Wistar Rat Wound –Healing Potential of Cnius Benedict-leaf Extract Assessed Pharmacological

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***Abstract*:**

Wound healing is the process of repair that injury to the skin and other soft tissues. Injury, an inflammatory response occurs and the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin) is regenerated. There are three stages to the process of wound healing: inflammation, proliferation, and remodeling. Medicinal plants involve continuous cell-cell interaction and cell-matrix interactions that allow the process to proceed in different overlapping phases and processes including inflammation, wound contraction, re-epithelialization, tissue remodeling, and formation of granulation tissue with angiogenesis.

Method: *Cnius Benedict-leaf Extract underwent phytochemical analysis to identify bioactive compounds. The Wound –Healing Potential was assessed using the Acute Dermal Toxicity Study. Study was confirmed via light FTIR, in-vitro and in-vivo methods respectively*.

Results: *Cnius Benedict-leaf ethanolic extract contained bioactive compounds like flavonoids, alkaloids, glycosides, steroids, carbohydrates, phenols, tannins, and terpenes. The Anto-microbial assay showed an IC50 value of 49 Percentage inhibition. The findings highlight the significant potential of Cnius Benedict-leaf Extract in developing natural Wound –Healing remedies, showcasing their diverse bioactive compounds and their associated benefits.*

Conclusion: *Cnius Benedict-leaf extract exhibits potent Wound –Healing activity by using Wistar Rat. This suggests its potential as a natural medicine for future therapeutic development in Wound –Healing.*

Keywords: *Wound healing, Cnius Benedict-leaf, phytochemical compositions, Health promotion, In-vito & in-vivo.*

1. Introduction:

Wound healing:

The dynamics of wound healing are complex. A thorough understanding of the normal healing process is a prerequisite for unveiling the pathology. Wound healing begins with homeostasis at the site of injury, progresses to an inflammatory phase followed by proliferation of the epithelial and matrix components, and ends with the formation of scar tissue marked by laying down of a highly organized collagen matrix (Bernhoft et al., 2010). Various factors, extrinsic and intrinsic to the injured tissue, affect the healing process. These are broadly categorized into local and systemic factors (Braiman et al., 2007). Factors directly influencing the immediate wound environment are considered to be local factors, while the overall health of the individual affecting his ability to heal constitutes the systemic factors Impaired wound healing is not an uncommon occurrence in clinical practice (Camacho et al., 2013). Both local and systemic factors are responsible for impaired healing and weak scar tissue

formation. Acute wounds heal following the normal sequence of the healing process. Acute wounds that fail to progress in a timely and orderly fashion through the normal stages of healing are described as chronic wounds (Chaudhari et al., 2006). Because of associated early and late complications, chronic wounds remain an intractable clinical problem and a frequent cause of morbidity and mortality (Collier 2002).

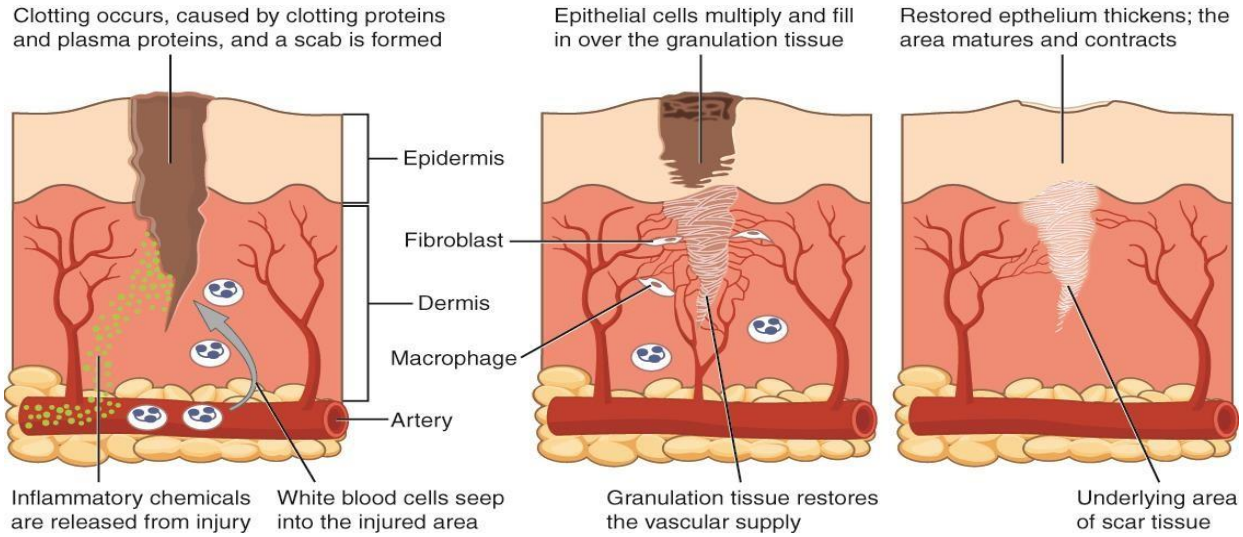


Figure 1: Wound healing

Process of Wound Healing

The sequence of events in normal wound healing has been widely studied and described in literature. Wound healing is a complex biological process that takes place in all tissues in all organs of the body (Collier et al., 2003). Various cell types, including keratinocytes, neutrophils, macrophages, lymphocytes, fibroblasts and endothelial cells, are involved in this process (Cooper et al., 2003). The necrotic tissue is either removed by scavenger cells or separated from living tissue by the process of phagocytosis. The wound healing process consists of four phases: hemostasis, inflammation, proliferation and remodeling (Fabricant et al., 2001).

Coagulation and Hemostasis: The initial step assists in the protection of the vascular system to maintain the functionality of the organ (Falanga et al., 2005). The clot formed as a result of coagulation provides a matrix for the cells involved in subsequent steps of hemostasis and inflammation (Gabbiani et al., 2003). Various pro-inflammatory cytokines and growth factors are released by the clot and wound tissue. Inflammatory cells then migrate to the wound site by the process of chemotaxis and promote the inflammatory phase (George et al., 2006).

Inflammation: The goal of the inflammatory phase is to fight potential bacterial contamination of the wound and to activate cytokine secretion. Uncontrolled inflammation can destroy the early migratory effect, leading to an arrest of the healing process (Godlewska et al., 2020).

Proliferation: The proliferation phase overlaps with the preceding inflammatory phase. It represents a proliferation of both epithelial and dermal elements which results in re-epithelialization of the wound and laying down of the primary extracellular matrix. Epidermal stem cells and bone marrow derived stem cells also play a role during this phase. Angiogenesis occurs secondary to endothelial progenitor cells, a derivative of hematopoietic stem cells (Gorvetzian et al., 2018).

