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Study of the Wound healing effect of *Nerium oleander* and *Ixora coccinea* Leaves extract in albino Wistar rats by Excision Wound Healing Techniques

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Abstract

Nerium oleander and *Ixora coccinea* are useful as medication in many abnormalities and diseases due to consisting of rutin, quercetin, kaempferol, and hesperidin. In this research, 50 percent hyrdo-methanolic extract of oleander leaves (HMEOL) and 50 percent hyrdo-methanolic extract of Ixora coccinea (HMEIL) were used to study the comparative evaluation for the wound healing activities for 16 days by the help of formulated ointments. Healthy Wistar rats were selected for this study. Phytochemical evaluation was helpful for the confirmation of Phytochemical chemical such as, Tannins, alkaloids, carbohydrate, glycosides, flavonoids, phenolic contents & protein. Three ointment such as 5 percent w/w HMEOL, 5 percent w/w HMEIL and 2.5 % w/w HMEOL + 2.5 % HMEIL were formulated for application of drug on animals' skin of three different test groups. Study for Acute dermal toxicity of selected drugs was done by following guidelines acute dermal toxicity study 402. Comparative examination of Wound healing effect of drugs was done on the 0, 4th, 8th and 16th day. Study for histopathological examination of tissues of different animals was done at the end of protocol of research. Formulated ointments of both drugs were found to be similar effective in treatment of open wound individually and their combined formulation found to be more effective as compare to individual.

Key words: HMEOL, HMEIL, wound healing, rutin, quercetin, kaempferol.

Introduction:

Skin: Skin is one of the most major organs of body. It provides support & Protection to other part of the body against outer harmful microorganism. It also acts as barrier to inner environment of body against external environment. Skin is categorized into major three layer; epidermis, dermis and hypodermis (Outer to inner Side).[1]

Skin Wound: This may define as any breakage into skin or unwanted changes in the continuity in homeostasis of skin. Wound by occur due to the reason of burns, surgery, trauma and some other complications. Skin wound may be simple or complicated.[2]

Classification of Skin Wound: On the basis of nature and anatomy of wound, Wound may open or closed.[3] In open wound, injury and bleeding can be see easily with naked eyes but this phenomenon is not seen in close wound because injury is covered with outer layer of skin.[4] Incised wound & Lacerated wound fall under the category of open wound, in another hand closed wound may be categorized into Contusion, Abrasion and Hematoma.[5] based on duration of wound and healing of wound, wound may be acute or chronic. Acute wounds have normal injury and healing occur in some hours to few days but chronic wounds are severe with compare to acute wound and they do not heal in specific duration.[6]

Wound Healing: It is defined as the recovery or reversible process to achieve their natural integrity. Process complied in several stages which are Homeostasis, Inflammation, Proliferation and maturation.[7]

Medication for wound healing: healing in wound achieved by the use of many natural or synthetic drugs. Natural drugs and natural therapy are applied for wound healing since ancient time but human started to prefer synthetic medication in earlier era but they found many complications to them. Due to this reason, people are started to show their faith in herbal drug again.[8-9]

Here In this study *Nerium oleander* and *Ixora coccinea* are taken which are specific plant consisting of chemical consisting chemical constituents such as rutin, quercetin, kaemferol, and hesperidin to show wound healing capability. *Nerium oleander* is commonly known as Rose Bay, Dog Bane and Sweat oleander.It belong to the Apocynaceae family. Another plant, Ixora coccinea (L.) Blume, belong to Rubiaceae family. It knowed to many name like Jungle Geranium, Passionate love, scarlet Ixora, west Indian Jasmine.[10-11]

Material Method:

Requirement of major chemical:

For Extraction methanol and water were taken. Povidone was used as a standard drug for wound healing and Xylocaine has been taken for producing local anesthesia .[12]

Requirement of Apparatus & Instrument :

china dish., Beaker., measuring cylinder, test tube, crucible, petridish, vernier calliper, digital weighing balance., water bath.,funnel, Soxchlet. Apparatus., grinder., dessicator., sieve, conical flask, R.B.flask. and HAV, Disection Kit, muffle furnace, rota-vapor., T.L.C chamber, U.v-cabinet.

