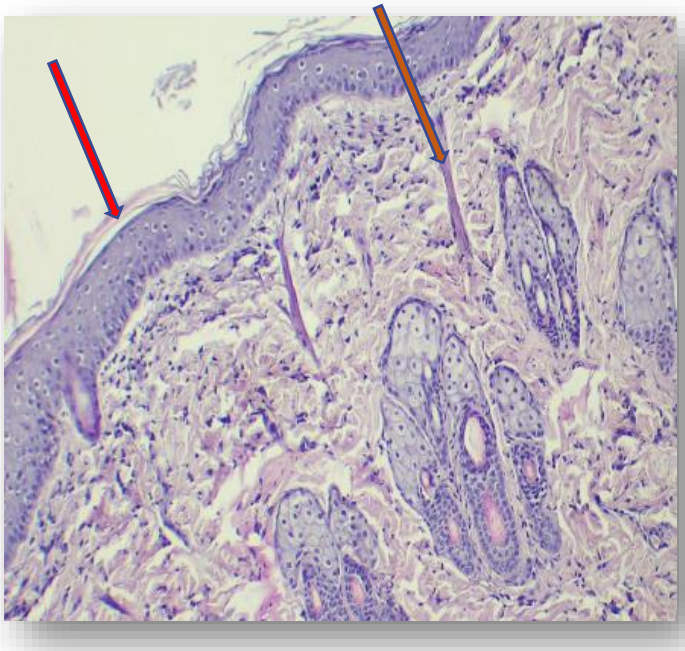

Fig. No.: 54

Photomicrograph showing healing pattern on day 14TH with N.S., betadine and GNP. Healing tissue in GNP Treated wound was better or as normal skin in comparisons to betadine and NS respectively (H & E stain 10X).

7TH DAY TREATMENT WITH N.S.-

- Some bleeding surface due to removal of Escher.
- Minimum epithelisation.
- Fibroblasts with infiltration of mononuclear cells like macrophages and few plasma cells.
- At some places neutrophils were also found indicating active chronic inflammation.
- Edematous area in the upper dermis was also seen, however process of epithelisation in some rat can be seen.
- Few or lack of ground substance (protein, collagen).

7TH DAY TREATMENT OF BETADINE-

- Epithelisation was evident, however mild amount of mononuclear cells were also seen in the dermis.
- The amount of collagen fibre and ground substance was less in comparison to GNP treatment rats.
- Adnexa (sweat, sebaceous, hair follicles) were present in initial stage of development.
- At some places ulcerative points were also evident with hyperkeratosis

7TH DAY TREATMENT OF GNP-

- Well-developed epidermis with mature collagen fibres and more amount of ground substances.
- Connective tissue fibres was more in comparison to betadine group of rats.
- Angiogenesis was more in comparison betadine.
- Somewhere in epidermis a very thick indicating severe mitotic activity of the basal cells of epidermis.
- Adnexa was well developed.
- Collagen fibres are haphazardly arranged.

14TH DAY OF TREATMENT N.S.-

- Epithelisation has occurred however the thickness of epidermis was less indicating slow mitotic activity of basal cells of epidermis.
- Somewhere parakeretosis was also seen.
- In the epidermis edemas still persistent with mild amount of inflammatory cells.
- Adnexa formation was in initiative stage.
- No of blood vessels are more in comparison to 7th group.

14TH DAY TREATMENT OF BETADINE-

- Complete epithelisation with small unhealed part at the center.
- The skin appears as normal.
- Mild inflammatory response.
- Well-developed adnexa present in the epidermis

14TH DAY TREATMENT OF GNP-

- Complete repair of the wound except very small unhealed part at the center.
- The skin appears as normal
- All the components of dermis were fully developed consisting of well matured collagen fibres, blood vessels, sweat gland, sebaceous gland, hair follicles, nerve bundles and corpuscles.
- However the collagen fibres was still haphazardly arranged but still in orientation.