Wound remodeling leading to scar formation: This phase marks the final step in tissue remodeling and differentiation leading to recovery of the skin and its aesthetic restoration. Reconstruction of the dermis occurs by reorganization of the matrix collagen. Fibroblasts differentiation into myofibroblasts, leading to wound contraction and closure (Guo et al., 2010).

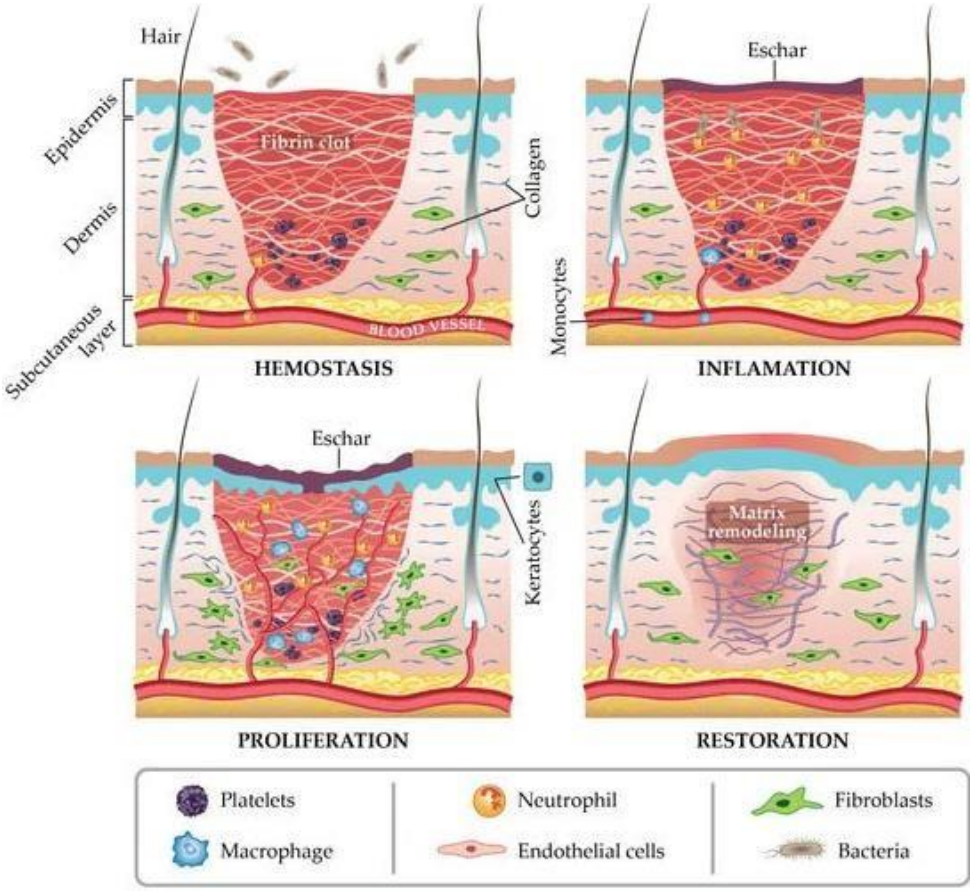


Figure 2: The four stages of wound repair

Physiology of wound healing :

The physiological process of wound healing is achieved through four temporarily and spatially overlapping phases: hemostasis, inflammation, proliferation, and remodeling phases. Immediately after injury, hemostasis occurs and is characterized by vasoconstriction and blood clotting, which prevents blood loss and provides the provisional matrix for cell migration. Platelets secrete growth factors and cytokines attract fibroblasts, endothelial cells, and immune cells to initiate the healing process (Kumar et al., 2007). The subsequent inflammation phase lasts up to 7 days. The predominant cells at work in this phase are phagocytic cells, such as neutrophils and macrophages. Neutrophils release reactive oxygen species (ROS) and proteases that prevent bacterial contamination and cleanse the wound of cellular debris. Blood monocytes arrive at the wound site and differentiate into tissue macrophages (Lazarus et al., 1998). The latter not only remove bacteria and nonviable tissue by phagocytosis but also release various growth factors and cytokines recruiting fibroblasts, endothelial cells, and keratinocytes to repair the damaged blood vessels. As the inflammatory phase subsides accompanied by apoptosis of immune cells, the proliferation phase begins (Li et al., 2018). This phase is primarily characterized by tissue granulation, formation of new blood vessels (angiogenesis), and epithelialization. The last phase occurs once the wound has closed and may last 1–2 years or longer. During this phase, the provisional matrix is remodeled into organized collagen bundles (Lu et al., 2014).

Pathophysiology of wound healing :

The complicated process of wound healing can be divided into three phases: remodeling, propagation, and allergic reaction (Martinez et al., 2008). A proliferative wound repair response that is further typified by vascular responses like blood coagulation and hemostasis is triggered by the inflammatory process (Menke et al., 2007). Leukocyte invasion with the release of cytokines and antibiotics is one of the cellular processes. Granulation tissue grows to cover the wound space after the proliferative process forms the epithelium to coat the wound surface. Fibroblast proliferation, the deposition of collagen and other extracellular matrix, and the creation of new blood vessels are all involved in the formation of granulation tissue. As the new tissue grows inside the wound, the remodeling process starts to restore the tissue's structural integrity and functional competency. The trio However, the three phases of wound healing are not straightforward linear processes; rather, they vary in length (Nagori et al., 2011). Acute wounds are ones that heal quickly, such as burns, other serious traumas, and wounds from surgery (Negut et al., 2018). A clean, uninfected incisional surgical wound that is approximated by operative sutures is an example of a normal acute wound. Although the goal of organized healing is to produce tissue that has the same structure and functions as retained skin, regeneration is uncommon (with some notable exceptions, including early fetal repair) (Onyekwelu et al., 2017). As a result, healing produces a product that is functionally and structurally adequate but not identical. Many growth factors and cytokines generated at the wound site tend to tightly regulate the wound healing processes.

Modifications that conflict with Recovery is delayed and tissue damage is increased by controlled, timely healing processes (Pg et al., 2001).

2. **Material and Methods**

PLANT PROFILE :

Taxonomic classification.

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: Cnicus

Species: Cnicus benedict

Binomial name :Cnicusbenedictus

Synonyms :Carduusbenedictus (L.) Thell. andCirsiumpugnaxSommier& Levier

Biological Description:

Thistle height ranges from 30 to 50 cm. The stems are pubescent, villous, thistle-like, highly branched, and sticky. The leaves are approximately reticulate, thorny-dentate, oblong, and emarginated to pinnatifid. Fruit and Flower: The single flower on the tops of the twigs is sessile and has a pale yellow composite bloom. There are tubular florets (Rea et al., 2009). In comparison to the many androgynous florets, the few sterile lateral florets have three-part margins and are smaller. There is an ovate epicalyx. A long, stiff, and pinnatifid thorn marks the end of the inner bracts. Upon termination, the outer bracts form a single thorn. They are wide, leafy, and have many web-like hairs that attach them to the epicalyx's cordate-oblong leaflets. There's a tuft of hair on the fruit (Reddy et al., 2020).

