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WOUND-HEALING PROPERTY OF THE FORMULATED OINTMENT USING EXTRACTED CURCUMIN FROM THE *CURCUMA LONGA* L. (FAM. ZINGIBERACEAE) POWDER AMONG DIABETES-INDUCED SPRAGUE-DAWLEY RATS

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ABSTRACT: Diabetes mellitus can lead to impaired healing of wounds and can cause life-long complications. Curcumin is reported as one of the promising therapeutic agents in the management of impaired wound healing in diabetics. This study aimed to formulate a wound-healing ointment from the extracted curcumin and evaluate its efficacy among diabetes-induced rats. The extract was subjected to organoleptic evaluation, phytochemical screening, pre-formulation studies, and thin layer chromatography. Three trial formulations of ointment were prepared with varying concentrations of the extract. Prior to wound excision, alloxan monohydrate (110 mg/kg) was administered intraperitoneally among thirty male Sprague Dawley rats to induce diabetes. The trial formulations of ointment were then applied in all treatment and control diabetic rat (FBG of > 200 mg/dL) groups for 14 days. The wound-healing activity was assessed through the following parameters: percent wound closure (PWC), period of epithelialization (PE), percentage of scar formed (PSF), and histopathological analysis. Among the trial formulations, 10% extracted curcumin ointment exhibited the highest PWC, fastest rate of wound closure, shortest PE, and comparable histopathological results with the positive control. Statistical analyses ($\alpha \leq 0.05$) further confirmed that 10% extracted curcumin ointment was significantly different from the positive control (Mupirocin) in terms of PE, and the negative control (placebo), in terms of PWC and PE. No significant difference was shown on the PSF of the treatment groups and the positive control. In conclusion, 10% extracted curcumin ointment has the highest wound-healing activity and was selected for the final formulation which passed all quality control parameters.

INTRODUCTION: Diabetes is a global epidemic with a rapidly increasing prevalence in middle- and low-income countries. In 2016, the World Health Organization projected that it will be the 7th leading cause of death in 2030 [4, 5].

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Diabetes mellitus is an increasingly prevalent chronic metabolic disease characterized by prolonged hyperglycemia that leads to long-term health consequences.

The hyperglycemic environment promotes the formation of biofilms and makes diabetic wounds difficult to treat and lead to life-long complications such as limb amputation [6]. To treat these wounds and prevent further infections, antibiotic ointment is topically applied on affected areas. Topical application has many potential advantages over systemic therapy that includes high and sustained

concentrations of drug directly at the infected site, low quantity of antibiotic needed, better compliance, fewer systemic side effects and potentially less chance of antimicrobial resistance [7]. Curcumin or diferuloylmethane is an active pharmacological agent with proven antioxidant, anti-inflammatory, and anti-infective properties [8].

It is extracted from the rhizomes of *Curcuma longa* L., commonly known as turmeric, an aromatic plant from the ginger family (Zingiberaceae) [9]. Studies to date have shown that topical application of curcumin exhibited more pronounced wound-healing effects compared to its oral administration due to the greater accessibility of the drug at the wound site [10].

Various types of topical formulations since then have been developed for curcumin targeting delivery at the wounds such as films, fibres, emulsions, hydrogels, and topical nanoformulations [3, 11].

However, there is still no existing formulation of a topically applied curcumin that has been tested to treat diabetic wounds at the time of this study. Furthermore, there is an upsurge in the use of alternative therapies and natural remedies to treat wounds; also, with the rise of the global threat in health and development, *i.e.* antimicrobial resistance, it could help to limit the misuse and/or overuse of topical antimicrobials. Hence, performing a study regarding an alternative treatment option that would tackle the efficacy of a formulated curcumin ointment for this specific condition may provide better patient outcomes. This study aimed to formulate a wound-healing ointment of the extracted curcumin from *C. longa* L. powder and evaluate its efficacy among diabetes-induced rats. Specifically, this study was conducted to determine the stability and compatibility of the extracted curcumin with the excipients; assess the quality of the formulated ointment in terms of appearance, color, odor, viscosity, spreadability, spatula feel and stability; measure the wound-healing property of the formulated ointment in terms of percent wound closure, period of epithelialization, percentage scar formation and histopathological analysis; and compare its wound-healing property against an antibiotic standard and placebo.