MASSON'S TRICRHOME STAINING

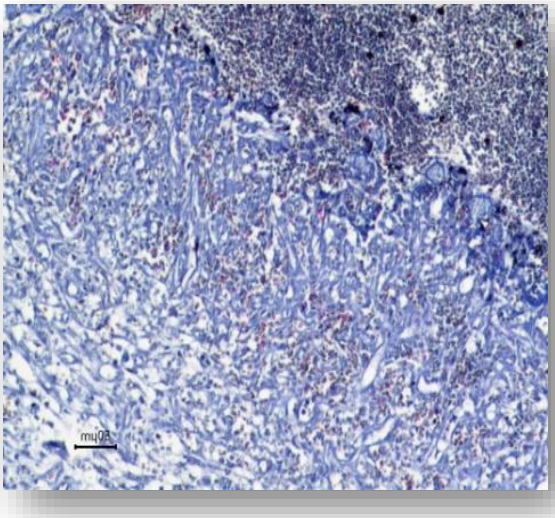

Fig. No.: 55

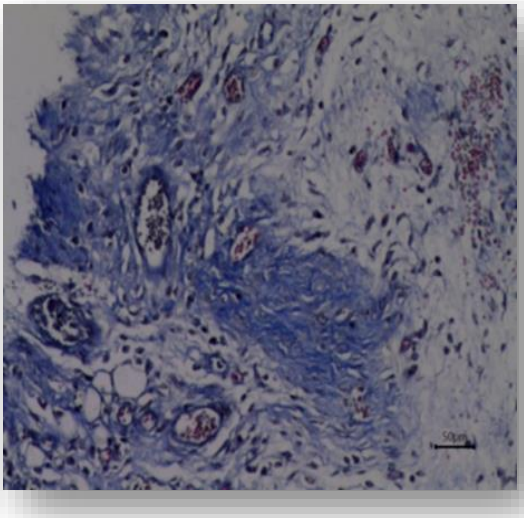

Fig. No.: 56

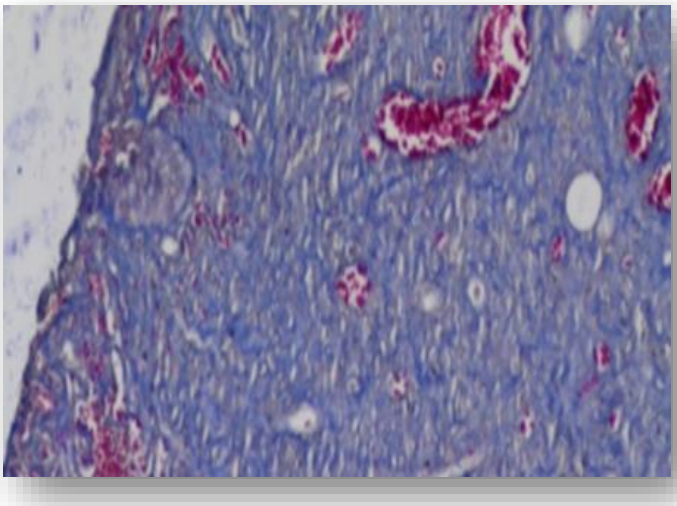

Fig. No.: 57

Photomicrograph showing the Angiogenesis pattern on day 7th with NS, Betadine and GNP. In comparison to NS and betadine wound has more dense and thick collagen fibre in GNP treatment group shown in MTS 10X)