Traditional uses : The leaves, stems, and blossoms of blessed or holy thistle have been used to improve bile secretion, strengthen the liver, reduce jaundice, lessen flatulence, and facilitate digestion (Richardson et al., 2013). Over the years, it was employed for this purpose in both Indian Ayurvedic treatment and traditional herbalism in Europe. Because of its emmenagogue function, it was also utilized to maintain and regulate the female reproductive system (Schultz et al., 1999). Additionally, it was utilized as a galactagogue to improve and enhance the flow of milk in nursing women. Blessed thistle flowers, stems, and leaves have long been used to "bitter" tonic cocktails and other oral treatments meant to improve digestion and appetite. Aside from its traditional uses as a general tonic, it has also been used historically as a diuretic, diaphoretic, emmenagogue, contraceptive, and antipyretic (Seifert, et al., 2012). Currently, its primary usage is as a bitter tonic for indigestion, flatulence, and dyspepsia; some herbalists also suggest it as a vulnerary, galactagogue, astringent (for bleeding or diarrhea), and a dysmenorrheal cure (Shakya et al., 2012).

Chemical constituents: Sesquiterpene lactone glycosides such as cnicin (0.2–0.7%), polyacetylen, and absinthin, as well as salonitenolide and artemisiifolin (Vachhrajani et al., 2020) , were among the chemical components of Cnicus Benedictus. Triterpenoids include multiflorenol acetate, a-amyrin acetate, a-amyrine, and aamyrenone. Linguan substances include arctigenin, nortracheloside, and trachelogenin. Flavonoids, such as astragalin, luteolin, and apigenin-7-O-glucoside (Velnar et al., 2009). The amount of tannins reached was 8%. n-nonane, n-undecane, n-tridecane, dodeca-l,ll-dien-3,5,7,9-tetrain (polyyne), p-cymene, fenchon, citral, and cinnamaldehyde are among the volatile and essential oils (0.3%) (Rajakumari et al., 2020). Benignans such cnicin and trachelogenin are responsible for the bitter properties of blessed thistle. In addition, the plant included triterpenes, coumarins, starch, glycosides, alkaloids, and phenolic chemicals. In central Europe, blessed thistle (Cnicusbenedictus L.) was used as a substitute oil crop (Tuhin et al., 2017). Early seeding produces fruit yields of about 2.0–2.5 t/ha and oil yields of 500–700 kg/ha. Linoleic acid (>70% of total fatty acids) and α-tocopherol (>90% of total tocopherols) can be found in good amounts in Cnicus Benedictus oil (Sankar et al., 2015). The oil seems appealing for use in technical applications (like coating agents) and cosmetics. It's

possible that Cnicus Benedictus oil is appropriate for human nutrition. Furthermore, arctiin, a lignan glycoside, can be obtained from Cnicus Benedictus oil cake (Dwivedi et al., 2017). Journal of Pharmaceutical and Chemical Research 131 Stosteryl-3 β-D-glucoside, salonitenolide, multiflorenolacetat, multiflorenol, αamyrenone, α-amyrine acetate, and αamyrine were obtained from the herba of Cnicus Benedictus by light petrol and chloroform extracts (Yao et al., 2017).

Pharmacological Effects: Reduced Inflammation Cicine was tested in a panel of cellular assays to see whether it inhibited nuclear factor κB (NF-κB), inducible nitric oxide synthase (iNOS), and reactive oxygen species in order to determine its anti-inflammatory activity. Cnicin demonstrated iNOS activity suppression and NF-κB inhibition with IC_{50} values of 1.8 and 6.5 μM, respectively (Bardaa et al., 2016).

Wound healing

50g of Vaseline and 10g of plant root powder were combined to create a lotion that was applied to rats' wounds. Rats' dorsal skin was shaved, and a 1 cm2 incision was formed in that location. The effect of Cnicus Benedictus root ointment on wound healing was monitored on a regular basis (Benkhaled et al., 2020). The findings demonstrate that when rats were treated with the test product extracted from the powdered root, their wounds completely healed within fourteen days, but the ointment control group (Baneocin®) showed a longer healing period (Sarhan et al., 2016). On the other hand, the Cnicus Benedictus cream shown a strong activity when compared to the reference product when comparing the plant powder's activity on rats, with a 98.81% reduction in wounds at day 14 compared to those treated by Ointment (Baneocin®) (95.69%) (Nicolaus et al., 2017).

Table 1: list of reagent and chemicals

S.No.	Reagents and chemicals	Company Name
1	Glacial Acetic Acid	Merck
2	Petroleum ether	Research lab
3	Conc. H2SO4	Fizmerck
4	Ethanol	Molychem
5	Nitroprusside	Merck
6	Sodium Hydroxide	Merck
7	Ammonia	Merck
8	95% Alcohol	Clorofiltind
9	Conc. HCl	Clorofiltind
10	Magnesium	Himedia
11	Chloroform	Himedia
12	1 % Copper Sulphate Solution	Himedia

2.1. Plant collection

300 grams of the medicinal herb Cnicus Benedictus were gathered. Following cleaning, the plant components (leaves) were dried for three days at room temperature in the shade and for a final three days at 45°C in an oven until completely dry (Nayak et al., 2006). To prevent contamination and deterioration, dried plant components were kept in airtight glass containers in a dry, cool environment (Silambujanaki et al., 2011). A plant taxonomist verified the authenticity of a chosen traditional plant, Cnicus Benedictus, to ensure its identity and purity (Roy et al., 2012).

2.2. Extraction

Plant material will be extracted for the current investigation using the Soxhlet apparatus and a continuous hot percolation process (Nayak et al., 2009). Cnicus Benedictus powder will be added to a thimble of Soxhlet equipment. The translation will be 60°.Peroxide is a non-polar solvent (Brito et al., 2018) . After drying, the exhausted plant material (marc) will be extracted again using an ethanol solvent. For every solvent, the extraction process will be repeated until the siphon tube shows no visible color change, and the lack of any residual solvent upon evaporation will prove the extraction's completion (alshlash et al., 2023). Using a rotating vacuum evaporator of the Buchi type, the obtained extracts will evaporate at 40°C (Eskandarinia et al., 2020). The following formula will be used to weigh the dried extract and calculate the percentage yield for each extract:

$$\%\text{Yield} = \frac{\text{Weight of extract}}{\text{Weight of Plant Material used}} \times 100$$

Prepared extracts will be stored in an airtight container and labeled for future usage after being examined for organoleptic characteristics (percentage yield, color, and odor).