MATERIALS AND METHODS:

Sample:

Collection and Preparation: *Curcuma longa* L. powder and raw plant sample were acquired from Green Spark Organic, an herbal powder manufacturer and distributor in the Philippines. The raw sample was authenticated at the Botany Division of the National Museum, Manila, Philippines. All the laboratory work where conducted at the College of Pharmacy, University of the Philippines Manila.

Extraction: Curcumin was extracted from *C. longa* powder using Soxhlet apparatus. In each set up, 20 grams of *C. longa* powder was placed in a thimble and was heated at reflux with ethanol as solvent. The set up was run for 7 cycles until the yellow color of the extractions faded. The solvent was removed in vacuo using a rotary evaporator.

Organoleptic Evaluation and Phytochemical Screening: Color, odor and pH of extract were evaluated. Phytochemical screening was conducted to test the presence of alkaloids, tannins, glycosides, saponins, flavonoids and carbohydrates in the extract.

Pre-formulation Studies

Stability of Extract and Compatibility Testing: The vials were divided into three groups: (1) without air, without light (NANL); (2) with air, without light (PANL); and (3) without air, with light (NAPL). Three (3) vials per group were placed at 8ºC (refrigerator), 28ºC (room temperature), and 40ºC (oven). Each of the 27 vials contained 100 milligrams of the extract. Compatibility of extract with white petrolatum and beeswax were also tested.

One hundred milligrams of the extract triturated with an equal amount of excipient was placed in 27 vials. The vials were divided into three groups and subjected to different temperatures as done in stability of extract testing. The vials were observed for signs of instability, such as changes in color, growth of mold among others, every week for four consecutive weeks. At the end of the fourth week, TLC analysis was conducted for each vial and the chromatograms were compared with the TLC profile of the original extract.

Thin Layer Chromatography: Ten microliters of standard, extract, and extract-excipient mixture were spotted on to TLC Silica gel 60 F254 plates. The plates were developed in an optimized solvent system containing Dichloromethane and Methanol (99:1). Detection of bands was done by using ultraviolet light at 254 nm and 366 nm.

Trial Formulation:

Excipients: The white petrolatum comprised of up to 100%, oleaginous ointment base, inherently stable with few incompatibilities, generally considered to be a non-irritant and nontoxic material while beeswax was made up of 5–20%, stiffening agent, incompatible with oxidizing agents, generally regarded as an essentially nontoxic and non-irritant material [11].

Formulation: Three trial formulations of ointment were prepared and were based on the concentration of the extract: 1, 5, and 10 (in % gram extract per gram ointment base).

Method of Preparation: On a water bath, white petrolatum and beeswax were melted separately in porcelain dishes until completely melted (61-65ºC). The excipients were mixed at constant temperature and the mixture was allowed to cool while continuously stirred until it congealed. The extract was triturated with the ointment base through geometric dilution until homogenous.

Wound Healing Assay:

Animal Model: Thirty male Sprague Dawley rats, weighing 110–200 grams, 8 to 10 weeks old, were used as animal models for this study. The rats were obtained from the Food and Drug Administration, which is situated in Civic Drive, Filinvest Corporate City, Alabang, Muntinlupa City, Philippines.

The rats were kept separately in polypropylene plastic cages for the whole study period and were acclimatized at a temperature and humidity of 22°C ± 2°C and 50–60%, in a 12-h-light/dark cycle environment for one week prior to diabetes induction. All the rats were fed with standard rat chow (B-Meg Integra 3000) and water *ad libitum*. The study was approved by the university's Institutional Animal Care and Use Committee (IACUC), prior to animal experimentation.

Induction of Diabetes: After a 16-hour fast, the rats were weighed using a calibrated balance. Prior to injection of alloxan monohydrate, baseline blood glucose levels of each rat were obtained. Alloxan monohydrate (Sigma-Aldrich) was dissolved in normal saline to a 10% w/v concentration. The prepared solution was dissolved and kept cool with the aid of an ice bath. A single intraperitoneal dose of alloxan monohydrate (110 mg/kg) in normal saline was used to chemically induce diabetes among all the male Sprague-Dawley rats. Seventy-two hours after alloxan injection, blood samples were withdrawn from each rat's tail vein. The blood glucose levels were measured by Advan BG-101 glucometer and readings of fasting blood glucose (FBG) levels had exceeded 200 mg/dL which confirmed that the rats were diabetic. Hence, the diabetic state of the rats were again confirmed with FBG measurement repeated on the day of euthanasia.