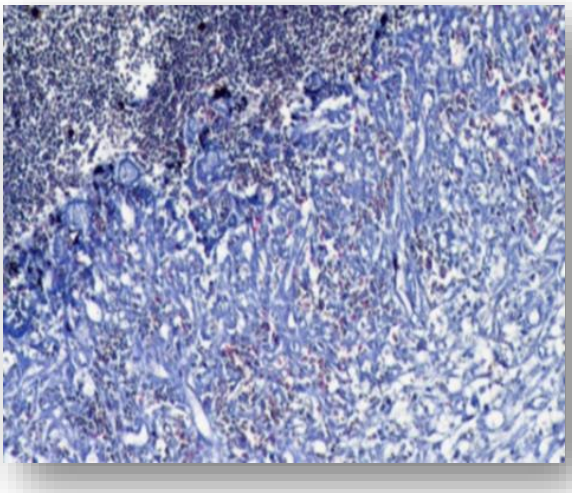

Fig. No.: 58

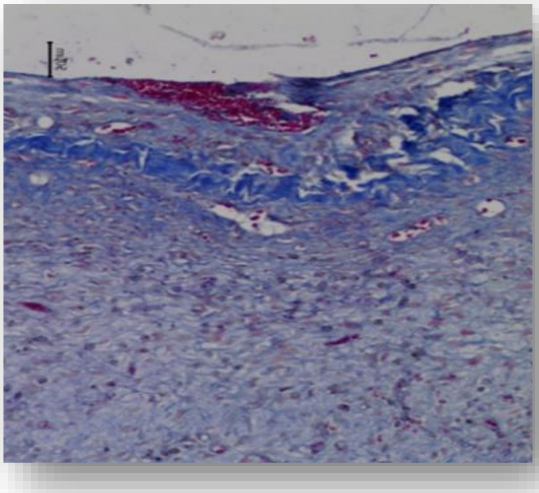

Fig. No.: 59

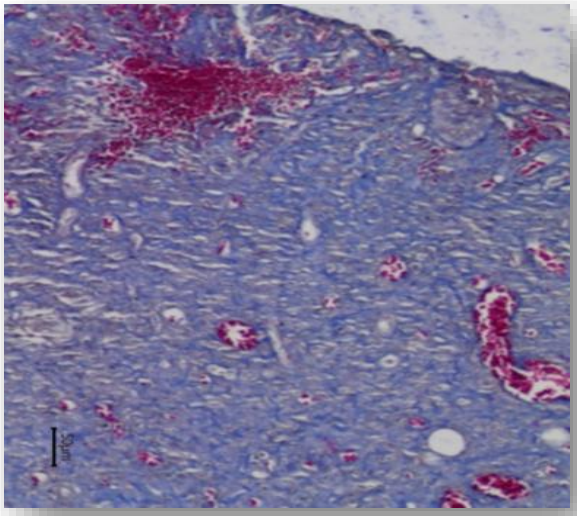

Fig. No.: 60

Photomicrograph showing healing pattern on 14th day of treatment showing moderate deposition of collagen fibres which are denser and thicker in the sections from GNP than betadine and NS groups (shown in MTS 10X)

7TH DAY OF STUDY-

In comparisons with N.S. and betadine, GNP treated animal group wounds had denser and thicker collagen fibres and showed more fibroblast of shown in photomicrograph.

14TH DAY OF STUDY-

The stained slides of masson trichrome N.S. treated group I has mild collagen deposition at wound center while in the betadine treated group thin collagen was seen at the wounded site, whereas the tissue sample of group III showed thick collagen which deposits at wound center as shown in photomicrograph.

5. DISCUSSION

This study was directed to evaluate the wound healing prospective of biofabricated gold nanoparticle with *Moringa oleifera* and its molecular dynamics to explain the healing process in full thickeners excisional wound in rat models. 42 rats were equally divided into three groups viz group I rats consist of control group, group II consist of rat treated with betadine and group III rats were treated with topical application of biofabricated gold nanoparticle.