Figure 3: Continuous Hot Extraction

2.3. Phytochemical investigation

2.3.1. A thorough qualitative phytochemical analysis was used to determine if certain phytoconstituents were present in the experiment or not. The precipitate formation or color intensity was used to gauge how the body would react to certain tests. Standard operating protocols were followed.

2.3.2. Test for Carbohydrates

2.3.2.1. **Molisch's Test:** Concentrated sulfuric acid (H2SO4) was applied dropwise along the test tube wall after the aqueous solution of the Cnicus Benedictus extract was reduced to one milliliter and combined with a few drops of the Molish reagent (naphthol). When two liquids combine, a purple color is formed at the intersection. It shows that there are carbohydrates presents.

2.3.2.2. **Fehling's Test:** 2 ml of the Cnicus Benedictus extract aqueous solution were added after equal amounts of Fehling A and Fehling B solutions—one milliliter each—were combined. Boil it in a water bath for 5–10 minutes. Precipitation that is reddish brown in color as a result of cuprous oxide production indicates the presence of decreasing sugar.

2.3.2.3. **Benedict's Test** A test tube containing an equal amount of Benedict's reagent and Cnicus

Benedictus extract was heated in a water bath for five to ten minutes. Looks green, yellow, or red, indicating the presence of reducing sugar, depending on the amount of reducing sugar in the test solution**.**

2.3.2.4. **Barfoed's Test:** 1ml of Benedict solution was added to the aqueous solution of Cnicus Benedictus extract and brought to a boil. As a result of the synthesis of cupric oxide in the presence of monosaccharides, red coloration was seen.

2.3.3. Tests for Alkaloids

2.3.3.1. **Dragendorff's Test:** 1 ml of an extract of Cnicus Benedictus was taken. A solution of alcohol, a few drops of acetic acid, and Dragendorf's reagent were combined and thoroughly agitated. The appearance of an orange red precipitate indicates the presence of alkaloids.

2.3.3.2. **Wagner's Test:** Cnicus Benedictus extract was dissolved in 1 ml of acetic acid. There were a few drops of Wagner's reagent added. Alkaloids were present, which explained the reddish-brown precipitate.

2.3.3.3. **Mayer's Test:** A small amount of Mayer's reagent was added to 1 ml of Cnicus Benedictus extract, which was dissolved in acetic acid. The production of a dull white precipitate showed the presence of alkaloids.

2.3.4. **Hager's Test:** The Cnicus Benedictus extract was dissolved in 1-2 milliliters of acetic acid. Three milliliters of Hager's reagent were applied to it, and the presence of alkaloids revealed the creation of a yellow precipitate.

2.3.5. Test for Saponins

2.3.5.1. **Froth Test:** Add 1 ml of Cnicus Benedictus extract to purified water and give it a good shake.Stable foam formation revealed the presence of saponin.

2.3.6. Test for Triterpenoids and Steroids

2.3.6.1. **Libermann-Burchard Test:** In chloroform, the Cnicus Benedictus extract was dissolved. It was cooked on a waterbath, cooled, and then 1 mL each of acetic acid and acetic anhydride were added. Next, a few drops of strong sulfuric acid were applied along the test tube's sides. The emergence of blue green color indicates the presence of steroids.

2.3.6.2. **Salkowski Test:** After dissolving the Cnicus Benedictus extract in chloroform, an equivalent volume of strong sulfuric acid was added. The development of a blue-to-cherry-red color inch chloroform layer and green fluorescence in the acid layer were signs that steroids were present.

2.3.7. Test for Tannin and Phenolic Compounds

- **Ferric Chloride Test:** The Cnicus Benedictus extract was dissolved in a certain amount of distilled water. A few drops of a diluted ferric chloride solution should be added to it. The development of a deep blue color suggested the presence of tannins.
- **Gelatin Test:** In the distilled water, a certain amount of Cnicus Benedictus extract was dissolved. 10 ml of a 1% gelatin solution with 10% sodium chloride were introduced. The formation of white precipitate indicates the presence of phenolic chemicals.
- **Lead Acetate Test:** A test tube was filled with distilled water and a small amount of Cnicus Benedictus extract. A few drops of lead acetate solution were then added to the mixture. White precipitate formation shows the presence of phenolic chemicals.

2.3.8. Test for Flavonoids

- **Shinoda's Test:** 1 ml of Cnicus Benedictus extract in alcohol was mixed with a few magnesium turns and tiny drops of strong hydrochloric acid. On a waterbath, it was heated.The appearance of reddish-pink color when it formed suggested the presence of flavonoids.

2.3.9. Test for Glycosides

- **Borntragers Test:** Three milliliters of the test solution were mixed with diluted sulfuric acid. After boiling it for five minutes, a filtrate was produced. Equal parts of either chloroform or benzene were added to the cold filtrate and shaken well. After the organic solvent layer had been separated, ammonia was added. The creation of a pink to red colored ammonical layer was indicated by the presence of anthraquinone glycosides.
- **Keller Killiani Test:** In a test tube, add two ml of the test solution, three ml of glacial acetic acid, and one drop of 5% ferric chloride. Add cautiously 0.5 ml of sulfuric acid concentrate. The development of a blue color in the acetic acid layer signified the presence of cardiac glycosides.

1.3.10 . Test for fats and oils

- **Solubility test**

➢ To 2-3 ml alcoholic solution of Cnicus Benedictus extract was added, along with a few ml of chloroform, and solubility was noted.

➢ To 2-3 ml of an alcoholic Cnicus Benedictus extract solution. Add a tablespoon of 90% ethanol, and solubility was noted.

2.4. DPPH

The antioxidant activity of *Cnicusbenedictus* extract was determined by using the DPPH free radical scavenging assay. 1mg/ml methanol solution of extracts/standard was prepared.

Different concentration of *Cnicusbenedictus* extracts /standard (20 – 100µg/ml) were prepared from 1mg/mL stock solution and 2mL of 0.1mM solution of DPPH was added. The obtained mixture was vortexed, incubated for 30 min in room temperature in a relatively dark place and then was read using UV spectrophotometer (Shimadzu 1700) at 517 nm. For control, Take 3 ml of 0.1mM DPPH solution and incubated for 30 min at room temperature in dark condition. Absorbance of the control was taken against methanol (as blank) at 517 nm **(**Athavale et al., 2012**).**

Percentage antioxidant activity of sample/standard was calculated by using formula:

%Inhibition=[(Ab of control-Ab of sample)/ Ab of control x100]

2.5. FT-IR

To establish the presence of the functional groups in the isolated fraction (A) of PI methanolic extract, FT-IR spectroscopy was performed using Perkin Spectrum BX spectrophotometer. The sample was dried and ground with KBr pellets and analyzed on Thermo Nicolet model 6700spectrum instrument. A disk of 100 mg of KBr was prepared with a mixture of 2% finely dried sample and then examined under IR-spectrometer. Infrared spectra were recorded in the region of 400-4,000cm^{-1}.