METHODS

Authentication Collection and drying of plant material

Both plants with leaves and other parts were collected from Naini and Jhalwa, local area of Prayagraj in January 2023 and their authentication of identities were done by a senior scientist of the Botanical Survey of India, Prayagraj

Physicochemical Study:

Physicochemical parameters such as moisture contents, calculation for tistal ash contents, water soluble ash, acid insoluble ash, alcohol and acid soluble extractive values were studies according standard protocol given in the standard book of pharmacognosy.[13]

Extract preparation:

Leaves of both selected plants were dried properly and converted into coarse powders by grinding then hydro-methanolic extraction were done using a continuous hot extraction method (Soxhlet apparatus) at 50 to 55 deg.C. Percentages yield of both extract were found to 7 % and 8% for HMEOL and HMEIL respectively.[14-15]

HMEOL – Hydro-methanolic extract of oleander leaves, HMEIL- Hydro-methanolic extract of *Ixora* leaves

Phytochemical Study:

Preliminary phytochemical Evaluation:

Preliminary phytochemical Evaluation for different components such as carbohydrates, amino acids, glycosides, alkaloids, proteins, fats, tannins, Flavonoids, and phenolic components present in **HMEOL and HMEIL** were performed by following standard protocol, mentioned in standard books and various research articles.[16]

Fluorescence Analysis:

One by one, Small amount of both extract were taken with few drops of different solvents, named ethanol, H_2SO_4, glacial acetic acid, NH_3 Sol, Conc. HNO_3, solution of NH_3, iodine, methanol, NaOH, ferric chloride and ethanol and they were Evaluated in the UV cabinet by passing UV light with the range of 254nm, 366 nm and 765nm.[17]

Examination with TLC:

Samples were prepared by dissolving both sample extracts in the methanol with a 1 mg/mL ratio. With the ratio of 1:2 (silica & water), the standard slurry of silica gel G was prepared. Thickness of slurry on TLC plates were adjusted to 0.25 mm thicker and sampling were done using microcapillary. Based on various article and books, solvent system of chloroform, methanol and ethyl acetate (With the ratio of 7:3:3) was used for examination. After elution, for clarification of the separated band of component and for calculation of Rf value, TLC plates were examined under the UV chamber by passing 365 nm UV light. Rf values were calculated using standard formula of Rf calculation; Rf= distance travel by solute/Distance travel by solvent.[18-19]

Ointment formulation

Ointments of both sample extracts were formulated by following standard steps of protocol given in British Pharmacopoeia's master guide and by following standard methods of ointment preparation, mentioned in standard research article.[20-21] 5 % wt./wt. ointments of MEOL &MEIL. (with a ratio of sample in whole ointment amount) were prepared by adding sample extracts in the other component of ointments, required for formulation of standard ointments. Other ingredients with their amount such as 10 gm of wool fat, 10 gm of hard paraffin, 170 gm of white soft paraffin and 10 gm of cetostearyl alcohol, were taken and melted on a water bath with continuous stirring. Then mortar pestle was used for providing proper homogenization. Now calculated amount of samples extract were added in other ingredient of ointment with continuous stirring on water bath so that complete and excellent homogenized ointment might be obtained. At the end, ointments were examined for their quality to meet up the standards mentioned in pharmacopeias.

PHARMACOLOGICAL STUDY:

Required Experimental animals

150 to 200 grams of healthy Wistar rats were bought from Chakraborty Enterprises, a registered and authorized seller in Kolkata. Before performing any protocol, animals were kept in quarantine, for 14 days for acclimatization. A standard environment with standard required amenities of food and drink was provided to animals. SIP, Prayagraj was approved to this research protocol.

Study of Oral Acute Toxicity for the MEOL &MEIL:

For the calculation of the effective dose of both plant's extracts individually, the protocol of this study was performed by following OECD Guidelines 402. OECD 402 is based on acute dermal toxicity study and helps to show the short-term result of any chemical applied to the dermal route. Rats were divided into 3 groups for this protocol and On dorsal back region of the animal, marking of one cm^2 patches were done. Then ointments of selected strength (5 percent HMEOL & 5 percent HMEIL and Combination HMEOL & HMEIL ointment) were applied to animals for 24 hours and evaluated out the exposures of patches of skin of rats for effect of drugs. This observation repeated for 72 hrs to examine the any deformities in examinations.[21]

Experimental Design

Grouping of animals:

Purchased Wistar rats were divided into five groups and each group consists of 6 rats with as following;

Group 1: Wound excision + for 16 days, normal saline water given to this group of rats, and no other treatments given to this group. This group is considered as Normal control group.