Creation of Excision: On the day of DM confirmation, diabetic rats were anesthetized by a single intramuscular injection of Zoletil (tiletamine + zolazepam) at a dose of 0.10 mL/kg body weight. Each rat was subjected to a prone position in a clean cloth, then the dorsal hair was trimmed with an electric clipper. The dorsum of all rats was rinsed with 10% povidone-iodine solution. One full thickness of wound excision was created on the dorsum of each rat with an approximate area ranging from 10 mm x 10 mm to 20 mm x 20 mm, and a depth of approximately 2 mm. Each rat was placed on a 37 °C heating pad to minimize hypothermia and aid in easy recovery from anesthesia, then the respective ointment concentrations (1%, 5%, and 10%), positive control (Mupirocin) and negative control (placebo) were applied on the wounds. Sterile gauze was used to cover rat wounds. After full recovery, rats were placed in individual cages. The sterile gauze was removed on Day 1 of wound observation.

Treatment of wounds and Animal House Management: Ointment application in all treatment and control groups were applied once daily for 14 days. Standardized amount of ointment was measured, approximately equivalent to 0.5 gram, and spread evenly onto the wound area of each rat throughout the study period.

Treatment group ointment formulations were composed of 1%, 5%, and 10% extract. Mupirocin (Microscot; 2% w/w) was set as positive control while placebo, composed of white petrolatum and beeswax, was set as negative control. Animal house was maintained to have temperature and humidity of 22°C ± 2°C and 50–60%. Rat cages were cleaned daily, and autoclaved wood shavings were replaced in each cage every day for 14 days.

Determination of the wound-healing Activity of Ointment:

Determination of Percent wound Closure: Photographs of the excisional wound areas were documented using an iPhone 6 camera fixed with a 15-cm ruler attached perpendicularly to one end of the fixture. The fixture enabled standardized photography, which made it suitable for pictures to be taken vertically from a 15-cm constant distance in every wound measurement on days 0, 3, 6, 9, 12, and 14. The areas of wounds in each group were analyzed using Image J software. Wound closure was expressed as percentage closure of the original wound and was calculated using the formula [12] as follows:

% Wound closure = (Area of Original Wound (0) -Area of the Remaining (n) / (Area of Original Wound (0) × 100

Determination of the Period of Epithelialisation: Epithelialization period was monitored by noting the number of days required for eschar to fall away, leaving no raw wound behind [13].

Determination of the Percentage of Scar Formed: Wounds sites were monitored visually for scar formation, redness, and hyperpigmentation all over the 14-day study period. The area of scar was measured using an iPhone 6 camera fixed with a 15-cm ruler attached perpendicularly to one end of the fixture and was analyzed using Image J software. The percentage of scar was determined by the following formula:

% Scar formed = (Area of scar) / (Wound area at Day 0) x 100

Histopathological Analysis: On the 14th day, the rats were euthanized by cervical dislocation. Three representative samples were taken from each of the five groups (3 treatment groups, 1 positive control and 1 negative control). Skin tissue samples, cut along with a 10 mm perimeter of surrounding unwounded skin, were collected for histopathological analysis. The formalin-fixed skin tissues were processed by the Department of Pathology and Research of the University of the Philippines, College of Medicine. The slides were stained with hematoxylin and eosin (H&E). Photomicrographs were taken with histopathologic microscope visualizer. Each slide was scored in a blind fashion by a pathologist using modified 0-to-4 Ehrlich and Hunt numerical scale, assisted by two researchers. The parameters evaluated were inflammatory cell infiltration, neovascularization, fibroblast ingrowth, and collagen deposition [14]. Each parameter was individually assessed by the histological grading scale assigned using the modified 0-to-4 Ehrlich and Hunt numerical scale.

Statistical Analysis: The results for the percent wound closure were analyzed using one-way analysis of variance (ANOVA). Shapiro-Wilk Test was used to determine the normal distribution of data. Bartlett's Test was used to determine the homogeneity of variances. After performing one-way ANOVA, Scheffe Test was used to determine which among the groups were significantly different from each other. On the other hand, Kruskal-Wallis Test was used to analyze the results for the period of epithelialization and percentage of scar formed. Dunn Test was then performed to determine which groups were significantly different from each other. The statistical softwares used to analyze the results were Stata/IC version 13 and IBM SPSS Statistics version 23.