Healing of wound is a complex and convoluted phenomenon consisting of three basic phases i.e. Inflammation, Proliferation and Remodeling (Karodi *et al.,* 2009). In excision wound, biofabricated GNP showed faster healing compared with betadine and normal saline respectively. Different wound healing medication have been directed towards fixed channel of these phases and consisting of many more molecular stream pathways in order to assay healing process. The healing process depends to a large extent on the regulated biosynthesis and deposition of new collagens and their subsequent maturation (Agarwal *et al.,* 2009). In the tissue repair process, inflammatory cells promote the migration and proliferation of endothelial cells, leading to neovascularization of connective tissue cells which synthesize extracellular matrices including collagen, and of keratinocytes resulting to re-epithelialization of the wounded tissue (Murthy *et al.,* 2013). Inflammation, collagen maturation, and scar formation are some of the many phases of wound healing, which run all together but independent of each other.

There are very few reports dilute in the area of tissue regeneration and wound healing in human and veterinary clinical practices by using biofabricated gold nanoparticle. As per Ayurveda term viz. Vranaropaka term explains the drugs of natural origin to be used for wound healing (Biswas and Mukherjee, 2003) and term Vranashotha means swelling or inflammatory reactions were appreciated for initial phases of wound healing as used in modern day medicine. Certain disease condition like diabetes ulcer due to venous ulcer, due to venous stenosis and use of drugs causes immunological compromise and due to this wound healing becomes challenging. As per Shukla *et al.,* 2020 wound healing is a crucial pathophysiological process which forecast collusion of some angiogenic and vasculogenic markers with other derivatives like cytokines and chemokine's.

Nanoparticles, contains superior surface-to-volume ratio, doing efficiently employed in countless medical applications, including wound therapy. Metal nanoparticles such as

silver, gold and zinc possess excellent properties in term of low toxicity and antibacterial activity, making them perfect candidates for integration in wound dressings. Lu *et al*., 2010, says that low concentrations of GNP enhance keratinocyte growth and differentiation, but higher levels were associated with cytotoxicity. Present study was also revealed that gold nanoparticle have wound healing property as well as antioxidative property.

Due to impairment of immune status chemo stasis, and endothelial and inflammatory cells migration to the wounded site have been compromised which leads to delayed wound healing. Additionally cutaneous wound healing activity enclose the involvement of all three phases viz hemostasis. Inflammation extracellular matrix formation, genesis of granulation tissue which include re-epithelisation, angiogenesis vasculogenic process and remodeling (Reinke and sorg., 2012 and Shankar *et al*., 2015)

Evolution of herbal wound healing products is a new encouraging domain for tissue rejuvenation and healing of wound in veterinary as well as human clinical practice. Development in the biomedicine research has broaden the scope to resolve the molecular intuition and gesture to some phytobiomolecule and also come up with an opportunity to popup the evaluation of new option in the field of regenerative medicine. The basic goal behind wound healing process is that the wound should have least scar formation. Ease of availability, low dermal toxicity and cost economics and effectiveness has promoted the use of medicinal plants and herbs in reparative and regenerative therapy in human as well as veterinary research (Nguyen *et al*., 2009).

Phytobiomolecules has been connected with several pharmacological attributes like antioxidative, anti-inflammatory properties etc. In addition to these aforesaid pharmacological attributes, many plant based biomolecules have antimicrobial quality and thus limit and control the growth of microbial population over the wounded site and prevent entry of microbes into contaminated wound form. Therapeutic bioactive representative that are able to promote fibroblast growth and expansion is being able to ameliorate or promote wound healing as in the case of our present study, we demonstrated the effect of aqueous extract and fractions of *M. oleifera* in enhancing the proliferation and viability of fibroblast cells in *in vitro*. Similar result were found Rathi *et al*., 2006 aqueous extract of leaves of *Moringa oleifera* was rationalized for excisional wound healing activity. It significantly increase in wound closure rate, skin-breaking strength. Granuloma breaking strength, granuloma dry weight and decrease in scar area was observed.