2.6. Acute Dermal Toxicity Study

The acute dermal toxicity test of the crude extract of *Cnicusbenedictus* was carried out as per OECD draft guideline number 404. Three female rats having normal skin surface were randomly selected and maintained in a cage individually and acclimatized to the working environment for a week prior to the commencement of the test. Around 10% of the body surface area fur was shaved from the dorsal area of the trunk 24 hours prior tostudy.10% w/w of the extract formulation was uniformly applied over the shaved area for 24 hours. Rats were housed individually during the exposure period.

The residual test substance was removed at the end of the exposure period and the rats were observed daily for any adverse skin reactions for 14 days (Guidelines for the Testing of Chemicals 2015**).**

2.7. Formulation of Extract Ointment and Simple Ointment

Simple ointment was formulated using white 170 g white soft paraffin, 10 g hard paraffin, 10 g cetostearyl alcohol, and 10 g wool fat**.** All the constituents of the ointment base were mixed and gently heated, stirring until homogeneous and cold ointment was obtained.

The extract ointment was prepared in different concentrations, i.e., in 5% (w/w) ointment (5 g of *Cnicusbenedictus* extract was mixed with 95gof ointment base B.P) and in 10%(w/w) ointment (10 g of *Cnicusbenedictus* extract was added in 90 g of ointment base B.P) (Tredget et al., 1997**)**. To compare wound healing potential of the extract, nitrofurazone ointment (0.2% w/wNF) was used as a standard drug.

2.8. Evaluation parameters

2.9.1.1. Organoleptic parameters:

Organoleptic parameters like color, odor and taste of all the formulations were carried out.

2.9.1.2. Determination of pH:

The pH value of a solution was determined by digital pH meter. The pH meter was operated according the manufacturer's instructions. First the apparatus was calibrated using buffer of 4, 7 and 9 pH. The electrodes were immersed in the solution and the pH was measured.

2.9.1.3. Homogeneity:

All the developed ointments were tested for homogeneity by visual inspection. They were tested for their appearance.

2.10. *In-vivo* wound healing activity Animals Protocol

IAEC Approval: All animal experiments were approved by Institutional Animal Ethics Committee (IAEC).

Animal used

Weight- 200±50 gm

Strain -Wistar rat

Sex -Either

Housing Condition: Animals were housed in a group of six in separate cages under controlled conditions of temperature (22 ± 2°C). All animals were given standard diet (golden feed) and water regularly.

2.11. Wound Healing activity (excision model)

The back of the animals was shaved and sterilized with 70% ethanol before 7 X 7 mm excision wound going to be create by a surgical blade from a predetermined shaved area on the back of each animal. The wound left undressed to the open environment and no local or systemic antimicrobial agents used (Chen et al., 2017**).** This model is used to monitor the rate of wound contraction. A progressive decrease in the wound area was monitored periodically at every 4^{th} day interval. Immediately after injury and on subsequent days, all the ointment was daily applied topically for 21 d or till complete epitheliazation whichever will earlier.

The wound contractions are measured by a tracing paper on the wounded margin and calculated as percentage reduction in wound area. The actual value is converted into percentage value taking the size of the wound at time of wounding as 100 %. The animals were randomly divided into 4 groups and each group containing 6 animals. The treatments of extract ointment (5%and10%) were applied topically once a day.

Group I: Control group.

Group II: Test group treated with extract ointment (5% w/w)

Group III: Test group treated with extract ointment (10%w/w)

Group IV: Nitrofurazone ointment (0.2%w/w) (Reference Standard Marketed Preparation).

Wound contraction rate

The wound contraction rate was measured. It is the percentage reduction of wound size. It can also be treated as a percentage of wound protection. By using a transparency paper and a suitable marker, at a specified interval, the decrease in size of wounds was monitored and accordingly, the percentage of wound closure is accessed, which indicates the formation of fresh epithelial tissue to heal the wound. Wound contraction is expressed as a reduction in the percentage of the original wound size.

$$\% \text{ of wound contraction} = \frac{\text{Initial area of wound on day 0} - \text{Area of wound on N}^{th}\text{ day}}{\text{Wound area on day 0}} \times 100$$

1.12 Antimicrobial Activity (Well Diffusion Assay)

1.12.1 Antibacterial Activity Preparation of Dilutions of the Samples:

The dilutions of the samples were made for the concentration as 100µg/ml, 150µg/ml, 200µg/ml, and 250µg/ml respectively of the sample, after that volume makeup was done with distilled water till 1ml.

Preparation of Nutrient Agar Media

28 g of Nutrient Media was dissolved in 1 liter of distilled water. pH of media was checked before sterilization. Media was sterilized in autoclave at 121 ^{o}C at 15 lbs pressure for 15minutes. Nutrient media was poured into plates and placed in the laminar air flow until the agar was get solidified.

Well Diffusion Assay

Culture of bacterial strains (*E. coli*) was spread on the Nutrient agar media (NAM). The wells were then formed for the inoculation of the samples (extract ointment) given in the different concentrations, volume make-up was done till 1 ml. 100 µl of the sample was loaded. The plates were allowed to incubate at 37 0

C for 48-72 hours for the best results. The bacterial suspension was standardized to10^{8} CFU/ml of bacteria and kept into the shaker. Then, 100µl of the inoculum from the broth (containing 10^{8} CFU/ml) was taken with a micropipette and then transferred to fresh and sterile solidified Agar Media Plate. The agar plate was inoculated by spreading the inoculum with a sterile spreader, over the entire sterile agar surface. Four wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. Each well was filled with different concentration (100µg/ml, 150µg/ml, 200µg/ml and 250µg/ml) of samples. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37o C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of tested compounds. The zone of inhibition (ZOI) was observed and measured in mm. Zones were measured to a nearest millimeter using a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, non-reflecting back ground. The diameters of the zone of complete inhibition (as judge by unaided eye) were measured, including the diameter of the well (Mohammadi et al., 2012).

3. Results and Discussion

3.9. Percentage Yield:

In phytochemical extraction the percentage yield is very crucial in order to determine the standard efficiency of extraction for a specific plant, various sections of the same plant or different solvents used. The yield of extracts received from the *Cnicusbenedictus*is shown in Table: 3.