Final Formulation and Quality Control of Ointment: The final formulation, which was considered as the prototype formula and prepared in larger batch size, was based on the concentration of ointment with the greatest significant wound-healing activity. The finished product was evaluated based on the following quality control parameters: appearance, color, odor, viscosity, spreadability, spatula feel and stability at 28ºC and 40ºC for three weeks.

RESULTS AND DISCUSSION: A total amount of 1,094 grams *Curcuma longa* L. powder was subjected to Soxhlet extraction which yielded 149. 8878 grams of extracted curcumin, which then represented 13.70%. The extract was dark yellow orange in color with distinct but milder scent to raw

Curcuma longa L. rhizome. The extract's pH was measured 6, which is favorable since degradation of curcumin in acidic condition is slower by 20%. Phytochemical screening confirmed the presence of alkaloids, cardiac glycosides, saponins, and carbohydrates. However, the test did not confirm the presence of tannins and flavonoids. Turmeric rhizome contains two main classes of pharmacologically active secondary metabolites: curcuminoids and essential oil. Curcuminoids (curcumin, demethoxycurcumin and bis-demethoxycurcumin) are most responsible for the biological activity of turmeric [15].

The extract showed no significant change in color and no mold formation after having been subjected to the different conditions: no air, no light; presence of air, no light; no air, presence of light (NANL, PANL, NAPL) and temperatures (8°C, 28°C, 40°C) for four consecutive weeks. Each extract-white petrolatum and extract-beeswax mixture also showed no significant change in color and no mold formation after being subjected to the different conditions (NANL, PANL, NAPL) and temperatures (8°C, 28°C, 40°C) for four weeks. This is one indication that white petrolatum and beeswax are compatible with the extract. Hence, to further assess the stability of extract in air, light, and temperature conditions, Rf values of the extract were compared to the standard. The Rf values of the extract-excipient mixture, was compared to the original extract to evaluate compatibility.

The standard used in the TLC analysis developed three yellow-orange spots visible under 254 nm with R_f values of 0.08, 0.16, and 0.32. An additional blue spot with R_f value 0.69 is visible when viewed under 366 nm. Factors identified as possible causes of the change in R_f values include plate activation time, overloading of sample, unsaturation of chamber, and variable technique of analyst in spotting. The extract subjected to various air, light, and temperature conditions developed similar yellow-oranges spots with similar Rf values to that of the standard. The extract-white petrolatum mixture also developed yellow orange spots like the reference extract with R_f values of 0.06, 0.15, and 0.32. However, R_f value of first spot in the three trials of NANL at oven changed from 0.08 to 0.09. The extract-beeswax mixture also developed yellow orange spots.

All spots of NAPL, NANL, and PANL trials under refrigerated condition have R_f values of 0.08, 0.13, and 0.30 which is similar to the reference extract. All spots of extract-beeswax mixture under room temperature have R_f values similar to the reference extract. However, the fourth spot of NAPL-2 and NAPL-3 changed from 0.70 to 0.80 and 0.60 respectively. All spots of extract-beeswax mixture kept in oven has Rf values of 0.09, 0.19, 0.34, and 0.70 which is like the reference extract. However, Rf values of the 4^{th} spot of NAPL trials 1,2,3, NANL trial 2, and PANL trial 2 changed from 0.70 to 0.67. The researchers used the R_f values of the spots of standard and extract in each plate as reference for that specific plate. Hence, it was concluded that the extracts and extract-excipient mixtures subjected to different air, light, and temperature conditions were stable and compatible.

The ointments were prepared using fusion method. A small-scale batch, composed of five 18-gram packaged extracted curcumin ointments of each formulation, was produced for the wound healing assay. The color of the formulation varies in lightness and darkness depending on the concentration of the extract used.

The marvelous healing power of curcumin is long known and its role in the improvement of several types of topical skin wounds is due to its antioxidant activity, improvement of the production of granulation tissue and new vascularization and increasing the process of reepithelialization of wound damage, but unfortunately all of those useful applications are limited by its poor oral bioavailability, low water solubility and rapid metabolism. Tailoring the suitable topical drug delivery systems for carrying curcumin improves its pharmaceutical and pharmacological effects [16, 17].

In the induction of diabetes, substantial measures like testing the baseline FBG levels of each rat was implemented to assure that hyperglycemic rats would not be subjected to alloxan injection which would more likely increase morbidity, leading to mortality of rats. Prior to alloxan injection, equipment for weight measurement was an optimized, calibrated balance since a rat's body weight is critical to the dose of alloxan to be administered.