Wound contraction

In excision wound, biofabricated GNP treated groups III rats showed faster wound contraction and take shape as normal in comparison to betadine treated groups II and normal saline treated group I respectively. Wound healing is a soundly arranged sequence of method which is done by intercellular links via a cytokine and growth factor (Diegelmann and Evans, 2004). The important equilibrium between the stimulating and inhibitory arbitrators of wound contraction procedure pivotal to attain an early and fast healing following injury. Fibroblast plays very crucial role for the synthesis, deposition and remodeling of the extracellular matrix (Singer and Clark, 1999). A fast wound contraction and reduced risk have always thought of beneficial for wound healing. The purpose of present study was that the topical application of biofabricated GNP significantly speed up the cutaneous wound healing in full thickness excisional wound in time dependent manner. GNP has strong antioxidative effects to put out free radicals such as DPPH (2,2-diphenyl-l-picrylhydrazyl), OH (hydroxyl), H2O2 (hydrogen peroxide), and NO (nitric oxide) (Esumi *et al.*,2003; Leu *et al*.,2012 and Medhe *et al*.,2014). Chen *et al*.,(2016), scrutinized the effects of GNP with other anti-oxidant agents for topical applications in diabetic wounds. Topical application of GNP significantly accelerated the healing processes through anti-inflammation and turns new blood vessels into mature non-leakage vessels. The GNP may creep into stratum corneum to increase the specific mixture absorption and the functional ability of anti-oxidants. Li *et al*., 2016 and Volkava *et al*., (2016) reported that GNP have potential in anti-inflammation, anti-oxidation, anti-microbial, and anti-angiogenesis properties to intensify proliferation of fibroblast and decrease cell apoptosis in wound healing process.

Histopathology-

The effect of both treatment in the wound healing was also evaluated through gross and histomorphochemical examination. The wound of group II and III showed proper homeostasis and absence of any bacterial infections. The tissue section collected at day seven and fourteenth of experiment were routinely stained with H & E stain and to demonstrate the collagen fibers special stain with MTS was done. The sections were qualitatively assayed under light microscope and observed in respect of fibroblast proliferation, Collagen fiber, angiogenesis, and epithelisation. This was also revealed that the extract containing GNP accelerated the ending of the inflammatory stage, forming of blood vessels (Krychowiak,*et al.*, 2014; Shankar *et al*., 2015.)

In present study there was rearly complete development of all the normal cells including epidermis, dermis and hypodermis components in rats treated with biofabricated gold nanoparticle group. The epithelisation time was markedly reduced in both groups II and III. Epithelisation was observed from the first day up to end of the experiment i.e. 14th day. Kundu *et al*. (2016) also reported that application of plant based product which corroborates the showed similar histoarchitectural restoration i.e. re-epithelialization and improved formation of hair follicles and considered it as healthy sign of healing process.

Tissue healing process, inflammatory cells promote migration and proliferation of endothelial cells which synthesize extracellular matrix including collagen and keratinocytes resulting re-epithelisation of wound tissue. Increased cellular proliferation and collagen synthesis are also assessed through hydroxyproline estimation of granulation tissue. Collagen is predominant extracellular protein in granulation tissue of healing wound and increased in the synthesis of this protein provides strength and integrity to tissue matrix. This results also revealed that the extract containing GNP accelerated the ending of the inflammatory stage, forming of blood vessels and remodeling of the collagen matrix, resulting in faster skin regeneration and improved healing of wounds than the control group. (Hajialyani, *et al*., 2018)

In this study orientation of thick, dense and final alignment of collagen fiber was visible in Masson trichome stained tissue in group III rats. Also, the adnexa i.e., sebaceous gland, hair follicles, elastic fibers, erector pili muscles etc. was evident in group III rats. The picture was markedly less in group II rats and reticular pattern in group III. Kundu *et al*. (2016) also reported that application of plant based product showed improved formation of hair follicles, adnexa and consider it as healthy sign of healing process. At fourteenth day the histopathological examination of the biopsy sample revealed continuous granulation tissue formation in the control group but in group III rats the deposition and orientation of the collagen fibers were near to the normal skin. Group II also showed that the original tissue regeneration was much greater in comparison to control group, but lesser than GNP treated rats. Shukla *et al*. (2020) also reported the role of deposition of collagen and sprouting of hair follicles, presence of sebaceous gland as reparative indicator of better healing potential.