Table 2: Percentage Yield of crude extracts of *Cnicusbenedictus* extract

S.No	Plant name	Solvent	Theoretical weight	Yield(gm)	%yield
1	*Cnicus benedictus*	Petether	298	1.40	0.46%
2		Methanol	287	6.55	2.28%

Table 3: Phytochemical testing of extract

S.No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	**Methanolic extract**
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer'sreagent test	Absent	Present
1.3	Wagner'sreagent test	Absent	Present
1.3	Hager'sreagent test	Absent	Present
2.	**Glycoside**		
2.1	Borntrager test	Present	Present
2.2	Legal's test	Present	Present
2.3	Killer-Killiani test	Present	Present
3.	**Carbohydrates**		
3.1	Molish's test	Absent	Absent
3.2	Fehling's test	Absent	Absent
3.3	Benedict's test	Absent	Absent
3.4	Barfoed's test	Absent	Absent
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Absent	Absent
5.	**Flavonoids**		
5.1	Alkalinereagent test	Absent	Present
5.2	LeadAcetate test	Absent	Present
6.	**Tannin and Phenolic Compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Present
8.2	Libbermann-Burchard's test	Present	Present

3.10. *Invitro* Antioxidant Assays

In the present investigation, the *in vitro* anti-oxidant activity of extracts of *Cnicusbenedictus was*

Evaluated by DPPH radical scavenging activity.

The results are summarized in Tables

3.10.1. DPPH1, 1-diphenyl-2-picrylhydrazylAssay

Table 4: DPPH radicals scavenging activity of Std. Ascorbic acid

Concentration(µg/ml)	Absorbance	%Inhibition
20	0.482	51.411
40	0.433	56.350
60	0.342	65.524
80	0.283	71.471
100	0.143	85.584
Control	0.992	
IC50		21.53

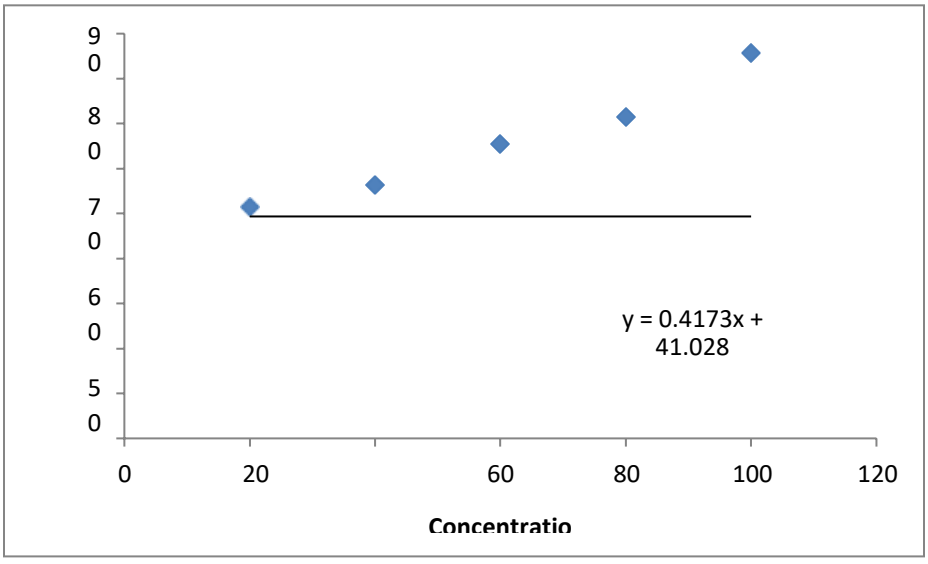


Figure 4: DPPH radical scavenging activity of Std. Ascorbic acid

Table 5: DPPH radical scavenging activity of methanol extract of *Cnicusbenedictus*

Concentration(µg/ml)	**Absorbance**	**%Inhibition**
20	0.518	43.939
40	0.464	49.783
60	0.453	50.974
80	0.412	55.411
100	0.366	60.389
Control	0.924	
IC50		49.27

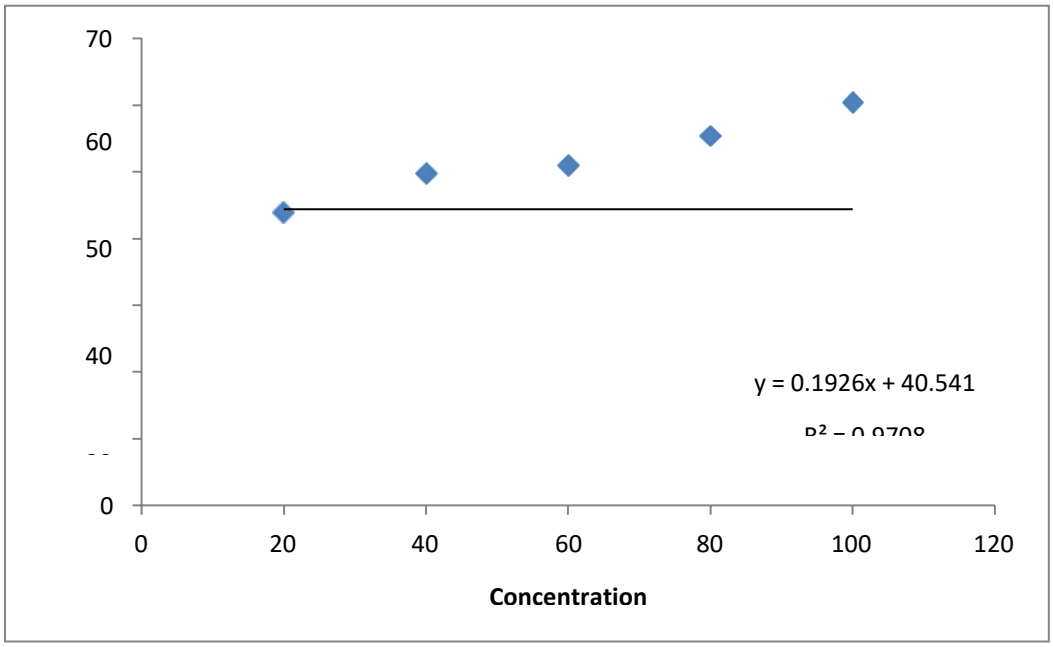


Figure 5: Represents the Percentage Inhibition *Vs* Concentration of extract of *Cnicusbenedictus*