Group- II : Wound excision + for 16 days 5% Povidone-iodine (Betadine)ointment was applied to this group of rats. This group is considered as standard control group.

Group- III: Wound excision + for 16 days, 5% Ointment containing hydro-methanolic extract of Nerium oleander leaves (**HMEOL**) was applied to this group of rats. This group is considered as **Treatment control group I (test Group 1).**

Group- IV: Wound excision + for 16 days, 5% Ointment containing hydro-methanolic extract of *Ixora coccinea* leaves **(HMEIL)**was applied to this group of rats. This group is considered as **Treatment control group II (test group 2**).

Group V: Wound excision + for 16 days, 5% Ointment containing a combination of HMEOL + HMEIL was applied to this group of rats. This group is considered as **Treatment control group III (test Group 3).**

Wound Excision:

All the selected groups of rats were anesthetized using ketamine through I.P routs. Shaving creams were applied on dorsal back area region and after hair removing, 400 mm square area was marked for excison. Then 2 mm deep excision was made using blades and scissor. At the end, animals were separated in different cages with clean and hygienic conditions. The day of excision is considered as 0 days and start to count the says of protocol of experiments from this same day.[22]

Parameter Evaluation:

Excision Wound Closure:

Following the experimental protocol, 5 % Povidone ointment and ointment of selected extracts with 5 % strength started to be applied on the open wounds of various groups of animals from the same day of excision. Wounds are marked clearly for visualization with naked eyes during ointment application. Recovery of wound excision might be observed with naked eyes on the 0^{th} day, 4^{th} day, 12^{th} day, 16^{th} day.[23]

Epithelization period measurement

Recovery in denuded surface of skin by formation of epithelial skin, is called as epithelization. This process required some days for the healing of open wound sufficiently. Duration of epithelization could be easily seen with naked eye and the total days should notes after final observation.[24]

Histopathological Study

On the last the day of study, the cut-down selected marked skin of animals of selected various groups and granulated tissue (0.5 x 0.5) were separated from those skin for histopathology. Those tissue specimens are stored in formalin solution and sent to an authorized animal laboratory for histopathological study. At the end,

histopathological examination of seleceted specimens were done on 40 x using compound microscope.[25]

RESULTS:

Physico-chemical parameter:

Table: 1: Physico-chemical parameter Examination of HMEOL & HMEIL

S.No.	Factor	(wt/wt percentage)	
		HMEOL	HMEIL
1	foreign impurities.	Nil	Nil
2	Presence of moisture.	6.6	7.0
3	total presence of ash values	4.4	5.8
4	water solubilized ash values.	3.6	4.2
5	acid insolubilized ash values.	3.8	4.6
6	W.S.E.V.	18.2	18.4
7	A.S.E.V.	21.8	21.6

Preliminary Phytochemical examination

Table: 2: Preliminary Phytochemical Examination

S. No.	Test	HMEOL	HMEIL
1	Tannins & phenolic compounds.	+	+
2	Alkaloids.	+	+
3	Steroidal test.	-	-
4	Oils & fats.	-	-
5	Carbohydrates.	+	+
6	**Flavonoids.**	+	+
7	Saponin glycosides.	+	-
8	Anthraquinone Glycosides	+	+
9	Cardiac glycosides	-	-
10	Amino acids & proteins.	+	+

Qualitative study by TLC:

TLC techniques were performed for qualitative investigation of selected sample (HMEOL & HMEIL). Many TLCs were performed on the behalf of previous published research article of Phyto-component for wound healing activities and hit & trial method. Best TLC investigation found with the solvent and their ratio of **Chloroform: Methanol: Ethyl acetate (7:3:3). Final Rf values were obtained to 0.55 for HMEOL and 0.46 for HMEIL.**

Table.3: Rf. values of HMEOL. & HMEIL. using TL.C at 365 nm.

S.N.	Methanolic extract	Mobile phase	Stationary Phase	Rf value
(1)	**HMEOL**	Chloroform+ Methanol+ Ethyl acetate (7:3:3).	Silica Gel G.	**0.55**
(2)	**HMEIL**	Chloroform+ Methanol+ Ethyl	Silica Gel G.	**0.46**

		acetate (7:3:3).		