Freshly prepared alloxan monohydrate dissolved in normal saline was prepared on an ice bath to promote dissolution and covered with aluminum foil to prevent alloxan degradation. Based on each rat's body weight, a dose of 110 mg/kg alloxan monohydrate was injected intraperitoneally. The assessment of the wound-healing activity of the different ointments to the treatment and control groups were examined with qualitative and quantitative parameters namely percent wound closure (PWC), period of epithelialization (PE), percentage of scar formed (PSF), and histopathological analysis [18]. For PWC, **Table 1**, Percent Wound Closure (PWC) of Rat in Treatment and Control Groups, summarizes the mean PWC in each of the treatment and control groups while **Table 2**, Rate of Wound Closure in Treatment and Control Groups, summarizes the rate of wound closure among the treatment and control groups.

TABLE 1: PERCENT WOUND CLOSURE (PWC) OF RAT IN TREATMENT AND CONTROL GROUPS

Treatment	Percent Wound Closure				
	3rd day	6th day	9th day	12th day	14th day
1% Ointment	25.654± 18.332	38.253± 13.597	69.660± 8.001	81.119± 9.155	98.967± 1.547
5% Ointment	21.165± 9.168	35.583± 6.211	62.711± 14.826	76.939± 12.019	98.955± 2.336
10% Ointment	20.528± 18.744	37.215± 10.170	73.615± 9.721	84.202± 7.014	99.439± 1.255
Positive control (Mupirocin-Microsccot:2%w/w	18.685± 10.646	29.035± 19.578	62.249± 23.628	78.236± 22.662	88.755± 13.803
Negative control (placebo)	9.339± 5.093	16.428± 12.824	25.477± 10.870	40.138± 12.238	50.763± 12.893

TABLE 2: RATE OF WOUND CLOSURE IN TREATMENT AND CONTROL GROUPS

Treatment	Rate of Wound Closure (PWC/hour)
1% Ointment	0.2814
5% Ointment	0.2913
10% Ointment	0.3056
Positive Control	0.2827
Negative Control	0.1572

All the treatment groups exhibited faster rates of wound closure than the negative control. However, only the 5% and 10% extracted curcumin ointment exhibited faster rates of wound closure than the positive control. Furthermore, the 10% extracted curcumin ointment showed the highest PWC among the treatment groups. Shapiro-Wilk test confirmed that the data among the treatment and control groups were normally distributed.

Barttlet's also confirmed that the data at days 3, 6, 9 and 12 showed homogeneity in variances. One-way ANOVA determined that there was no significant difference among the groups at days 3 and 6, while the groups at days 9 and 12 had significant difference among each other. Scheffe test then confirmed that the treatment groups and the positive control significantly differed from the negative control at days 9 and 12. On the other hand, Kruskal-Wallis test determined that there is significant difference among the groups at day 14. Dunn test then confirmed that the treatment groups were significantly different from the negative control. For PE, **Table 3**, Period of Epithelialization of Rat in Treatment and Control Groups, summarizes the mean PEs of the treatment and control groups. The treatment group that showed the shortest PE was the 10% ointment.

TABLE 3: PERIOD OF EPITHELIALIZATION OF RAT IN TREATMENT AND CONTROL GROUPS

Treatment	Period of Epithelialization (Days)
1% Ointment	8.6 ±0.548
5% Ointment	8.2 ±0.837
10% Ointment	7.6 ±0.548
Positive Control	11.8 ±2.049
Negative Control	11.0 ±1.000

Shapiro-Wilk test confirmed that the data among the treatment and control groups were normally distributed. However, Barttlet's test confirmed that the variances of the groups under PE were heterogenous. Kruskal-Wallis test determined that there was significant difference among the groups under PE. Dunn test then confirmed that 10% extracted curcumin ointment significantly differed from the positive and negative control. The 10% extracted curcumin ointment showed the fastest PE among the groups which was consistent with the results of PWC and rate of wound closure and this indicates that a fast rate of wound closure would

result to a shorter PE. All the treatment groups showed lower mean PSFs than the positive control, while they showed higher mean PSFs than the negative control. This shows that the scars that were formed among the treatment groups were smaller than those among the positive control. Shapiro-Wilk test confirmed that the data among the treatment and control groups were normally distributed. However, Barttlet's test confirmed that the variances of the groups under PSF were heterogenous. Kruskal-Wallis test determined that there was significant difference among the groups under PSF. Dunn test then confirmed that the positive control significantly differed from the negative control.