3.11. Functional group identified by FTIR Study

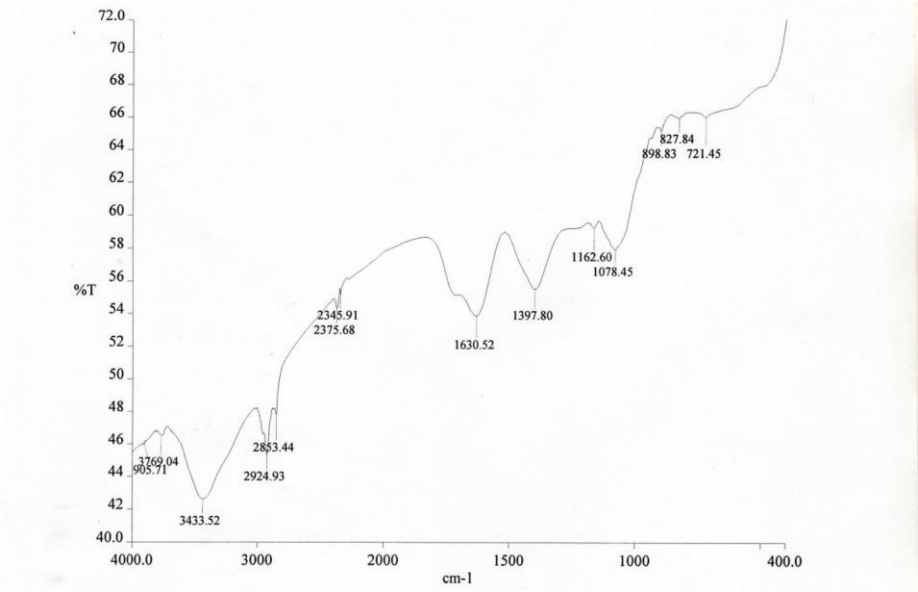


Figure 6: FTIR of *Cnicusbenedictus*

3.1 Evaluation parameter of extract ointment

Table7: Organoleptic properties

S.No	Parameters	Results
1.	Appearance	Oily or greasy consistency
2.	Colour	Slightly yellowish grey ointment
3.	Homogeneity	Absence of aggregates

An evaluation of the extract ointment, including colour, appearance and homogeneity, was conducted. Extract ointment was discovered to have a slightly yellowish grey colour to it when tested. Extract ointment exhibited the same colour, and Appearance as the I.P. requirements for these characteristics and the results were listedinTableNo.7.

3.1.1 Measurement of pH

Table 8: pH

S.No	Formulation	Results
1.	Extractointment(5%w/w)	6.1
2.	Extractointment(10%w/w)	6.3

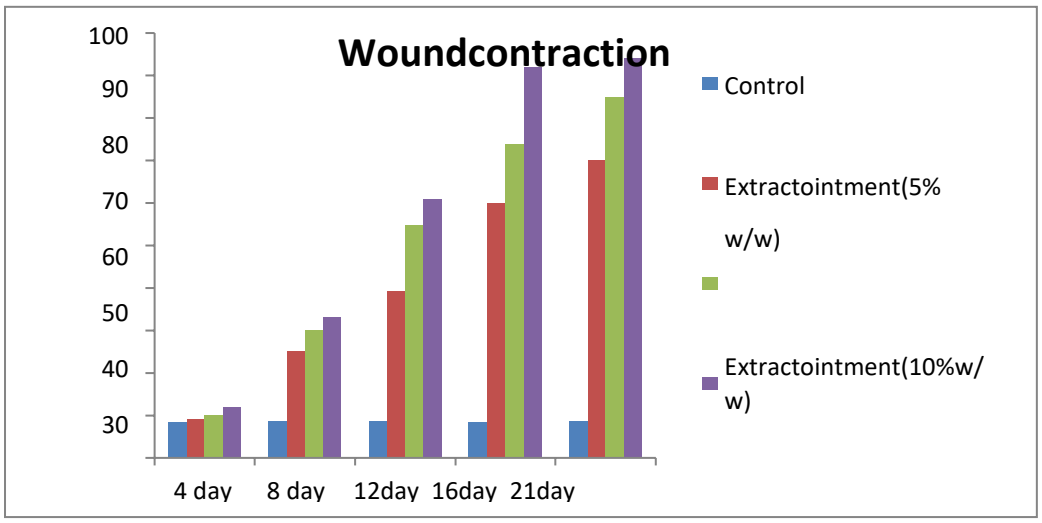
The pH of all prepared formulation rangedfrom6.1-6.3. The pH of the prepared extract ointment formulation wasconsidered to be acceptable to avoid the risk of irritation upon application to the skin. The results were shown in Table No.8.

3.2 Wound contraction studies

Wound contraction is another parameter used to assess wound healing. Significant wound contraction was shown in table.

Table 9: Percentage wound closure in various treatment groups

Sr.No.	Formulation	Area of wound during different days of observation (%)				
		4day	8day	12day	16day	21day
1	Control	8.31±0.7123	8.45±0.8144	8.49±0.7824	8.39±0.8849	8.42±0.9810
2	Extractointment (5%w/w)	8.98±0.4049	25.12±0.4356	39.20±0.7073	59.95±0.5422	69.95±0.5534
3	Extractointment (10%w/w)	10.05±0.7823	29.98±0.5239	54.76±0.5519	73.86±0.5532	84.76±0.5824
4	ReferenceStandard(Nitrofur azoneointment)	11.99±0.7437	33.03±0.7279	60.99±0.6429	91.98±0.6431	93.99±0.2523