Hydroxyproline assay

Our study revealed significant improvement in collagen deposition in reparative wounds as evident by the significant increase in hydroxyproline value among the group III

which was treated with biofabricated gold nanoparticle as compared to group I and group II treated with normal saline and betadine respectively. Syndicate of collagen fibres furnish strength and firmness to the granulation tissue. Hydroxyproline at first hand correlates with the stability, tensile strength and inter chain syndicate through ester linkage between polypeptide chains. The carbonyl group of the adjoining peptide part forms a syndicate with hydroxyl group i.e.-OH portion of hydroxyproline molecule (Kilarski *et al.*, 2009). Amount of collagen present in wound is directly proportional to the value of Hydroxyproline content. Together extent it has been entirely accepted that hydroxyproline and collagen has bit part in later stages of wound homeostasis and re-epithelisation process (Badr, 2013).

Higher concentration of hydroxyproline designed speedy healing that also considered by the higher cellular DNA content during the re-epithelisation process (Roy *et al*., 2012). Cellular differentiation and quickened wound healing is also determined by assessment of total protein and DNA content in the lesioned tissue. Studies of Hozzein *et al*. (2015) and Kundu *et al.* (2016) also tell about the improvement in values of Hydroxyproline, and DNA and total protein following treatment with different plant extracts in wound healing models. Rathi *et al*., 2006 also revealed that aqueous extract of leaves of *Moringa oleifera* accelerated the excisional wound healing activity by increasing the hydroxyproline content hence higher the concentration of hydroxyproline indicates faster rate of wound healing activity. Biochemical analysis revealed that increased hydroxyproline content, which is a reflection of increased cellular proliferation and there by increased collagen synthesis. Present study also signify that wound healing potential of gold nanoparticle which was biofabricated with *Moringa oleifera* as evidenced by significant increased values of DNA and hydroxyproline content as compared to other betadine and normal saline group respectively.

Discussion for COX

Cyclooxygenase (COX) enzymes are a class of enzyme that is responsible for formation of prostaglandins from arachidonic acid. Two kind of Cox is normally present Cox-1 and Cox-2. Cox-1 is expressed as a housekeeping enzyme in most tissues during homeostasis, whereas Cox-2 is generally unregulated only in pathological conditions such as inflammation and cancer (Moon *et al*, 2020). The COX-2 is not normally expressed in the most cells, but is rapidly induced in response to inflammatory stimuli producing prostaglandins, such as prostaglandin E2 (PGE2). COX-2 activity increases within 3 hours after injury and reach to peak after three days (Futagami, *et al.*, 2002). In our study also

COX-2 activity decreases after GNP and betadine treatment on 7th and 14 days. This down regulation of COX expression may be due to inhibitory effect of both GNP and betadine. Further, COX-2 expression more down regulated in GNP treated group as compare to betadine treated on both 7th and 14th days. It indicates that GNP having more inhibitory effect COX-2 on than betadine. Romana-Souza (2016) demonstrated that the reduction of COX-2 protein expression has a beneficial effect on wound healing through the reduction of iNOS and PGE2 protein expression.

6. SUMMARY AND CONCLUSION

Summary

This study was planned to see the wound healing efficacy and molecular perception of wound healing process following topical application of biofabricated GNP in full thickness excisional wound in rat models. In this study 42 rats were divided randomly and equally into three groups viz group I (healthy control), group II (betadine treated) and group III (biofabricated GNP) with 14 rats in each group. Experiment was conducted for 14 days and wound healing were assessed in animals of each group (n=6) at weekly interval of 7^{th} and 14^{th} day.