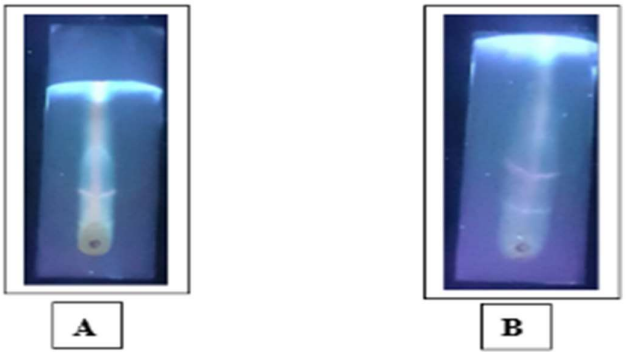

Fig:1. TLC plate for HMEOL (A) & HMEIL (B)

1. Evaluation for ointment:

Three ointments with strength of 5 % were freshly prepared by taking different extract with the first one was 5% w/w HMEOL, another was 5 % HMEIL and third was prepared for their combining effect by addition of both HMEOL & HMEIL

Table 4: parameter and their for quality of ointments:

S. N.	Parameter	5% w/w HMEOL	5% w/w HMEIL	2.5 % w/w HMEOL + 2.5 % w/w HMEIL
(1)	Colour.	Pale greenish white	greenish white	greenish white
(2)	Odour.	none	none	none
(3)	Homogeneity.	good	good	good
(4)	Grittiness	no	no	no
(5)	pH	6.50	6.70	6.60
(6)	Phase separation	No	No	no

Pharmacological Evaluation and Comparison:

Study for acute dermal toxicity:

Study for acute dermal toxicity was done by following the direction, mentioned in OECD 402 guidelines. Selected ointments of extract with strength of 5% w/w HMEOL, 5% w/w HMEIL, 2.5 % w/w HMEOL + 2.5 % w/w HMEIL, applied the dorsal back area of distributed group Wistar rats. After 24 hrs, effects of ointment were examined and found to show favored effect. No anaphylaxis or any other unwanted effect found to observed. Finally, effect were found to be favored, and examined after 72 hrs again.

Comparison and evaluation for wound healing:

Table.5: Comparison and evaluation for wound healing effect 5% w/w HMEOL, 5% w/w HMEIL, 2.5 % w/w HMEOL + 2.5 % w/w HMEIL

Post-wounding days	Control	Standard	Test 1 (HMEOL)	Test 2 (HMEIL)	Test 1 + Test 2 (HMEOL+ HMEIL)
Day-0	382.6±3.20 (0 %)	378.2 ± 2.40 (0%)	384.8±2.50 (0%)	380.6±3.40 (0%)	388.0±3.20 (0%)
Day-4	356.8±4.40 (6.4%)	344.6±2.70 (8.9%)	351.0±3.50 (8.26%)	348.6±3.0 (8.40%)	348.2±4.20 (10.25%)
Day-8	304.0±3.30 (20.54%)	262.4±3.80 (30.6%)	277.3±2.20 (27.9%)	272.3±2.60 (29.20%)	274.3±3.4 (29.3%)

Day-12	234.4±3.60 (38.73%)	178.3±3.4^{z} (52.85%)	188.4±4.2^{C} (51.0%)	179.2±3.30^{C} (52.9%)	181.4±3.3^{C} (53.28%)
Day-16	144.5±2.60 (62.23%)	32.4±2.4^{z} (91.4%)	58.3±2.2^{C} (84.80%)	41.2±3.8^{C} (89.3%)	40.8±4.7^{C} (89.5%)
Epithelization period (day)	33.0±0.8	17.4±0.4^{z}	19.2±0.3^{b}	18.3±0.6^{b}	17.3±0.6^{b}

The data represent as mean ± SD of 6 animals in each group ^{Z}p < 0.0001 compared to Normal control. ^{c}p<0.0001, compared with diabetic control group

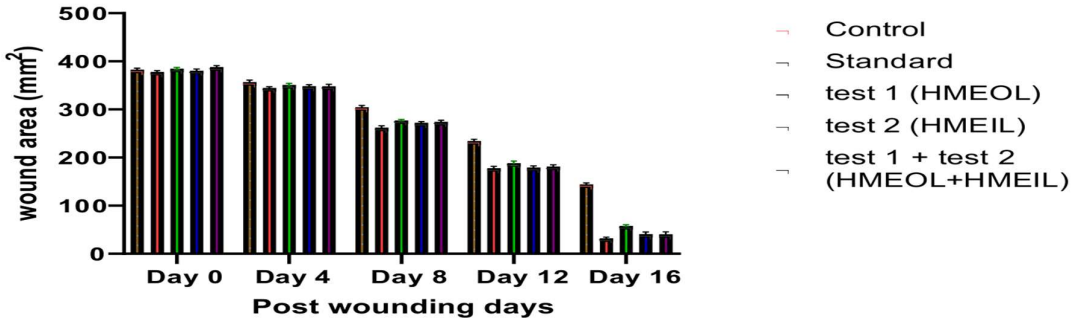

Fig: 2. Graph for wound healing area in mm^{2}

Description:

The outcome data of practical show 5% w/w HMEOL, 5% w/w HMEIL, 2.5 % w/w HMEOL + 2.5 % w/w HMEIL found to effective in wound healing as compare to the normal control group and standard control group. 5% w/w HMEOL ointment and 5% w/w HMEIL found to effective individually as compare to standard and normal control group. Supra-additive effect was observed with combination of ointment (2.5 % w/w HMEOL + 2.5 % w/w HMEIL). Open wound reduced to 58.3±2.2 from 384.8±2.50 for test 1 Group with showing 84.80 % wound healing effect, P^{C} <0.001.Test group 2 Showed 89.3% wound healing activity, Open wound reduced to 41.2±3.8, P^{C} <0.001 and test group 3 found to showed 89.5% recovery, P^{C} <0.001.Epithilzation period for standard group was 17.4±0.4 days and 19.2±0.3 for the treatment group 1; P^{b} <0.001, 18.3±0.6 for the treatment test group 2;P^{b} <0.001 and 17.3±0.6 for the test group 3;P^{b} <0.001.

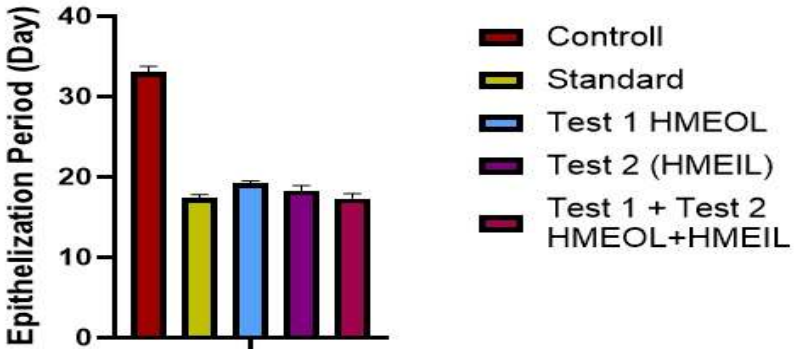

Fig:3. Graph for epithelization period of wound healing

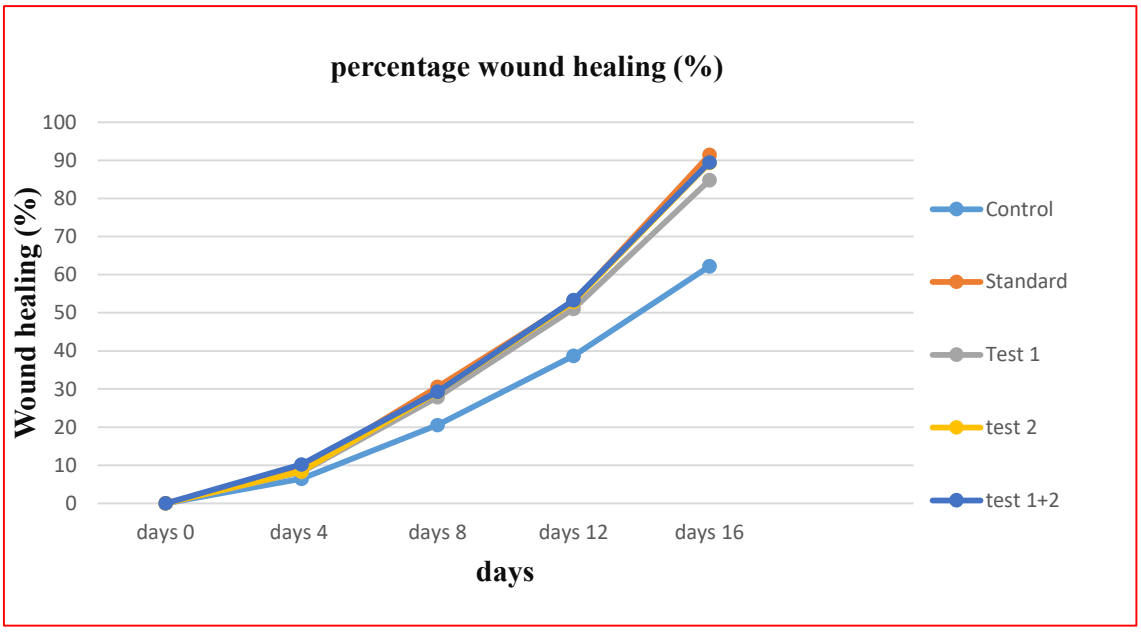

Fig:4. Graph for percentage wound healing

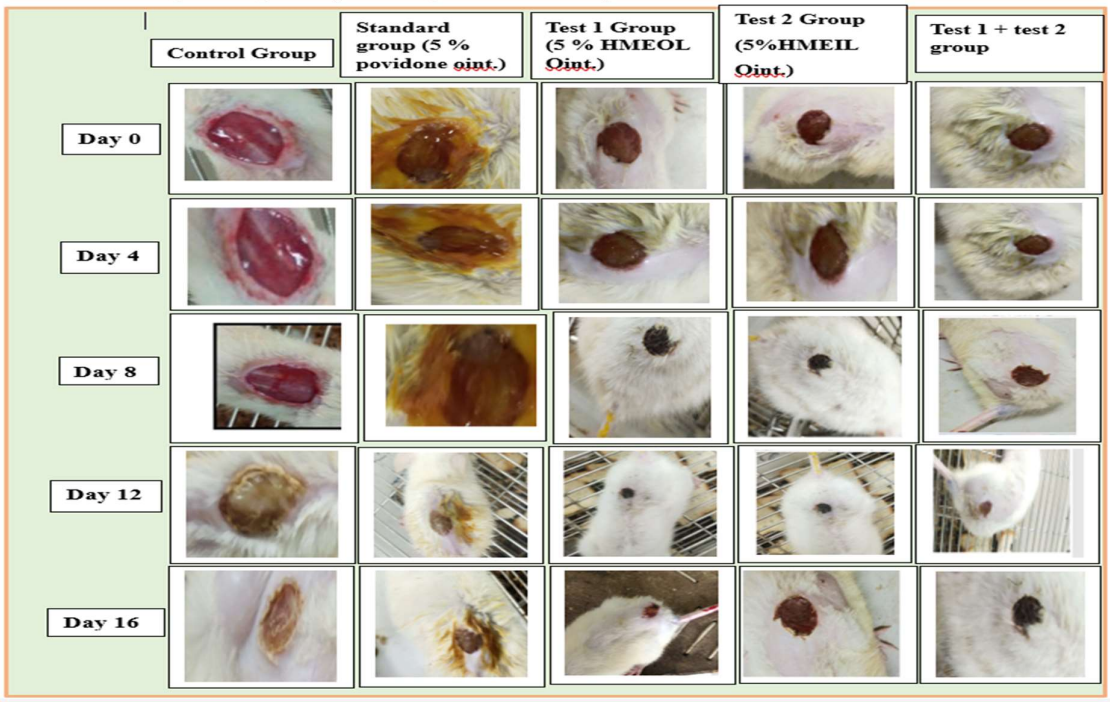

Fig:5. Photo of skin of various groups of animals for wound healing

Histopathological comparison and evaluation:

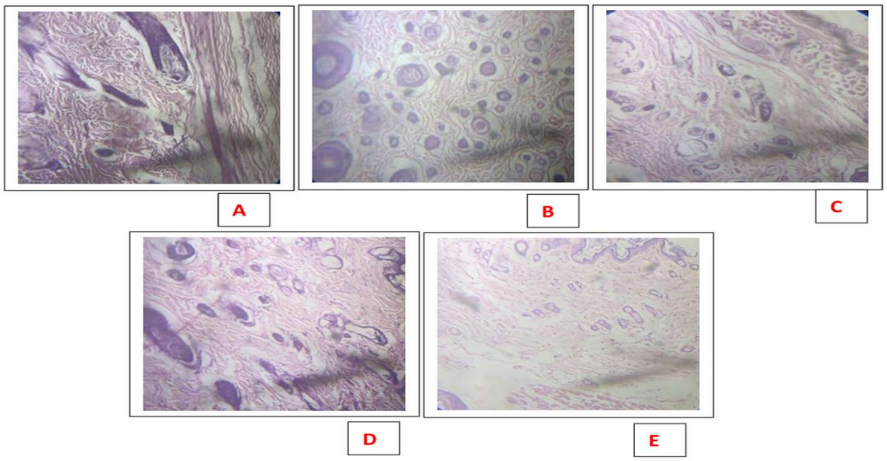

Fig 6. Photo of histopathological report

Description:

The control group showed decreased number of collagen matrix, fibroblast matrix. Number of inflammatory cells increased (A). Standard group showed excellent recovery, and easily could be seen in their histology picture. Density and number of collagen & Fibroblast matrix are increased with compare to control group; pinkish mature granulated tissue found in injured tissues. (B). Treatment group 1 show that epithelial and matrix components proliferate sufficiently as like to standard group. Mass of Collagen tissues and fibroblast increased as like to standard group. (C). For the test group 2, histopathological report found to be similar as test group 1. epithelial and matrix components proliferate sufficiently, mass of Collagen tissues and fibroblast increased and blood vessels appeared to recovered. (D). Histopathology for Test group 3 show significantly recovery in number of epithelial cell and blood vessel structure as similar to standard group. Mass of collagen tissue and fibroblast could be seen sufficiently and more with compare to test 1 and 2. (E)

Discussion:

The wound may be defined as any breakage into skin or unwanted changes in the continuity in skin homeostasis. Wound by occur due to the reason of burns, surgery, trauma and some other complications. Skin wounds may be simple or complicated. Wound is defined as the recovery or reversible process to achieve its natural integrity. Process complied in several stages: Homeostasis, Inflammation, Proliferation and maturation. There are many synthetic and semisynthetic drugs are used for treatment of wounds but they have too many complications so people of new era started to show their faith in natural sources of drugs again.

In this research, comparative study of wound healing was done, with extract of 5% Ointment containing hydro-methanolic extract of Nerium oleander leaves (HMEOL), 5% Ointment containing hydro-methanolic extract of Ixora *coccinea* leaves (HMEIL) and 5% Ointment containing a combination of HMEOL + HMEIL. Flavonoids such as Rutin, quercetin, kaempferol, and hesperidin are major chemical constituents which are presents in leaves of Nerium oleander and *Ixora coccinea*. These extracts are helpful to show wound healing properties. In-vivo wound healing study was done by taking these three ointments and study was done for 16 days.

Positive result in preliminary phytochemical study, Examination of total flavonoid contents and qualitative study with TLC, have confirmed the availability of flavonoids which are responsible to show wound healing effect. OECD Guidelines 402 has been followed for the examination of acute dermal toxicity study and no toxic effect found with 5 % ointments of these plants extract. So, these ointments of selected extract were applied to the various test group of animals. Excision was done on dorsal area of animals and ointments were started applied on those area for 16 days.

Measurements of diameter of post wound healing effect were evaluated from 0 to 16 days and epithelization duration were also studied for final comparison of wound healing effect of extract at the ends. These extracts have properties to reduced open wound and inflammatory conditions. All the three testing groups were compared to show wound healing activities and percentage of their effectiveness were measured. Both extracts have excellent wound healing properties but testing group having treated with combined form of both plants extract (HMEOL + HMEIL) found to show more effectiveness in wound healing activities.

Histopathology study was done to measure the cells of collagen matrix, fibroblast matrix. Number of inflammatory cells. In normal case, number of cells of collagen matrix & fibroblast were shown to decreased and inflammatory cells were increased in open excision wound. The control group showed decreased number of collagen matrix, fibroblast matrix. Number of inflammatory cells increased but Treatment group 1 & 2 show that

epithelial and matrix components proliferate sufficiently as like to standard group. Mass of Collagen tissues and fibroblast increased as like to standard group both testing found to show sufficient recovery as compare to control group. Histopathology for Test group 3 show significantly recovery in number of epithelial cell and blood vessel structure as similar to standard group. Mass of collagen tissue and fibroblast could be seen sufficiently and more with compare to test 1 and 2.

Conclusion:

After examine, overall data and result of comparative study of wound healing effect, it was found that 5% Ointment containing HMEOL and HMEIL have excellent properties to give wound healing properties individually and when combined form of ointments 2.5 % HMEOL + 2.5 % HMEIL was given then it was found to more effective with compared to individual extract. Wound healing diameter, epithelization and histopathology study during research found to give Excellent wound healing effect with both selected extract individually and found more effective when their combined form was given.

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