Graph 1: Evaluation of wound healing activity

3.3 *In-vitro*antimicrobialactivity

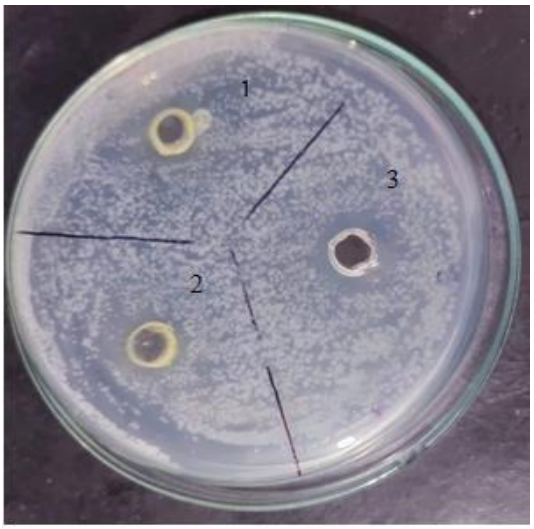


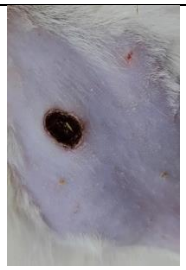
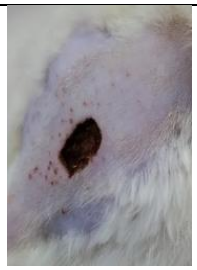
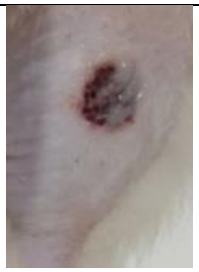
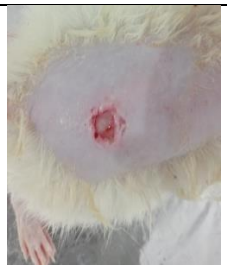
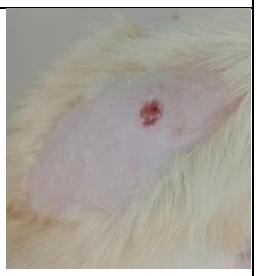
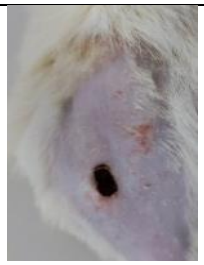
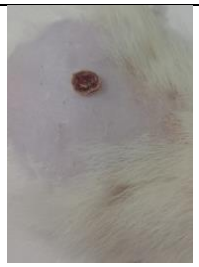
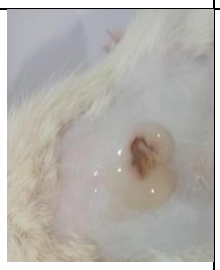
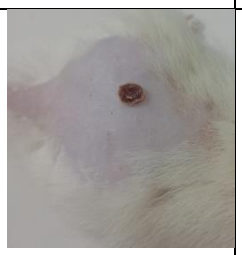
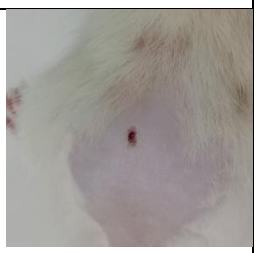
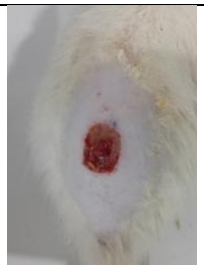
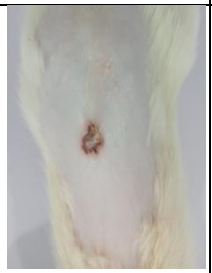
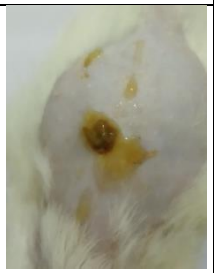
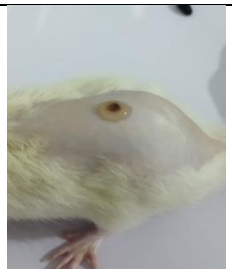
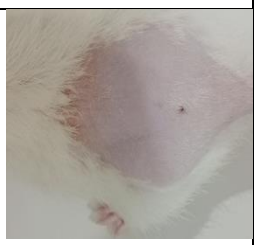
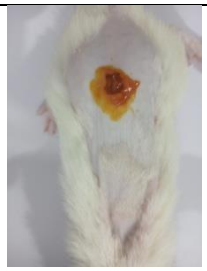
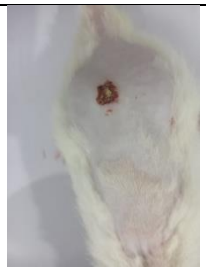
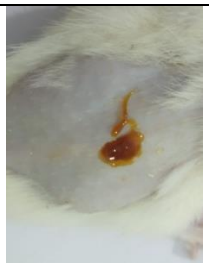
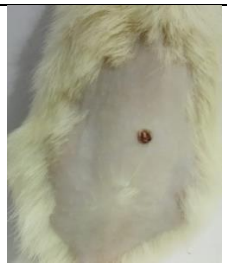
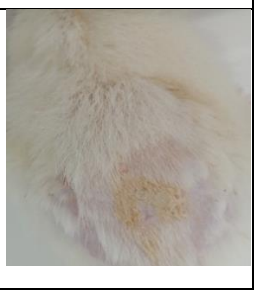
Figure 5: Antimicrobial activity

Table 10: Antimicrobial activity

S.No.	**Sample Name**	**Zone of Inhibition(mm)**
1.	Extract ointment (5%w/w)	7mm
2.	Extract ointment (10%w/w)	12mm
3.	Reference Standard (Nitrofurazone ointment)	16mm

3.4 Images of wound closure

Table 11: Images of wound closure in various treatment groups

Group	4Day	8Day	12Day	16Day	21Day
Control					
Extract ointment (5%w/w)					
Extract ointment (10%w/w)					
Standard(Nitrofurazone ointment)					

3.5 DISCUSSION

Wounds are major case of physical disabilities. A wound which is disturbed state of tissue caused by physical, chemical, microbial (or) immunological insults (or) typically associated with loss function. According to the wound healing society wounds are physical injuries that results in an opening (or) break of the skin that cause disturbance in the normal skin anatomy and function. Wound healing is an interaction of complex cascade of cellular and bio chemical actions healing to the restoration of structural and functional integrity with regain of strength of injured tissues. Involves continuous cell – cell interaction and

cell matrix interactions that allow the process to proceed in different over lapping phases and process including inflammation wound contraction, re-epithelialization tissue, remodeling, and formation of granulation tissue with angiogenesis.

Qualitative phytochemical screening of *Cnicusbenedictus is* showed the presence of active metabolites such as Alkaloids, flavonoids, glycosides, triterpenoids, steroids, tannin and phenols is present. After 24 hours of application of 10% formulation of the extract, there was no dermal toxicity (inflammation, irritation, or redness) observed. There were no also signs and symptoms as well as mortality manifested when the animals were monitored for 48 hours and for 14consecutive days of cage side observation.

The pH of all prepared formulation ranged from 6.1- 6.3. The pH of the prepared ointment formulation was considered to be acceptable to avoid the risk of irritation upon application to the skin.

Further we were performed the wound healing studies like contraction of wound model for 21days. For wound healing activity, the extracts were loaded in the ointment. The development wound curing activity by excision model was evaluated by wound shrinkage of the excision wound of different groups Group I: Control group, Group II: Test group treated with Extract ointment (5% w/w), Group III: Test group treated with Extract ointment (10% w/w), Group IV:Nitrofuraz oneointment (Reference Standard Marketed Preparation).The wound contraction studies revealed that the wound contraction increases on increasing the concentration of herbal extract. The study also reveals that the better activity of polyherbal formulation may be due to the synergistic action of the plant's constituents present in the formulation. Thus, the prepared topical ointment possesses a versatile approach in healing the wound contraction.

3.1 CONCLUSION

Therefore, it is suggested that the wound healing activity of extract of leaf of Cnicus benedictus is related to the presence of flavonoids and glycoside. In conclusion, the results of this study showed that the Cnicus benedictus extract may accelerate wound healing by enhancing epithelization and collagen deposition. The present investigations also offer scientific evidence to the folkloric use of Cnicus benedictus.

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