Shown in **Fig. 1** are photomicrographs of representative slides for each treatment and control group under scanner objective were compared with normal skin, and the gross examination of wound from each group at Day 14 in **Fig. 2.**

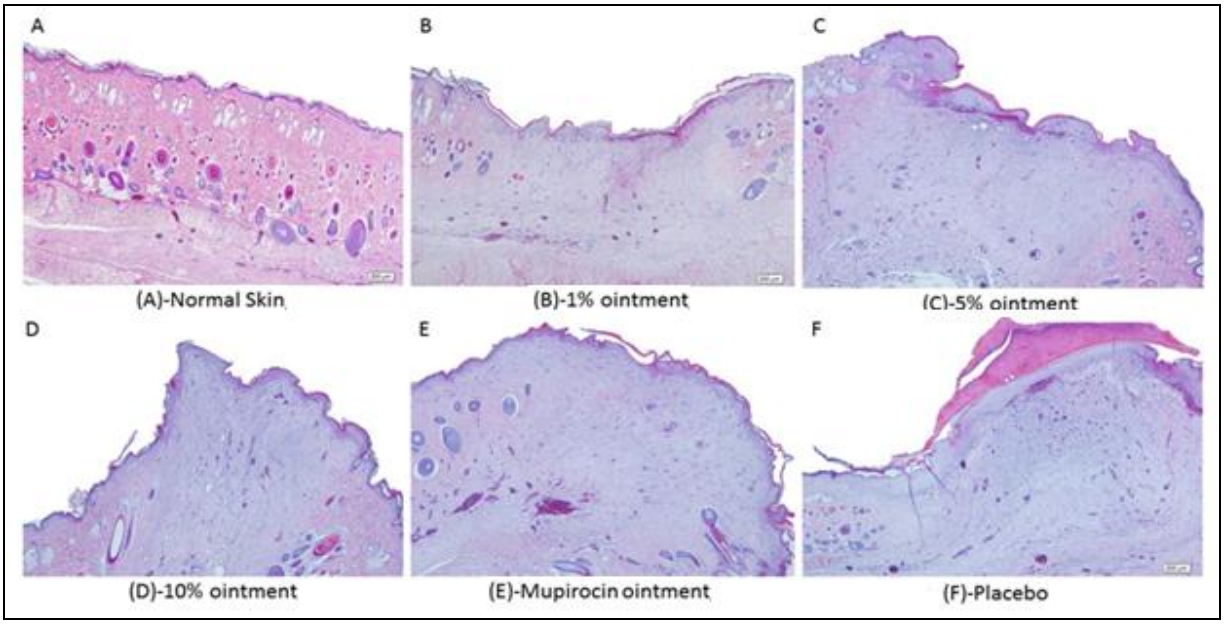

FIG. 1: PHOTOMICROGRAPHS TAKEN UNDER SCANNER OBJECTIVE

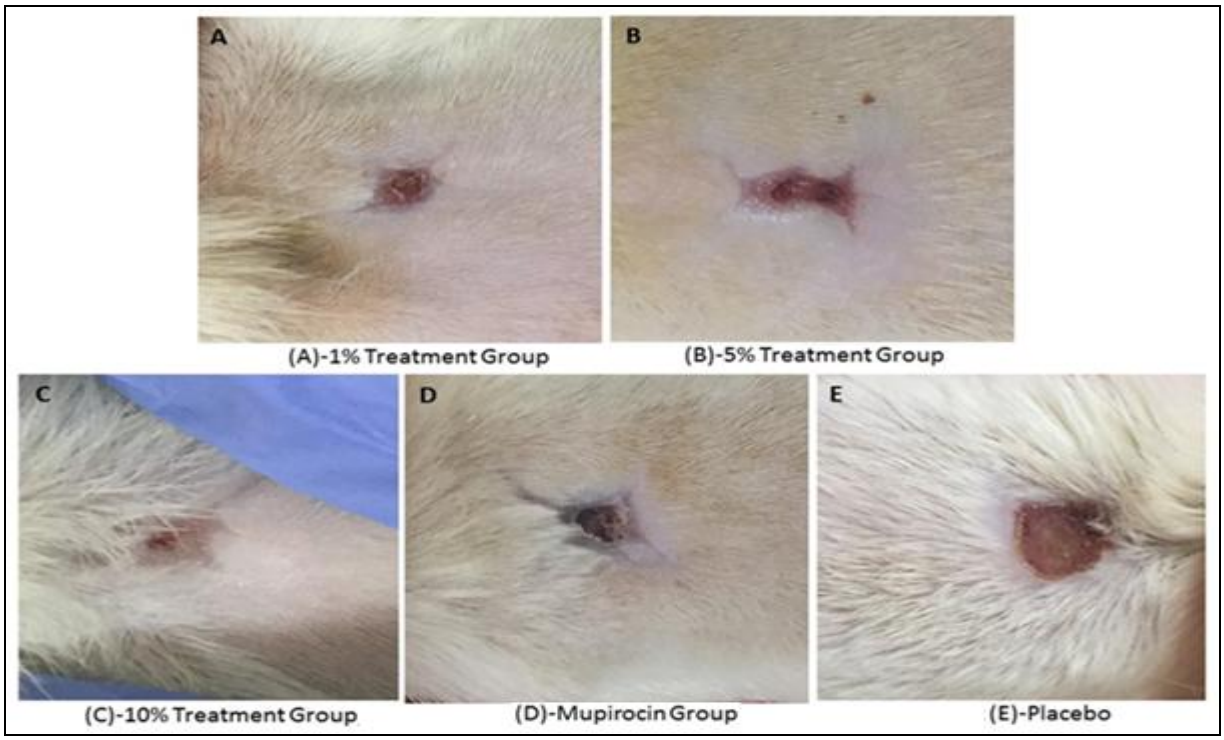

FIG. 2: GROSS EXAMINATION OF WOUND AT DAY 14 FROM REPRESENTATIVE GROUPS

The 10% extracted curcumin ointment treatment group produced comparable results with positive control (Mupirocin) which exhibited mild number of inflammatory cells. Consequently, it is evident that in all treatment (1%, 5%, 10%) and positive control (Mupirocin) groups, there was abundance of fibroblast ingrowth and collagen formation.

The 10% extracted curcumin ointment was used as the concentration for the final ointment formulation. It exhibited the greatest wound healing activity based on four parameters: percent wound closure, period of epithelialization, percent of scar formed, and histopathological analysis. A batch size (400 grams) of 10% extracted curcumin ointment was produced, filled into 18-gram content and packaged in ointment jars. The finished product passed the following quality control parameters: appearance, color, odor, viscosity, spreadability, spatula feel and stability at 28ºC and 40ºC for three weeks as shown in **Table 4** [18] (Finished Product Quality Control Test of Ointment).

TABLE 4: FINISHED PRODUCT QUALITY CONTROL TEST OF OINTMENT

Parameter	Theoretical Result	Actual Result	Remarks
Appearance	Homogenous mixture	Homogenous mixture	Passed
Color	Yellow-orange	Yellow-orange	Passed
Odor	Distinct *C. longa* scent	Distinct *C. longa* scent	Passed
Viscosity	Sheer-thinning (16,000 cP)	Sheer-thinning	Passed