Wound contraction (%) was calculated by using the Wilson's formula value of wound contraction (%) in animals of group I, II and III, on 7^{th} day were 10.88 ± 2.09, 28.81 ± 1.97 and 61.66 ± 1.71 respectively. On 7^{th} day % of wound contraction in group III (treated with biofabricated GNP) was significantly higher as compared to group II and group I which was treated with betadine and normal saline respectively. On 14^{th} days of experimental trial the contraction of wound were 39.91 ± 2.68, 80.35 ± 0.96 and 93.11 ±0.74 I, II, and III respectively. Significant increase in wound contraction (%) was noticed in animals of group III treated with biofabricated GNP. Animals of group III showed almost proper healing and showed as normal in comparisons to group I and group II on14th day of experimental trial.

In the present study on 7^{th} days of experimental trial, the value of hydroxyproline content in groups of animal's I, II and III were 3.93±0.14, 5.05±0.13 and 6.58±0.04 µg/mg of tissue. Significant increase in hydroxyproline content in collected tissue sample of rats which was treated with biofabricated GNP as compared to group II and group I treated with betadine and normal saline respectively. Maximum hydroxyproline content was noticed in animals if group III. On 14^{th} days of experimental trial, the value of hydroxyproline content in group I, II and III were 4.67 ± 0.10, 6.30 ± 0.11 and 8.16 ± 0.07 µg/mg of tissue collected from wounded site respectively. Significant increase in hydroxyproline content in collected tissue sample in group III was noticed which showed faster healing of wound in comparison to group II and group I.

On 7^{th} days of experimental trial, the value of DNA content in groups of animal's I, II and III were 53.18 ± 1.46, 301.33 ± 6.32 and 698.29 ± 9.56 µg/ml of tissue respectively. Significant increase in DNA content in collected tissue sample in group III, which was treated

with biofabricated GNP in comparisons to group II and group I which was treated with betadine and normal saline respectively. Maximum DNA content was noticed in group III on 7th day of treatment. On 14th days of experimental trial, the DNA content in group of animal's I, II and III were199.13 ± 1.82, 415.73 ± 68.60 and 907.60 ± 73.93 µg/ml of tissue collected from wounded site respectively. Significant increase in DNA content of collected tissue sample in animals of group III showed faster healing in comparisons to group II and group I.

In the present study, effect of gold nanoparticle which is biofabricated with *Moringa oleifera* on wound healing was examined. Topical application of biofabricated GNP on full thickness excisional wound showed that advantageous effect in comparisons to betadine and control group respectively. In gene expression gene was down regulated by 6.54 fold in MGNP treated group whereas it was 1.75 fold down regulation in Betadine treated group on 7th day. After 7th day of treatment the gene expression was going to be higher but still it was down regulated. The amplitude of the fold change was -0.93 and -0.37 on day 14th in MGNP and Betadine treated groups in comparison to the control group.

In histopathological examination GNP treated group showed fast epithelisation, vascularization, angiogenesis, thick collagen fibre, better collagen fibre arrangements and density of collagen fibre was maximum in respect to betadine and control group showed in Haemotoxylin and eosin staining and masson trichome staining.

Therefor our findings proposed that group of rats which is treated with biofabricated GNP which was more effective to contract the wound and performed fast healing because of fast formation of adnexa (sweat gland, sebaceous gland, hair follicles etc.) collagen and higher expression of COX-2 in initial phase of full thickness excisional wound in rat models performed well in histological examination and rt-PCR reaction. To a greater extent controlled in vitro and in vivo case on nanoparticle on different oligos expression and needed to expand our knowledge on the exact expression seen in different phases of wound healing

Conclusion

Betadine application can potentiate the wound healing in rat. Application of biofabricated gold nanoparticle (GNP) in wounds treatment potentiate better epithelisation, angiogenesis and collagen formation. Highest COX-2 expression in RT-PCR in GNP treated wound support that GNP has better wound healing capacity in compare to betadine and normal saline.

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