Spreadability	Easily spreadable	Easily spreadable	Passed
Spatula Feel	Smooth feel	Smooth feel	Passed
Stability (28◦C)	No change in color; No mold growth	No change in color	Passed
Stability (40◦C)	No change in color; No mold growth	No mold growth	Passed

CONCLUSION: It was confirmed that the extracted curcumin from *Curcuma longa* L. is stable when subjected to various light, air, and temperature conditions. Moreover, it is compatible with white petrolatum and beeswax that were used as excipients. The wound healing activity of the formulated extracted curcumin ointment was investigated among male Sprague-Dawley rats. Diabetes was successfully induced among the rats before wounding them and the set conditions were found to be effective. The wound-healing assay showed that the three treatment groups (1%, 5%, and 10%) exhibited better wound-healing activity than the positive control (Mupirocin) and the negative control (placebo) in terms of percent wound closure, rate of wound closure, and period of epithelialization. Among the treatment concentrations, it was the 10% extracted curcumin ointment that exhibited the highest wound-healing activity since it has the highest percent wound closure, fastest rate of wound closure, shortest period of epithelialization, and comparable histopathological results with the positive control. The statistical results also confirmed that the 10% extracted curcumin ointment was significantly different from the positive control, in terms of period of epithelialization, and the negative control, in terms of percent wound closure and period of epithelialization. Statistical analyses also showed that there was no significant difference in the effect of the treatment groups and the positive control on the percentage of scar formed.

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